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der Christian-Albrechts-Universität zu Kiel

**Manufacture, isolation and characterization of antibacterial peptides from rainbow trout
(*Oncorhynchus mykiss*)**

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Abstract:

Trout by-products were used as a source of antibacterial peptides, which were obtained by enzymatic hydrolysis using trout pepsin. The protein hydrolysates exhibited different antibacterial activities against gram-positive and gram-negative bacteria that cause food spoilage and fish diseases. The degree of hydrolysis was found to exert a considerable influence on antibacterial activity, with a significant increase in the observed inhibitory effect at the beginning of hydrolysis. The growth phase gradients were also affected by the degree of hydrolysis. After hydrolysis for 150 minutes (Degree of Hydrolysis (DH) 25 %), the hydrolysates demonstrated no further increase in antibacterial activity ($P > 0.005$). The highest antibacterial activity against most bacterial species was obtained at a DH % of 30 %. In contrast, a growth-promoting effect on the bacteria was observed for the raw substrate and for hydrolysates with low degrees of hydrolysis.

Depending on the bacteria, a peptide concentration of 47 mg/ml to 2 mg/ml resulted in complete growth inhibition when an incubation time of 24 h and a bacterial concentration of $1-2 \times 10^6$ cfu/ml were used. The highest inhibitory effect was indicated against *Flavobacterium* sp. and *R. salmoninarum*, which cause the so-called “bacterial coldwater diseases” (BCWD) and “bacterial kidney disease” (BKD); the minimal inhibitory concentrations (MIC) were 2 mg/ml and 5 mg/ml, respectively. The trout protein hydrolysate (DH 30 %) exhibited a higher antibacterial activity than nisin against most of the examined bacteria, whereas the antibacterial activity of the hydrolysate was up to 75 times lower than that of piscidin-1. In general, the antibacterial effect on food-borne microorganisms was lower than the antibacterial effect on fish farming pathogens.

To isolate antibacterial peptides from trout protein hydrolysate (DH 30 %), a combination of ion-exchange chromatography and gel filtration was used. The isolated peptides exhibited approximately nine-fold higher activity than the hydrolysate against the examined bacteria. Cationic peptides with molecular weights below 1 kDa showed the broadest spectrum of activity. These peptides had the highest content of amino acids with hydrophobic side chains (66 %). The peptides predominantly exhibited a prolongation effect during the lag phase of bacterial growth. Depending on the bacteria, peptide concentrations ranging from 1 mg/ml to 5 mg/ml resulted in complete growth inhibition of bacterial suspensions at concentrations of $1-2 \times 10^6$ cfu/ml.

One drawback of the use of enzymatic digestion is the high cost of enzymes; thus, the use of the fish's own pepsin, which is currently not subject to industrial use, offers a cost-reducing alternative to the use of the microbial or mammalian enzyme. A prerequisite for utilization of this enzyme is determination of its enzymatic properties. Therefore, trout pepsin was isolated via the

conventional enzyme isolation method (chapter 2), and a detailed enzyme characterization was performed. The isolation method consisted of a series of chromatographic separations preceded by ammonium sulphate precipitation.

Using this method, three pepsinogen isolates (PG-I till III) containing seven pepsinogen isoforms (PG-I a, b; PG-II; and PG-III c, d, e, f) were purified from the stomach of rainbow trout (*Oncorhynchus mykiss*). The pepsinogen isolates exhibited different optimal conditions (pH, temperature), catalytic constants (K_M and k_{cat}) for the proteolysis of acid-denatured haemoglobin, mechanisms of pepsinogen activation and N-terminal amino acid sequences. Pepsinogen II made up 60 % of the pepsinogens isolated. Electrophoretic tests indicated that pepsinogen I contained two pepsinogen isoforms with different molecular weights (45 kDa (PG-I a) and 44 kDa (PG-I b)) and that pepsinogen III comprised four isoforms with different isoelectric points (3.73 (PG-III a), 3.78 (PG-III b), 4.0 (PG-III c) and 4.15 (PG-III d)). It was shown that all trout pepsins are active at low (30-40 °C) temperature and over a wide range of pH and have higher catalytic efficiency than porcine pepsin. Based on these findings, the fish's own pepsin shows high potential as an alternative enzyme for enzymatic hydrolysis of different proteins.

Apart from a detailed enzyme characterization, the development of an enzyme purification method with good scaling-up properties, low cost and high enzyme yield is important for increasing the potential of fish pepsin as an enzyme source for industrial use (chapter 3). Via aqueous two-phase (ATPs) extraction and polyelectrolyte precipitation with pectin, a cost-reducing method with high enzyme yields (25-30 %) was developed.

The two aqueous phases were generated by mixing solutions of polyethylene glycol (PEG) and $MgSO_4$. This system separates into two phases, both water-rich, when a critical concentration of polymer or salt is achieved (chapter 3); pepsinogen showed a high affinity for the PEG-rich phase. The limiting concentrations, which result in high enzyme yield (87 %) with a high purification factor (23), were 20 % PEG with a molecular weight of 1500 and 20 % $MgSO_4$. The separation of pepsinogen from PEG was achieved by polyelectrolyte precipitation with pectin (2 % w/v). Under acid conditions (pH 2.5), pepsinogen was separated from the soluble ATPs extract and partially converted to pepsin. The formation of insoluble macroaggregates between pectin molecules and the enzyme was monitored by turbidimetric measurement. Subsequent separation of the components, which are predominantly held together by electrostatic forces, was performed by re-suspension in distilled water and concentrating them by pouring the suspension into a dialysis tube that was then embedded in PEG 20000, and separating the redissolved protein from the polyelectrolyte-containing sediment by centrifugation.

Kurzdarstellung:

Aus den Nebenprodukten der Forellenzucht (inneren Organe) wurden antimikrobielle Peptide über eine enzymatische Hydrolyse mittels Forellenpepsin isoliert. Die erzeugten Hydrolysate wiesen eine antibakterielle Wirkung sowohl gegenüber gram-positiven als auch gram-negativen Bakterien auf. Es konnte aufgezeigt werden, dass der Hydrolysegrad die antibakterielle Wirksamkeit der Hydrolysate signifikant beeinflusste. Mit steigendem Hydrolysegrad konnte ein Anstieg der antibakteriellen Wirksamkeit (bis zu einer Hydrolysezeit von 150 min (Hydrolysegrad 25 %)) beobachtet werden. Darüber hinaus konnte eine wachstumsfördernde Wirkung bei dem Rohsubstrat und den Hydrolysaten mit einem geringen Hydrolysegrad (< 10 %) aufgezeigt werden.

Je nach Bakterienart führte eine Peptidkonzentration von 47 mg/ml bis 2 mg/ml zu einer vollständigen Inhibierung des bakteriellen Wachstums über eine Inkubationszeit von 24 h bei einer Bakterienkonzentration von $1-2 \times 10^6$ KBE/ml (koloniebildende Einheiten). Die höchste antibakterielle Wirksamkeit wiesen die Hydrolysate gegenüber *Flavobacterium* sp. und *Renibacterium salmoninarum* auf (Krankheitserreger die häufig in der Fischzucht auftreten). Die minimale Konzentration welche zu einer vollständigen Inhibierung des bakteriellen Wachstums führt (MIC) lag jeweils bei 2 mg/ml (*Flavobacterium* sp.) und 5 mg/ml (*Renibacterium salmoninarum*). Verglichen mit Nisin, zeigte das Forellenproteinhydrolysat (DH 30 %) eine höhere antibakterielle Wirksamkeit, jedoch war die Wirksamkeit im Vergleich zu Piscidin-1 deutlich (75-fach) geringer. Generell konnte eine höhere antibakterielle Wirksamkeit gegenüber Krankheitserregern der Fischzucht als gegenüber Lebensmittelverderbskeimen aufgezeigt werden.

Für die Isolation der antibakteriellen Peptide aus dem Proteinhydrolysat wurde eine Kombination aus Ionen-Austauschchromatography und Gelfiltration angewandt. Die isolierten Peptide wiesen durchschnittlich eine 9-fach höhere antibakterielle Wirksamkeit verglichen zum Hydrolysat auf. Je nach Bakterienart führte eine Peptidkonzentration von 1 mg/ml bis 5 mg/ml zu einer vollständigen Inhibierung des bakteriellen Wachstums über eine Inkubationszeit von 24 h bei einer Bakterienkonzentration von $1-2 \times 10^6$ KBE/ml. Kationische Peptide mit einem Molekulargewicht < 1kDa wiesen das breiteste antibakterielle Spektrum sowie den höchsten Gehalt an hydrophoben Aminosäuren (66 %) auf. Die antibakteriellen Peptide führten zu einer deutlichen Verlängerung der lag Phase des bakteriellen Wachstums.

Ein Nachteil der enzymatischen Hydrolyse liegt in den hohen Kosten der üblicherweise verwendeten mikrobiellen Enzyme. Die Verwendung der fischeigenen Protease Pepsin, welche

derzeit keiner industriellen Nutzung unterliegt, würde daher zu einer deutlichen Kostenreduktion führen.

Zur genauen Enzymcharakterisierung, welche die Voraussetzung für eine industrielle Anwendung darstellt, wurde die konventionelle Enzymisolationmethode (Kapitel 2) durchgeführt. Diese Isolationmethode besteht aus einer Ammoniumsulfat Fällung mit anschließenden Säulenchromatographien.

Drei Pepsinogenisolate (PG I-III) bestehend aus sieben Pepsinogen Isoformen konnten mittels dieser Methode aus dem Magen der Regenbogenforelle isoliert werden. Die Pepsinogenisolate zeigten deutliche Unterschiede in ihren Enzymoptima (pH und Temperatur), katalytischen Konstanten (K_m und K_{cat}) für die Hydrolyse von Hämoglobin, dem Enzymaktivierungsprozess und der N-terminalen Aminosäuren Sequenz auf. Mengenmäßig war Pepsinogen II mit 60 % die Hauptgruppe. Elektrophoretische Untersuchungen zeigten, dass Pepsinogen I zwei Isoformen mit unterschiedlichen Molekulargewichten (45 kDa PG-Ia, und 44 kDa PG-IIa) und Pepsinogen III vier Isoformen mit unterschiedlichen isoelektrischen Punkten (3.73 (PG-III a), 3.78 (PG-III b), 4.0 (PG-III c) und 4.15 (PG-III d)) enthält. Es konnte gezeigt werden, dass alle Forellenpepsine verglichen zu Schweinepepsin eine höhere Aktivität bei geringen Temperaturen und über einen breiteren pH Bereich sowie eine höhere katalytische Effektivität gegenüber Hämoglobin aufwiesen. Diese Ergebnisse zeigen das hohe Potential von Forellenpepsin für eine Verwendung bei enzymatischen Hydrolysen von unterschiedlichen Nahrungsproteinen.

Die Voraussetzung für eine industrielle Anwendung von Fischpepsin ist die detaillierte Enzymcharakterisierung (Kapitel 2) sowie die Entwicklung einer Isolationmethode mit guter up-scaling Eigenschaft, hohen Enzymausbeuten und einem geringem Kosten- und Zeitaufwand (Kapitel 3). Die wässrige 2 Phasen Extraktion mit anschließender Polyelektrolyt Präzipitation mittels Pektin ermöglichte Enzymausbeuten von bis zu 30 % und zeigte darüber hinaus die geforderten Kriterien einer guten up-scaling Eigenschaft und einem geringen Kosten- und Zeitaufwand. Die wässrigen 2 Phasen wurden durch eine Mischung aus Polyethylenglycol (PEG) und $MgSO_4$ erzeugt. Sobald die kritische Konzentration an PEG und $MgSO_4$ erreicht wurde, erfolgte die Trennung der Phasen, wobei Pepsinogen eine hohe Affinität zur PEG Phase zeigte. 20 % PEG mit einem Molekulargewicht von 1500 und 20 % $MgSO_4$ führte zu hohen Enzymausbeute (87 %) mit einem hohen Aufreinigungsfaktor (23). Mittels einer 2 % igen (w/v) Pektinlösung erfolgte die Separation des Pepsinogens vom PEG, wobei unter den sauren Milieubedingungen während der Präzipitation Pepsinogen teilweise zu Pepsin umgewandelt wurde. Die Bildung der unlöslichen Makroaggregate zwischen Pepsinogen und Pektin sowie zwischen Pepsin und Pektin

wurden mittels turbidimetrischer Messung beobachtet. Die anschließende Separation der Komponenten, welche vorwiegend über elektrostatische Kräfte miteinander verbunden sind, erfolgte durch eine Resuspendierung in destilliertem Wasser. Nach einer Konzentrierung der Komponenten (einfüllen der Suspension in ein Dialyseschlauch und einbetten in PEG 20000) wurde das polyelektrolythaltige Sediment mittels Zentrifugation vom enzymhaltigen Überstand abgetrennt.

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List of abbreviations

AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AFS	Ammonium sulphate fractionation
AMPs	antimicrobial peptides
ANOVA	Analysis of Variance
ATP	Aqueous two phase
ATPs	Aqueous two phase system
B	the amount of HCl or NaOH
BME	β -mercaptoethanol
Cfu	colony formin units
Chi	chitosan
Da	Dalton
DEAE	diethylaminoethanol
DH	degree of hydrolysis
E/S	enzyme/substrate
E-64	trans-Epoxysuccinyl-L-Leucylamido-(4-Guanidino)butane
EDTA	Ethylenediaminetetraacetic acid
FAO	united nations Food and Agriculture organization
h_{tot}	total protein amount
H ₁	Hydrolysates with DH 10 %
H ₂	Hydrolysates with DH 20 %
H ₃	Hydrolysates with DH 25 %
H ₄	Hydrolysates with DH 30 %
HCl	hydrochloric acid
ID	identity
IEF	isoelectric focusing

k_{cat}	turnover number
KBE	koloniebildende Einheiten
kDa	kilo Dalton
K_E	partition coefficient of enzyme in the upper phase
K_M	Michaelis constant
K_P	partition coefficient of protein in the upper phase
LC	liquid chromatography
M	mole
MgSO_4	magnesium sulphate
M_H	hydrophobic moment
MIC	minimum inhibitory concentration
mM	millimolar
MP	protein concentration of the substrate
MW	molecular weight
NaOH	sodium hydroxide
Nb	normality of the solutions
OD	optical density
P	pepsin
PC	phosphatidylcholine
PEG	polyethylene glycol
PF	purification factor
PG	pepsinogen
pI	isoelectric point
PVDF	polyvinylidenedifluoride
rpm	revolutions per minute
SA	specific enzyme activity (unit/mg protein)

SD	standard deviation
SDS	Sodium dodecyl sulfate
<i>sp</i>	species
TCA	trichloroacetic acid
TNBS	trinitrobenzenesulphonic acid
v/v	volume/volume
v/w	volume/weight
w/w	weight/ weight
V_{Max}	maximum velocity
V_R	volume ratio (upper/lower)
BCWD	bacterial coldwater diseases
BKD	bacterial kidney disease

1. General Introduction

1.1. Introduction

1.1.1. Modivation

Amounting to more than 370,000 tons, rainbow trout is one of the most commonly used fish species in European aquaculture (FEAP, 2015). In the course of processing, 60 % of the total biomass consists of by-products that are either processed into fishmeal and fish oil or expensively disposed of without being put to any use (Benjakul & Morrissey, 1997; Chen & Jaczynski, 2007; Khosravi et al., 2015). The large quantity of processing waste – 222,000 tons – from this species offers a valuable, continuously available source of ① fish proteins from which antibacterial peptides can be liberated by enzymatic digestion and ② digestive enzymes such as pepsin that could be used for enzymatic hydrolysis (Figure 1.1).

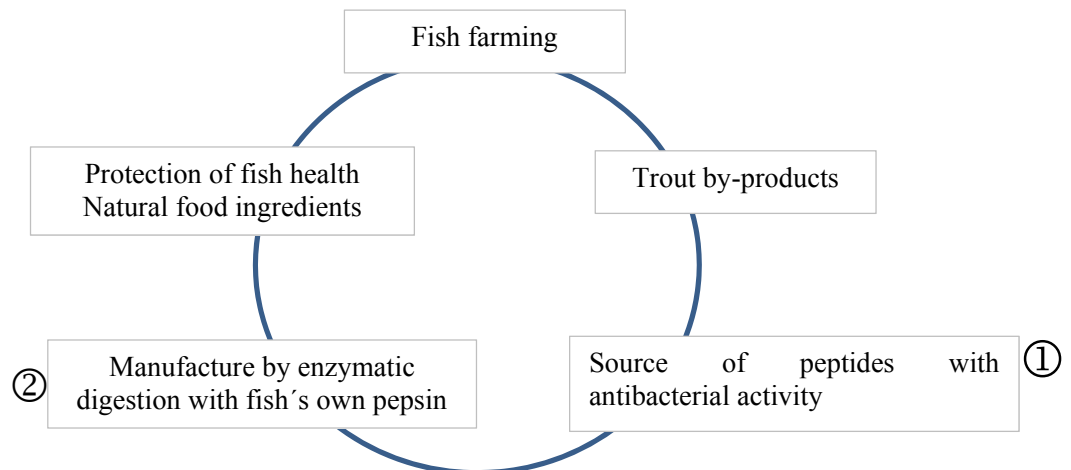


Figure 1.1: Use of trout by products

As demonstrated by Sila et al. (2014), Beaulieu et al. (2013), Doyen et al. (2012) and Robert et al. (2015) fish by-products represent sources from which antibacterial peptides can be isolated. These peptides are attractive to the nutraceutical and food industries because they can be used to improve disease resistance in farmed fish (Rajanbabu & Chen, 2011) and to keep food safe and unspoiled by microorganisms (Jabeen & Khanum, 2014). The broad spectrum of activity against gram-positive and gram-negative bacteria and the natural sources of these antibacterial peptides

demonstrate the high potential of these peptides for industrial purposes. Additionally, the increased amount of antibiotic-resistant bacteria and a growing interest in the use of natural substances for food preservation underlines the high interest in antibacterial peptides (Beaulieu et al., 2013; Lin et al., 2013).

To date, enzymatic hydrolysis has mainly been performed using microbial enzymes such as Protamex® and Alcalase® (both proteases from *Bacillus licheniformis*) and Flavourzyme® (protease from *Aspergillus oryzae*), rendering the hydrolysis process cost-intensive (Benhabiles et al., 2012). The use of the fish's own pepsin enzymes offers a cost-reducing alternative to the aforementioned approach. Pepsin exhibits high cleavage specificity for proteins and polypeptides, and the risk of bacterial contamination during the hydrolysis process is reduced due to the acidic hydrolytic pH (Benhabiles et al., 2012; Bougateg et al., 2009; Nalinanon et al., 2011). Additionally, fish pepsins, especially those from cold-adapted fish species, often exhibit lower activation energy and denaturation temperatures than mammalian pepsins as well as high activity over a wide range of pH and temperature conditions, making them attractive for this purpose. In contrast to marine and freshwater fish species, farmed fish species possess the advantage that their treatment (feeding, feeding time, quantity of food and living environment), which has a great influence on the activity of the digestive enzymes, can be controlled (Cao et al., 2011; Chen et al., 2007; Gildberg, 1988; Gildberg, 1992; Nalinanon et al., 2010; Shahidi & Kamil, 2001; Wu et al., 2009).

In this thesis, the antibacterial activity of hydrolysed trout by-products was evaluated. For the enzymatic digestion, the fish's own pepsin was used. Two methods for the isolation of pepsin from the stomach of *Oncorhynchus mykiss* were applied, with the objectives of (1) detailed enzyme characterization and (2) a cost reduction method with high enzyme yields and good scaling-up properties.

The antibacterial activities of trout protein hydrolysates were evaluated based on their degree of hydrolysis, and the effectiveness of the peptides was illustrated in relation to their charge (cationic/anionic), size (> 3 kDa, 1-3 kDa, < 1 kDa) and amino acid composition. The active peptides from the trout protein hydrolysate were isolated via a combination of ion-exchange chromatography and gel filtration.

The effectiveness of the hydrolysates against pathogens affecting fish farming and those that induce food spoilage was investigated. For comparison, the peptides piscidin-1 and nisin and the antibiotics oxytetracycline and erythromycin were used.

In this thesis, four main hypotheses were proposed:

- It is hypothesized that trout by-products contain antibacterial peptides that can be released via enzymatic hydroxylation by the fish's own pepsin. The effectiveness of the isolated peptides against gram-positive and gram-negative bacteria is primarily influenced by the degree of hydrolysis.
- It is hypothesized that differences in the composition of these peptides (amino acid content, molar mass and charge) result in differences in their antibacterial activities.
 - Therefore, active peptide fractions can be obtained from the hydrolysate via ion-exchange chromatography and gel filtration.
- It is hypothesized that trout pepsin provides an alternative to porcine pepsin for enzymatic hydrolysis of food proteins; trout pepsin exhibits higher cleavage activity at low temperatures, and protein denaturation occurs at lower temperatures compared to porcine pepsin.
- It is hypothesized that trout pepsinogen can be separated from polyethylene glycol (PEG) via polyelectrolyte precipitation with pectin.

1.1.2. Objectives:

The main objective of this study was to evaluate the use of trout by-products (especially trout viscera) as a potential source of antibacterial peptides that can be released by enzymatic digestion with fish pepsin. This included (1) the production of antibacterial peptides by treatment of trout by-products with trout pepsin; (2) the characterization of trout pepsinogen and pepsin; (3) the development of an enzyme isolation method with good scaling-up properties and high enzyme yields; and (4) the isolation and characterization of the antibacterial peptides obtained from the protein hydrolysate. The study therefore comprised the following:

1. Isolation and characterization of pepsinogen from the stomach of *Oncorhynchus mykiss* by the conventional method (chapter 2). The characteristics of the enzyme, including pH and temperature optima, substrate affinity, sensitivity to various protease inhibitors, molecular weight and n-terminal amino acid sequence, were analysed.

2. Isolation and purification of pepsinogen from the stomach of rainbow trout using aqueous two-phase extraction (ATP) with subsequent polyelectrolyte precipitation with pectin and determination of the characteristics of the enzyme obtained (chapter 3). The influence of PEG (polyethylene glycol) molecular weight, PEG concentration and salt concentration on the phase separation was systematically tested. To separate pepsinogen from PEG, pectin was tested as an alternative polyelectrolyte and compared to chitosan. Finally, the use of trout pepsin for the enzymatic digestion of plant proteins (potato and pea proteins) and animal proteins (fish by-products, fish meal) was examined.
3. Investigation of the antibacterial activity of trout protein hydrolysate digested by trout pepsin (chapter 4). The effectiveness of the hydrolysate against gram-positive and gram-negative bacteria was evaluated based on its degree of hydrolysis.
4. Isolation of active peptides from the trout protein hydrolysate using a combination of ion-exchange chromatography and gel filtration (chapter 5). The effectiveness of the peptide in relation to its charge (cationic/anionic), size (> 3 kDa, 1-3 kDa, < 1 kDa) and amino acid composition was illustrated.

1.2. Background

1.2.1. Fish by-products

The United Nations Food and Agriculture Organization (FAO) estimates that fish production has increased constantly since 1950. Thus, the global production of fish products increased to 66.6 million tons in 2014. Consequently, each year a considerable amount (20 million tons from the total production of marine capture fisheries) of fish waste, including muscle remains, heads, viscera, skin, bones and scales, are discarded. The viscera, which represent 20 % of the fish waste, are the most important by-product (Bougatef, 2013; FAO, 2014). The term by-products is not clearly defined to distinguish these materials from waste, and in many instances by-products are identified as leftovers (Bougatef, 2013; Kim & Mendis, 2006).

One of the most commercially important farmed fish species in the EU is rainbow trout (*Oncorhynchus mykiss*), which had an annual production of more than 370,000 tons in 2013 (FEAP, 2015). Trout processing produces approximately 40 – 70 % by weight of by-products (Fiori et al., 2012; Kristinsson & Rasco, 2000; Opheim et al., 2015); these are either used to produce fish meal, fish oil or fish silage (Klomklao et al., 2007) or wasted (Chalamaiah et al., 2010), resulting in environmental problems. To date, most of these recycled products have been considered to possess low economic value (Arvanitoyannis & Kassaveti, 2008; Zhao et al., 2011).

These by-products represent an important source of enzymes such as pepsin and proteins from which peptides with bioactive and functional properties can be liberated (Ben Khaled et al., 2012; Bougatef et al., 2010; Choi et al., 2009; Hoyle & Merritt, 1994; Robert et al., 2015). The transformation of fish proteins into biologically active peptides by enzymatic digestion provides an interesting alternative to the production of fish meal, fish oil or fish silage (Barkia et al., 2010). Use of these methods would also minimize the environmental problem of fish waste and provide a source of novel food additives and low-cost proteinases for industrial application (Bougatef, 2013).

1.2.2. Fish protein hydrolyzates

The functionality of protein hydrolysates is mainly influenced by the production method; solvent extraction and alkaline and acidic hydroxylation generally produce protein concentrates of low functionality, whereas enzymatic hydrolysis results in protein hydrolysates with desirable functionality (Barkia et al., 2010; Diniz & Martin, 1997; Najafian & Babji, 2012). In the food and pharmaceutical industries, enzymatic hydrolysis is the preferred method of production of protein hydrolysates because it does not leave residual organic solvents or toxic chemicals in the products, in contrast to other methods (Najafian & Babji, 2012). Recent studies have shown that upon the enzymatic hydrolysis of fish proteins, peptide sequences with bioactive properties that are hidden inside the proteins can be released and that the functional and nutritional properties of food proteins can be modified (Ben Khaled et al., 2012; Bougatef et al., 2010; Choi et al., 2009; Hoyle et al., 1994; Najafian et al., 2012). These studies showed that the hydrolysates exhibited higher solubility than the untreated proteins (Hoyle et al., 1994; Quaglia & Orban, 1987; Vieira et al., 1995) and better emulsifying, foaming and water-binding properties (Choi et al., 2009; Gbogouri et al., 2004; Kristinsson et al., 2000; Onodenalore & Shahidi, 1996; Shahidi et al., 1995; Vieira et al., 1995); fat absorption properties were also demonstrated (Onodenalore & Shahidi, 1996; Shahidi et al., 1995). In addition, bioactive peptides with ACE inhibitory effects (Ben Khaled et al., 2012; Byun & Kim, 2001; Darewicz et al., 2014) and antioxidative (Bougatef et al., 2010; Jiang et al., 2014) and antibacterial activities (Beaulieu et al., 2013; Robert et al., 2015; Sila et al., 2014) were released from fish by-products.

Based on their properties, these hydrolysates could be used in the food industry as (1) form and emulsion stabilizers (Choi et al., 2009; Kristinsson et al., 2000; Shahidi et al., 1995; Thiansilakul et al., 2007), (2) antioxidants (Barkia et al., 2010; Robert et al., 2015; Sila et al., 2014; Wu et al., 2003), or (3) preservatives. Furthermore, they could also be used in pharmaceutical and biotechnological applications as drugs with ACE inhibitory effects (Ben Khaled et al., 2012; Byun et al., 2001; Nasri et al., 2013) or to enhance bacterial growth by serving as a source of nitrogen (Dufosse et al., 2001).

Apart from the extraction method and the enzyme and substrate used, the functionality of the hydrolysates depends on the degree to which the protein has been hydrolysed. Limited hydrolysis has been shown to improve the foaming and emulsifying properties of proteins (Gbogouri et al.,

2004), whereas extensive hydrolysis increases their solubility and releases bioactive peptides with antioxidative, antibacterial and ACE inhibitory effects (Cheng et al., 2013; Spellman et al., 2003).

The substrate specificity of the enzyme used also affects the functionality of the hydrolysate because it influences the molecular size and the hydrophobic/hydrophilic balance. Gauthier et al., (1993) suggested that increased specificity results in smaller peptides, increasing the complexity of the peptide profile. In general, peptides with surface activity (emulsifying and good foaming properties) should have a minimum length of > 20 residues, whereas bioactive peptides are predominantly smaller < 10 kDa (Chi et al., 2015; Gauthier et al., 1993; Kristinsson et al., 2000; Saidi et al., 2014).

A number of studies on the optimization of enzymatic hydrolysis conditions using various microbial or mammalian enzymes have been published (Kamara et al., 2010). These studies illustrate the drawbacks associated with the high retail prices of these enzymes, which create a further hurdle for the utilization of fish by-products. Therefore, the use of fish enzymes for the hydrolysis would be a great benefit (Benhabiles et al., 2012).

Apart from the work of Tabarestani et al. (2010), in which the extraction of collagen and gelatine from the skin of rainbow trout was investigated, no further studies on the isolation of functional and bioactive components from rainbow trout by-products have been published.

1.2.3. Degree of hydrolysis

To obtain desirable organoleptic and function properties, hydrolysis must be carried out under strictly controlled conditions (Adler-Nissen, 1979). For this reason, the determination of the degree of hydrolysis (DH) (%), which is defined as the percentage of the total number of peptide bonds in a protein that have been cleaved during hydrolysis, is a useful way of monitoring the extent of protein degradation (Adler-Nissen, 1986; Kristinsson et al., 2000; Sila et al., 2014; Spellman et al., 2003).

The DH (%) can be quantified by various methods, which are based on four different principles:

(1) determination of the soluble protein content after precipitation with trichloroacetic acid (TCA) (Choi et al., 2009; Hoyle et al., 1994; Hung et al., 1984; Kamara et al., 2010; Silva et al., 2014; Wang et al., 2014);

(2) determination of the free amino groups released during hydrolysis by formol titration (You, et al., 2009) or by using compounds that react specifically with amino groups, such as trinitrobenzenesulphonic acid (TNBS) (Nuanmano et al., 2015; Phanturat et al., 2010), o-phthaldialdehyde (Roslan et al., 2014), ninhydrin or fluorescamine;

(3) measurement of the change in the freezing point of the protein solution using osmometry (Spellman et al., 2003); and

(4) titration of the H⁺ or OH⁻ ions that are released after the rupture of peptide bonds (pH stat method) (Cândido & Sgarbieri, 2003; Diniz et al., 1997; Gbogouri et al., 2004; Klompong et al., 2007).

General, no consensus as to the best method of determining the DH of protein hydrolysates has been reached (Rutherford, 2010), but methods 1 and 4 are predominantly used for the determination of the degree of hydrolysis of fish protein hydrolysates (Cândido et al., 2003; Choi et al., 2009; Diniz et al., 1997; Gbogouri et al., 2004; Hoyle et al., 1994; Hung et al., 1984; Kamara et al., 2010; Klompong et al., 2007; Silva et al., 2014; Wang et al., 2014). These methods are described in more detail below.

Method 1:

The DH (%) can be quantified by determining the amount of nitrogen released during hydrolysis; the released nitrogen is soluble in trichloroacetic acid. The calculation of the degree of hydrolysis is based on the percentage of protein that is soluble in a solution of TCA (10 %) relative to the total protein content of the sample; protein amounts can be determined by the method of Lowry, Rosebrough, Farr, and Randall (1951). The degree of hydrolysis can be calculated as follows:

$$DH (\%) = \frac{\text{soluble protein content in 10 \% TCA (mg)}}{\text{total protein content (mg)}} \times 100$$

This technique for the determination of the degree of hydrolysis was used in the studies of Choi et al. (2009), Hoyle et al. (1994), Hung et al. (1984), Kamara et al. (2010), Silva et al. (2014) and Wang et al. (2014).

Method 4:

The pH stat technique, according to Adler-Nissen (1986), is the simplest and most commonly used method; it allows monitoring of the degree of hydrolysis in real time by direct determination

of the number of peptide bonds that have been cleaved during hydrolysis (DH (%)) (Adler-Nissen, 1986; Rutherford, 2010; Tibbetts et al., 2011). When peptide bonds are broken, protons are liberated or consumed based on the pH of the medium, as shown in Figure 1.2 (Adler-Nissen, 1986).

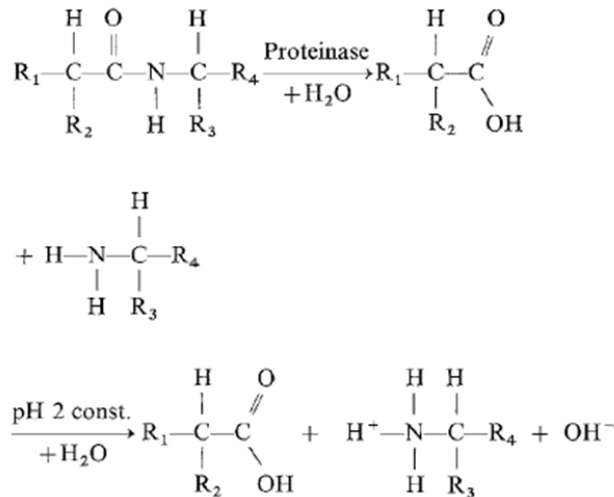


Figure 1.2: Consumption of H⁺ under acid conditions (Diermayr & Dehne, 1990)

The release or consumption of positively charged hydrogen ions causes the reaction mixture to become more acidic or more alkaline. To counteract this pH change, alkali or acid solutions (the most commonly used pH-control solutions are sodium hydroxide (NaOH) and hydrochloric acid (HCl)) are added to the reaction mixture to maintain the target pH (Fernández & Kelly, 2016). The volume of HCl or NaOH required to maintain the target pH, combined with various other data, is then used to calculate the degree of hydrolysis as shown in the following (Tibbetts et al., 2011):

$$\text{DH (\%)} = \frac{h}{h_{\text{tot}}} \times 100 = \frac{B \times \text{Nb}}{\text{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{\text{tot}}} \times 100 \qquad \alpha = \frac{10^{\text{pH}-\text{pK}_A}}{1+10^{\text{pH}-\text{pK}_A}}$$

The calculation includes the total protein amount (h_{tot}), the amount of HCl or NaOH (B) used to maintain the pH value during hydrolysis, the normality of the solutions (Nb) and the protein concentration of the substrate (MP). Use of the pH stat method to monitor protein digestibility has several advantages: it employs stable reaction conditions, the response is specific, and the

method is safe and does not require complex equipment (Tibbetts et al., 2011; Yasumaru & Lemos, 2014).

This technique for the determination of the degree of hydrolysis was used in the studies of Cândido et al. (2003), Diniz et al. (1997), Gbogouri et al. (2004) and Klompong et al. (2007).

1.2.4. By-products as natural source of antibacterial peptides

In recent years, the widespread use of antibiotics has led to a rapid increase in the prevalence of antibiotic-resistant bacteria. In recent decades, this development and the desire of consumers to buy food products that contain only natural ingredients have resulted in research into novel antimicrobial agents (Beaulieu et al., 2013; Lin et al., 2013).

Aquaculture, which averaged 8.6 per cent growth per year between 1980 and 2012, has exhibited more rapid growth than other animal production sectors (FAO, 2014; Santos & Ramos, 2016). The practice of intensive and semi-intensive production leads to a higher concentration of animals in small spaces, which results in more rapid spread of infectious diseases. To prevent these outbreaks and the spread of infectious diseases in fish, large amounts of antibiotics such as oxytetracycline, erythromycin, florfenicol, sarafloxacin and sulphonamides have been used in aquaculture (Acosta et al., 2013; Santos & Ramos, 2016). As reviewed by Santos & Ramos (2016), the major concerns associated with this practice are the presence of antimicrobial residues in the edible tissues of treated animals and the emergence of microbial antibiotic resistance.

Consequently, this development has triggered a great interest in the study of natural antimicrobial peptides (AMPs). AMPs such as piscidin, defensins, cathelicidins and nisin are an important part of the natural defence systems of human, animal and microbial organisms (Campagna et al., 2007; Marshall & Arenas, 2003). In addition to these proteins, it is well known that peptides with antibacterial activity can be released from certain food proteins by enzymatic digestion. In recent years, it was shown by Sila et al. (2014), Beaulieu et al. (2013), Doyen et al. (2012) and Robert et al. (2015) that AMPs with antibacterial activity could be isolated from fish and crustacean by-products by enzymatic digestion ((barel muscle proteins (*Barbus callensis*), snow crab by-products (*Chionoectes opilio*) and tilapia by-products (*Oreochromis niloticus*)).

These antibacterial peptides showed high activity against gram-positive and gram-negative bacteria. They exhibit low molecular weight (< 10 kDa), predominantly amphiphilic structure,

high content of hydrophobic amino acids and mostly cationic properties at physiological pH. These peptides are often enriched in asparagine, glutamine, lysine, leucine, glycine, phenylalanine, and proline (Beaulieu et al., 2013; Cheng et al., 2013; Robert et al., 2015; Yeaman & Yount, 2003). In addition to the cationic peptides, anionic peptides with antibacterial activity have also been isolated from fish protein hydrolysates (*Cancer irroratus* and *Chionoecetes opilio*) (Beaulieu et al., 2013; Beaulieu et al., 2009). The AMPs that have been produced from fish proteins to date have demonstrated growth inhibition of a number of species of food-spoiling bacteria, including *Escherichia coli*, *Enterococcus* sp., *Micrococcus luteus*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Listeria monocytogenes* and towards fish pathogenic bacteria such as *Aeromonas* sp., *Yersinia ruckeri* and *Edwardsiella tarda* (Beaulieu et al., 2013; Robert et al., 2015; Sila et al., 2014).

The biological activity of the peptides released from fish proteins depends on the protein and enzyme used and is significantly influenced by the hydrolysis conditions, as described by Adler-Nissen (1986) and Kristinsson et al. (2000). Peptide hydrophobicity, charge, size and several other factors, as shown below, determine the antibacterial activity of the peptides and can differ significantly depending on the degree of hydrolysis (Cheng et al., 2013; Yeaman et al., 2003).

Antibacterial peptides should produce (1) rapid bacterial killing that occurs in a much shorter time than the doubling time of the bacteria (therefore, it is useful if the antibacterial peptides interact with the bacterial surface instead of the cell interior); (2) broad antibacterial spectra; (3) selectivity of the antibacterial peptides for bacterial membranes; (4) and no easily developed resistance. According to Matsuzaki (1999), this could be possible if the targets of the peptides are important components of the bacteria. In addition, the high diversity of AMPs and their diverse mechanisms of action against their target cells offers a potential strategy for preventing or delaying the acquisition of bacterial resistance to these AMPs (Yeaman et al., 2003).

To compare the effectiveness of AMPs that are released from food proteins by enzymatic digestion, AMPs that are a part of the natural defence systems of organisms can be used. Examples of AMPs that form parts of natural defence systems are given below.

Piscidin was the first antibacterial amphipathic cationic peptide family to be identified in fish; piscidin-1 possesses the highest antibacterial activity of the members of this family. Piscidins-1 and 3 were originally isolated from hybrid striped seabass (Lee et al., 2007). Piscidin exhibits a

broad spectrum of activity against bacteria, fungi and viruses. Since then, many other antibacterial peptides in fish have been identified, including hepcidin, cathelicidin and oncorhyncin (Alvarez et al., 2014; Broekman et al., 2011; Fernandes et al., 2004; Rajanbabu et al., 2011).

Defensins are a family of cysteine-rich peptides that have been isolated from molluscs, acari, arachnids, insects, mammals and plants. Based on their structural diversity, the defensins have been grouped into families. Defensins isolated from invertebrates and plants are characterized by three or four disulphide bridges, respectively; their common structure consists of an α -helix linked to a β -sheet by two disulphide bridges (Marshall et al., 2003). Defensins exhibit antimicrobial, insecticidal and slight haemolytic activities (Reddy et al., 2004).

Cathelicidins are a group of small, cationic peptides that show diversity in structure. They can be categorized into peptides with α -helical, extended helical, and loop structures and β -sheet peptides with 2-3 disulphide bridges. Due to the high similarity of their proregions to cathelin, a 12-kDa protein from porcine leukocytes, this family was named cathelicidins. Cathelicidins are active against gram-negative and/or gram-positive bacteria, fungi, parasites and enveloped viruses (Ramanathan et al., 2002).

Nisin, synthesized from *Lactococcus lactis*, belongs to the group of lantibiotics. This antibacterial peptide consists of 34 amino acids and exhibits a broad spectrum of activity against gram-positive bacteria such as *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Micrococcus* and *Lactobacillus*. Nisin contains unusual amino acids with intermolecular thioether rings such as lanthionine and β -methyl-lanthionine that protect nisin against proteolytic degradation (Alkhatib et al., 1959).

1.2.4.1. Fish farming and food spoilage bacteria

The emergence of microbial resistance against conventional antibiotics, such as has occurred in *Aeromonas* sp. (Hatha et al., 2005) and *Weissella* sp. (Figueiredo et al., 2012), as well as the movement of animals between farms, results in the spread of diseases. For example, Scottish aquaculture production, which predominantly involves salmon and rainbow trout, has been affected by a range of diseases, including bacterial kidney diseases caused by *Renibacterium salmoninarum*, which typically cause approximately 30 % mortality (Murray et al., 2011).

As reported by Starliper (2011), Hatha et al. (2005) and Figueiredo et al. (2012), *Flavobacterium* sp. (*F. columnare*, *F. branchiophilum*, and *F. psychophilum*), *Aeromonas* sp. and *Weissella* sp. play an important role in diseases of humans and animals (Table 1.1). *Flavobacterium* sp., *Weissella* sp. and *Aeromonas* sp. are common pathogens in fishes (Figueiredo et al., 2012; Starliper, 2011); *Aeromonas* sp. (Hatha et al., 2005) are unwanted in food because they are active spoilers of fish and meat at ambient temperatures and at temperatures ranging from 2-13 °C. Moreover, Figueiredo et al. (2012) and Hatha et al. (2005) reported that *Weissella* sp. and *Aeromonas* sp. exhibited resistance to conventional antibiotics such as sulphonamides (Beaulieu et al., 2013; Lin et al., 2013; Murray et al., 2011).

The consumption of meat products, which are known to be an ideal substrate for the growth of several species of spoilage and pathogenic bacteria, can result in serious food-borne diseases (Turgis et al., 2012). Some bacteria that cause food-borne diseases are listed in Table 1.1.

Bacillus cereus, *Escherichia coli* and *Micrococcus luteus* are often involved in diseases related to food poisoning and food spoilage, producing classical symptoms such as diarrhoea (Caamaño-Antelo et al., 2015; Eley, 1992). Moreover, they are occasionally responsible for (1) food-borne infections such as epidemic tonsillitis and/or (2) septicaemia and meningitis and/or (3) urinary tract and wound infections (Eley, 1992). Histamine-forming bacteria such as the enteric bacteria *Proteus mirabilis* and *Citrobacter freundii* and the gram-negative bacteria *Pseudomonas fluorescens* and *Aeromonas* sp. are also often involved in food-borne poisoning as the causative agents of scombroid poisoning (Cebrian et al., 2012; Chen et al., 2008; Khan et al., 2010).

Table 1.1: Fish farming and food spoilage bacteria

Fish farming bacteria
Flavobacterium sp.
<i>Aeromonas</i> sp. (<i>A. salmonicida</i> , <i>A. hydrophila</i> , <i>A. caviae</i> , <i>A. sobria</i>)
<i>Renibacterium salmoninarum</i>
<i>Weissella</i> sp.
Food spoilage
<i>Micrococcus luteus</i>
<i>Bacillus cereus</i>
<i>Citrobacter freundii</i>
<i>Echerischia coli</i>
<i>Enterococcus faecalis</i>
<i>Proteus mirabilis</i>
<i>Pseudomonas fluorescens</i>
<i>Aeromonas</i> sp.

1.2.4.2. Mechanism of action of antibacterial peptides

Despite the fact that the mechanism of action of AMPs is not well established, various model systems for their action have been described in recent years. There is evidence that many AMPs interact with the lipid bilayer of the bacterial membrane (Figure 1.3 and 1.4 step a) in such a way as to increase the membrane's permeability. With respect to the mechanism by which the peptides break down the membrane permeability, the peptides may induce complete lysis by forming pores in the membrane, destabilizing the bacterial membrane by lipid displacement or blocking the ionic gradient across the membrane; however, none of these mechanisms alone cause an antibacterial effect (Dathe et al., 1997; Yeaman et al., 2003).

The first model system, the "barrel-stave model", describes the formation of transmembrane pores (Figure 1.3 step c) composed of bundled α -helices (Figure 1.3. step b); the formation of these pores leads to membrane permeabilization. First, the peptide monomers bind to the cell membrane (Figure 1.3 step a) and insert themselves into the hydrophobic core of the membrane (Figure 1.3 step c), resulting in the formation of pores and causing leakage of cytoplasmic material. This leads to the death of the bacterial cell (Figure 1.3 step c) (Reddy et al., 2004).

This model system was proposed for the following antimicrobial peptides: methicillin, ceratotoxins, and distinctin (Bessin et al., 2004; Campagna et al., 2007).

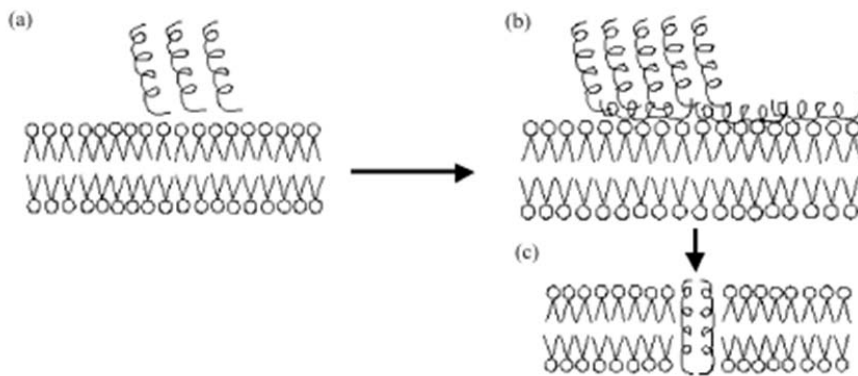


Figure 1.3: Pictorial representation of the barrel-stave model system (Reddy et al., 2004)

The second model system, the “toroidal pore” or “wedge-model”, is based on the storage of lipids between helices to form a mixed pore. This mechanism has been described for magainin. suggest that the antibacterial peptide piscidin-1 acts more like the toroidal model system than the barrel-stave model.

In the third model system, the peptides act as a detergent, and the membrane is eventually disrupted due to the formation of transitory pores (Figure 1.4 step c). This system has been called a “carpet-like” mechanism. After the interaction of peptide monomers with the bacterial membrane (Figure 1.4 step a), the peptides bind to the membrane surface (Figure 1.4 step b), reorient themselves towards the hydrophobic core of the membrane (Figure 1.4 step c) and disrupt the membrane by resolution (Figure 1.4 step d) (Campagna et al., 2007; Reddy et al., 2004).

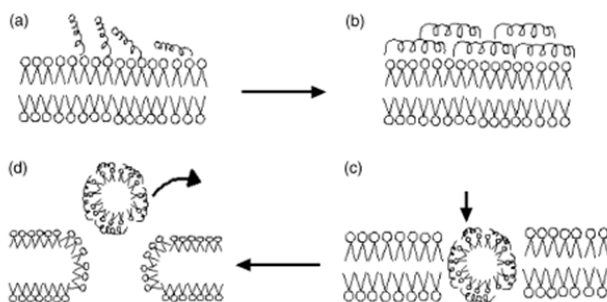


Figure 1.4: Pictorial representation of the carpet model system (Reddy et al., 2004)

In contrast to the above, several additional hypotheses regarding the way in which peptides exert their antibacterial effects have been proposed. These hypotheses include peptide penetration into cells, induction of hydrolases that break down the cell wall, disturbance of membrane function,

inhibition of synthesis of essential components of the cell wall, nucleic acids, and proteins and even the inhibition of enzyme activity. Inhibition of cell wall synthesis, as well as the formation of pores due to the binding to Lipid II, a cell wall precursor, has been described for the antibacterial peptide nisin (Alkhatib et al. 2012). Furthermore, a variety of peptides have been identified that can cause bacterial death without detectable cell lysis (Campagna et al., 2007; Epanand & Vogel, 1999; Marshall et al., 2003; Matsuzaki, 1999).

Most antibacterial peptides are cationic, and this is undoubtedly important for the initial electrostatic attraction between the peptides and the negatively charged phospholipid membranes of bacteria. Furthermore, this interaction provides a ready explanation for the specificity of the peptides for bacterial membranes. The bacterial membrane, in contrast to mammalian cell membranes, contains anionic molecules that are oriented towards the exterior of the cell (Figure 1.5). Thus, cationic peptides preferentially bind to the negatively charged bacterial membranes but not to the zwitterionic amphiphilic mammalian plasma membrane, as shown in Figure 1.5 (Epanand et al., 1999; Matsuzaki, 1999). The specificity of antibacterial peptides for bacterial membranes is illustrated in the following figure prepared by Matsuzaki (1999):

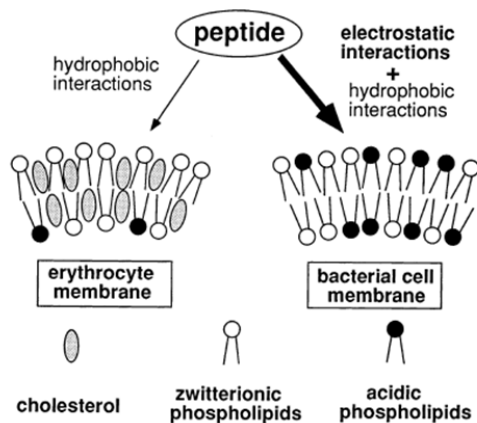


Figure 1.5: Molecular basis for the membrane interaction of antibacterial peptides

Via electrostatic interactions, cationic peptides preferentially bind to anionic phospholipids in the bacterial membrane. In contrast, the peptides have only a low affinity for the mammalian cell membrane, which is composed of zwitterionic phospholipids (Matsuzaki, 1999).

Antimicrobial peptides can be classified into various groups based on their biochemical (net charge, amino acid composition) and/or structural (α helical, β sheet) features (Marshall et al., 2003). The key properties influencing the mechanisms of action of antimicrobial peptides are shown in Figure 1.6 and described below. Generally, extremes of certain properties, such as hydrophobicity, amphipathicity and peptide charge, tend to decrease the antibacterial activity of peptides (Yeaman et al., 2003).

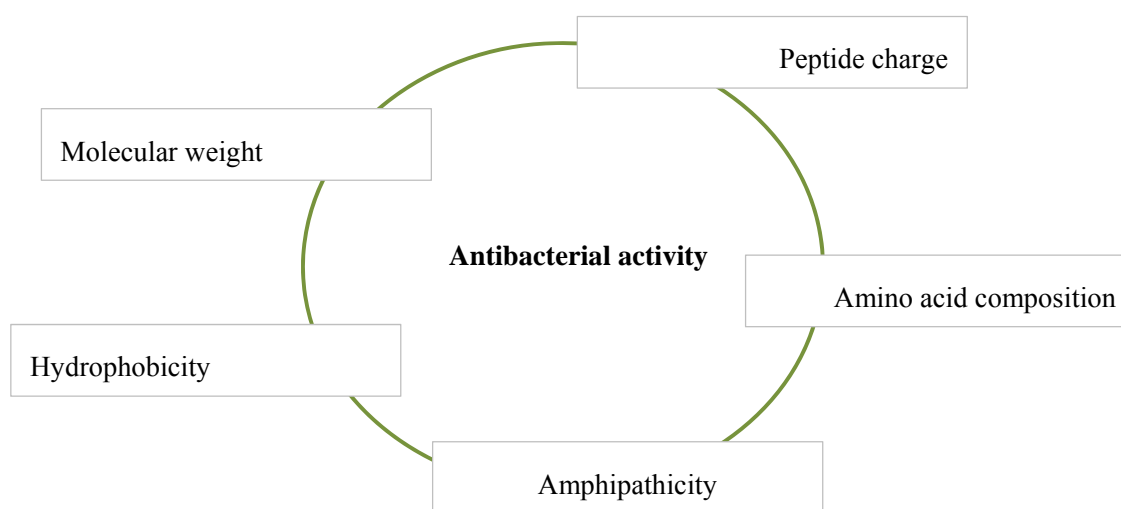


Figure 1.6.: Key properties influencing the mechanisms of action of antimicrobial peptides

Positive charge

Most antibacterial peptides possess a net charge of from +2 to + 9, and many of these peptides contain highly defined cationic domains. As already described, the cationic properties of peptides are undoubtedly important for the initial electrostatic attraction of antibacterial peptides to the negatively charged phospholipid membranes of bacteria. This relationship is not strictly linear; furthermore, at a certain level, the antibacterial activity no longer increases with increased positive charge of the peptides (Yeaman et al., 2003). Dathe et al. (1997), for example, indicated that the limit value for magainin is a net charge of + 6 to + 7.

Amphipathicity:

On interaction with the target bacterial membrane, nearly all peptides form amphipathic structures (Yeaman et al., 2003). Apart from the fact that increasing amphipathicity increases the antibacterial activity of peptides due to an increase in membrane permeabilization, it also affects,

perhaps to a much higher degree, haemolytic activity against zwitterionic or neutral membranes. As reported by Dathe et al. (1997), a higher degree of amphipathicity resulted in increased toxicity to cells composed of natural phospholipids, whereas peptide interactions were only slightly affected (Yeaman et al., 2003). Additionally, Pathak et al. (1995) suggested that the antimicrobial activity of peptides is more affected by their amphipathicity than by their hydrophobicity or their content of α -helical structures.

Hydrophobicity

For partitioning into the lipid bilayer of the bacterial membrane, peptide hydrophobicity is an essential feature. This explains the fact that most antibacterial peptides contain approximately 50 % hydrophobic residues (Yeaman et al., 2003). Increasing hydrophobicity, which is determined by the percentage of hydrophobic amino acids, leads to increased membrane permeability by peptides, as demonstrated using model peptides and target membranes (Wieprecht et al., 1997; Yeaman et al., 2003). Chen et al. (2007) demonstrated that increasing peptide hydrophobicity resulted in higher antibacterial activity up to a certain threshold. Beyond this threshold, hydrophobicity could lead to greater self-association of the peptides, which could prevent the peptides from passing through the cell walls of prokaryotic cells. It was also shown that protein hydrolysates exhibit antibacterial effects at certain degrees of hydrolysis (Cheng et al., 2013; Sila et al., 2014), which Cheng et al. (2013) attributed to a hydrophobicity threshold. The relationship between hydrophobicity and antibacterial effect is not linear, as shown in the investigations of Pathak et al. (1995) and Cheng et al. (2013).

Amino acid composition

A high content of several amino acids such as asparagine, glutamine, lysine, leucine, glycine, phenylalanine, and proline was identified in peptides with antibacterial activity isolated from various fish by-products (Beaulieu et al., 2013; Cheng et al., 2013; Robert et al., 2015; Yeaman et al., 2003). Furthermore, some antibacterial peptides contain a high content of amino acids such as cysteine (defensin), proline, glycine, histidine and glycine. Most of these antibacterial peptides exhibit activity against gram-positive and gram-negative bacteria and yeast (Alvarez et al., 2014; Dolashka et al., 2011).

Some amino acids, such as lysine and arginine, are known to interact strongly with phosphate groups in lipid bilayers via electrostatic forces (Mavri & Vogel, 1996). Bessalle et al. (1992)

demonstrated that the addition of 10 or 20 lysine residues enhanced the antibacterial effect of magainin. The presence of these amino acids increases the positive charge of the peptides.

Molecular weight

Antibacterial peptides are small proteins with molecular weights < 10 kDa (Alvarez et al., 2014; Battison et al., 1992). The peptides in active fractions isolated from fish and/or crustacean protein hydrolysates (*Chionoecetes opilio*, *Nile tilapia*, *Barbus callensis*, and *Cancer irroratus*) had molecular weights of between 200 and 3,624 Da (Beaulieu et al., 2013; Doyen et al., 2012; Robert et al., 2015; Sila et al., 2014). Based on the average weight of an amino acid of approximately 110 Da (Beaulieu et al., 2013), the peptides isolated by Robert et al. (2015), Beaulieu et al. (2013), Sila et al. (2014) and Doyen et al. (2012) can be assumed to contain approximately 2 to 33 amino acids.

1.2.5. By-products as natural source of Fish enzymes

In recent years, a number of studies have shown that fish by-products are a rich source of digestive enzymes such as pepsin, trypsin and chymotrypsin; these enzymes are used in the food industry, for example in cheese production and to determine the number of nematodes in fish fillets, and also as washing agents (Bougatef et al., 2008; Cao et al., 2011). The recovery of proteinases from fishery by-products would therefore not only minimize the environmental problem of fish waste but would also help provide novel low-cost proteinases for industrial application (Bougatef, 2013).

1.2.5.1. Digestive enzymes

Proteases are the most important group of industrial enzymes, accounting for 50 % of the total industrial enzyme market. They are mainly derived from animal, plant and microbial sources, and they have various applications in a wide variety of industries, including the detergent, food, agrochemical and pharmaceutical industries. Viscera, one of the most important by-products of fish production, constitute approximately 20 % of the by-products and are known to be a rich source of proteases. The most important digestive enzymes of fish viscera are the acid stomach enzyme pepsin and the alkaline intestinal enzymes trypsin, chymotrypsin and elastase (Bougatef, 2013).

Fish enzymes, especially those from cold-adapted fish species, exhibit some interesting advantages such as higher enzymatic activities at low temperatures and lower thermal stability compared to enzymes from warm-blooded animals; in addition, they often show high enzyme activity over a wider range of pH. These characteristics make them attractive for the aforementioned applications (Asgeirsson et al., 1989; Bougatef, 2013; Kristjansson, 1991; Simpson & Haard, 1987).

In enzyme nomenclature (EC), pepsin, trypsin, chymotrypsin and esterase belong to subclass 3.4, whereas pepsin (as an endopeptidase) and trypsin, chymotrypsin and esterase (as exopeptidases) are further classified as EC numbers 3.4.23.1-2 and EC 3.4.21, respectively. Endopeptidases hydrolyse the polypeptide chain at certain peptide bonds in the middle of the peptide or protein, whereas exopeptidases cleave the polypeptide chain one amino acid from the N-terminus (aminopeptidases) or the C-terminus (carboxypeptidases) (Bougatef, 2013).

The alkaline proteases trypsin, chymotrypsin and esterase are active between pH 8.0 and 10.0 and at 35-40 °C (Bougatef, 2013; Zhao et al., 2011). These serine proteases are characterized by the presence of serine, histidine and aspartic acid residues at their active sites (Bougatef, 2013).

Trypsin, the major enzyme of the intestine, has endopeptidase activity (EC 3.4.21.4.) and acts by cleaving ester and peptide bonds involving the carboxyl groups of arginine or lysine. The molecular weight of trypsin ranges from 22-28 kDa (Bougatef, 2013).

Pepsin (EC 3.4.23.1-2) belongs to the aspartic proteinase family, which is named for the presence of aspartic acid at the active site. Pepsin is present in fish as the precursor pepsinogen, which is synthesized by the gastric mucosa and secreted into the lumen as required. Pepsinogens contain a hydrophobic signal sequence 15-16 amino acids in length at the N-terminus. This signal sequence stabilizes the inactive form of the enzyme and prevents entry of the substrate to the active site during transport (Bougatef et al., 2008). Pepsin acts by cleaving peptide bonds involving the aromatic amino acids tyrosine, phenylalanine and tryptophan. Pepsin isolated from various fish species exhibited molecular weights of 31-38 kDa, whereas pepsinogen showed molecular weights of 38-42 kDa (Arunchalam & Haard, 1985; El-Beltagy et al., 2004; Nalinanon et al., 2010; Zhao et al., 2011; Zhou et al., 2007). In the acid environment of the stomach, pepsinogens are autocatalytically converted to pepsins by removal of the N-terminal prosegment by the one-step or the two-step pathway (Bougatef et al., 2008; Cao et al., 2011; Tanji et al., 2007). Pepsinogens are classified into five groups: pepsinogen A, B, C (progastricisin), pepsinogen F and prochymosin (Bougatef et al., 2008; Kageyama, 2002; Tanji et al., 2007); according to Foltmann (1981), pepsinogen A, C and prochymosin represent the major pepsinogen groups. The pepsinogen isoforms differ in their primary structures and in the enzymatic properties of their activated forms (Kageyama, 2002). The highest enzymatic activity for pepsin is achieved between pH 2.0 and 4.0 and between 37 and 40°C (Arunchalam et al., 1985; El-Beltagy et al., 2004; Nalinanon et al., 2010; Zhao et al., 2011; Zhou et al., 2007).

In recent decades, pepsin, trypsin and chymotrypsin have been isolated from a number of marine and freshwater fish species and characterized with regard to their physical and catalytic properties, including enzyme stability (Bougatef, 2013; Zhao et al., 2011). Trypsin has been extracted, for example, from stomachless bonefish, *Carassius auratus gibelio* (Jany, 1976), Atlantic cod, *Gadus morhua* (Amiza et al., 1997), yellowfin tuna, *Thunnus albacores* (Klomklao

et al., 2006); chymotrypsin has been obtained from rainbow trout, *Oncorhynchus mykiss* (Kristjánsson & Nielsen, 1992), and pepsin from chinook salmon, *Oncorhynchus tshawytscha* (Kurtovic et al., 2006), Atlantic cod, *Gadus morhua* (Gildberg et al., 1990), Antarctic rock cod, *Trematomus bernacchii* (Brier et al., 2007), albacore tuna, *Thunnus alalunga* (Nalinanon et al., 2010), smooth hound, *Mustelus mustelus* (Bougatef et al., 2008), European eel, *Anguilla anguilla* (Wu et al., 2009), Japanese seabass, *Lateolabrax japonica* (Cao et al., 2011), giant catfish, *Pangasianodon gigas* (Vannabun et al., 2014), and mandarin fish, *Siniperca chuatsi* (Zhou et al., 2008).

1.2.5.2. Methods for the isolation of trout pepsin

The process of isolating and purifying fish pepsin involves three steps: (a) extraction of pepsinogen, which involves the preparation of fish stomach and crude extract; (b) purification of pepsinogen by the conventional method or by an aqueous two-phase system; and (c) activation of pepsinogen to pepsin. To prevent enzyme denaturation, all steps are conducted at low temperatures (Zhao et al., 2011).

In the conventional enzyme isolation method, the purification process usually consists of a series of chromatographic separations preceded by ammonium sulphate precipitation, which allows the separate of different enzyme isoforms and results in high enzyme yields (Bougatef et al., 2008; Brier et al., 2007; Cao et al., 2011; Klomklao et al., 2007; Nalinanon et al., 2010; Tanji et al., 2007; Wu et al., 2009). Ammonium sulphate fractionation (ASF) is a general method for enzyme purification in which selective precipitation of proteins at two or more relative saturations of $(\text{NH}_4)_2\text{SO}_4$ solution is used to achieve high enzyme purification. Saturations of 20-40 % can be used to precipitate unwanted proteins, which are then discarded by centrifugation, whereas higher saturation (60-70 %) is applied to the supernatant for pooling of desired proteins (Zhao et al., 2011). For further protein purification and to separate the different pepsinogen and pepsin isoforms, ion exchange chromatography is used (Klomklao et al., 2007; Nalinanon et al., 2010; Zhao et al., 2011; Zhou et al., 2007). Gel filtration, which separates molecules based on their size, is used as a purification step for further purification (Zhao et al., 2011).

The conventional enzyme purification method is usually expensive (50-90 % of the total expenditure) because it involves several steps, the reactants are costly, and the methods are difficult to scale up (Boeris et al., 2009). In addition, some of the target molecules are lost at each step, resulting in a large overall loss; Spelzini et al., (2005) attribute this to protein denaturation during the purification process (Raja et al., 2011).

Aqueous two-phase extraction is an alternative method for isolating enzymes with good up-scaling properties; it is not costly in terms of time or material expenditure (Boeris et al., 2009; Nalinanon et al., 2009; Raja et al., 2011). The two aqueous phases can be generated by mixing solutions of two different water-soluble polymers or a polymer and a salt solution. This system separates into two phases, both water-rich, when a critical concentration of polymer or salt is achieved. The limiting concentrations as described by Raja et al. (2011) depend on the type of

phase-forming components and on the pH, ionic strength and temperature of the solution. Polyethylene glycol (PEG) is often used as one of the phase-forming polymers because it forms a two-phase system with other neutral polymers as well as with salts and is available at low cost (Raja et al., 2011). PEG can also significantly enhance the refolding of proteins, resulting in recovery of their activity (Cleland et al., 1992). The choice of ATPs depends on the type of biomolecules and economic considerations; so far, PEG- salt systems have been used for the isolation of pepsin and pepsinogen (Klomklao et al, 2005; Nalinanon et al., 2009). Aqueous two-phase systems consisting of polymer/salt are generally preferred over polymer/polymer systems because of the high cost and high viscosity of the polymer/polymer systems (Raja et al., 2011). In addition, polymer/salt systems have larger differences in density, greater selectivity, and larger relative drop sizes (Cunha & Aznar, 2009; Raja et al., 2011).

The affinity of enzymes for the PEG-rich phase increases with increasing negative charge of the enzyme, whereas affinity for the salt-rich phase increases at pH values below the isoelectric point (Boeris et al., 2009; Gautam & Simon, 2006). Thus, at a pH of 7.0, pepsinogen, with an isoelectric point of approximately 4, as well as pepsin, transfer into the PEG-rich phase, whereas undesired proteins predominantly transfer into the salt phase. This is schematically shown in Figure 1.7.

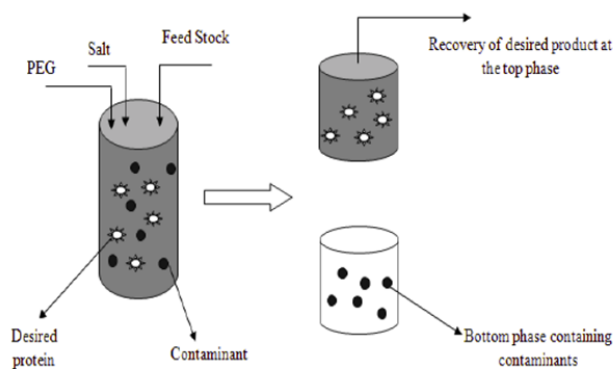


Figure 1.7: Model system of enzyme separation by the ATPs method (Raja et al., 2011)

The degree of purification and the separation of other proteins depend on various factors such as PEG concentration, PEG molecular weight, salt concentration and type of salt, as described by Lee and Lee (1981), Suppasith Klomklao et al. (2005) and Nalinanon et al. (2009). The following diagram explains the relationship between individual parameters (PEG concentration, PEG

molecular weight and salt concentration) and the phase separation (Figure 1.8) (modified vision of a figure from Raja et al. (2011)). This diagram also provides information about the ratio of phase volumes. The bimodal curve divides a region of component concentrations that will form one phase (below the curve) and those that will form two immiscible aqueous phases (above the curve) (Raja et al., 2011).

The following diagram shows that with increasing PEG molecular weight a lower concentration of salt and PEG are necessary for the destabilization of the thermodynamic system (Lee et al., 1981). High concentrations of salt also lead to a reduction in the volume of the upper phase (the PEG-rich phase), which Nalinanon et al. (2009) and Farruggia et al., (2004) ascribed to the rearrangement of water molecules around the PEG molecules. The formation of an aqueous layer around the cations creates a more compact molecular structure with minimal PEG volume (Farruggia et al., 2004; Nalinanon et al., 2009).

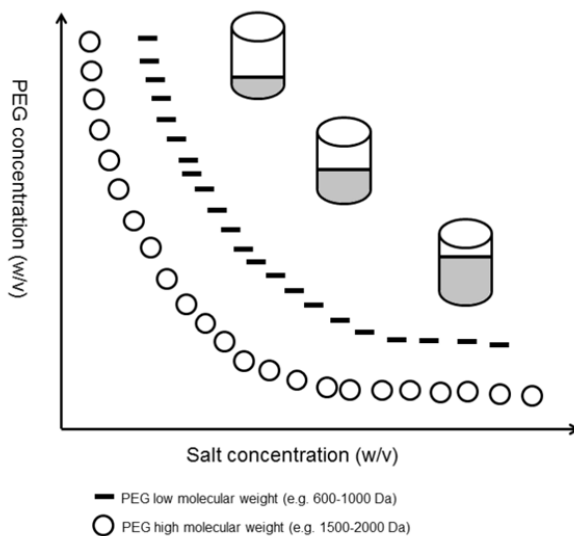


Figure 1.8: Influence of PEG concentration and salt concentration on phase separation

An increase in the salt content also led to a reduction in the enzyme yield. According to Nalinanon et al. (2009) and Antov et al., (2006), enzyme activity losses could be attributed to denaturation of the protease caused by the salting-out effect. This behaviour was also observed in the studies of Klomklao et al. (2005). In addition, the enzyme yield increased with decreasing PEG molecular weight. This may be related to the “excluded value effect”; with increasing molecular weight, there is a more compact PEG molecular structure, resulting in lessening of the

interaction of PEG with proteins (Arakawa & Timasheff, 1985; Spelzini et al., 2005; Yang et al., 2008). At high PEG concentrations, a reduction of this effect could be determined; Spelzini et al. (2005) attribute this to the penetration of PEG into the hydration shell of the protein, allowing interaction between the PEG and the protein's hydrophobic regions (Lee et al., 1981; Spelzini et al., 2005).

The formation of a complex between pepsinogen and PEG at a pH of 7.0 may be attributed to electrostatic interaction between the biomolecules and PEG (Raja et al., 2011).

The high binding affinity between pepsinogen and PEG often hampers subsequent separation by simple dialysis methods or added salts, as in the approach used by Teotia et al. (2004) for chitinase purification (Boeris et al., 2009; Rito-Palomares, 2004). Using polyelectrolyte precipitation with chitosan, Boeris et al. (2009) described a method whereby up to 40 % of the porcine pepsin could be separated from the polymer. An economical alternative with excellent chemical properties is the negatively charged polysaccharide pectin (Boeris et al., 2009; Gautam et al., 2006), which offers the advantage of a low precipitation pH at which PEG seems to be loosely associated with pepsin through hydrogen bonding of pepsin-COOH groups with the ether groups of PEG, as shown below (Kokufuta & Nishimura, 1991) (Figure 1.9).

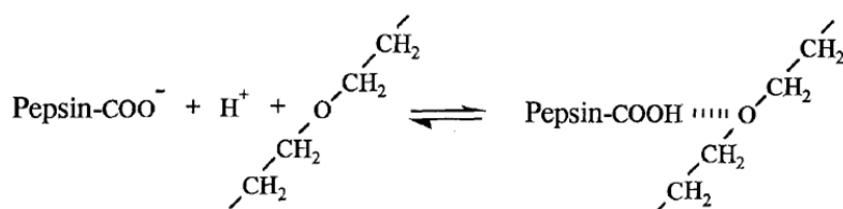


Figure 1.9: Complex formation between pepsin and PEG by the hydrogen bonding of COOH with the ether group in PEG (Kokufuta & Nishimura, 1991).

Pepsin exhibits high stability in acid environments. Xia et al. (1993) analysed complex formation between PEG and pepsin under acid conditions (pH 3.0). Pepsin seems to associate with PEG in three possible ways; in Figure 1.10, (a) shows the cooperative case, in which protein binds to protein and the number of proteins bound per polymer (n) is constant; (b) indicates anti-cooperative binding (n must decrease when PEG increases); and (c) illustrates the formation of an “interpolymer” complex in which multiple PEG molecules bind to one protein (Raja et al., 2011).

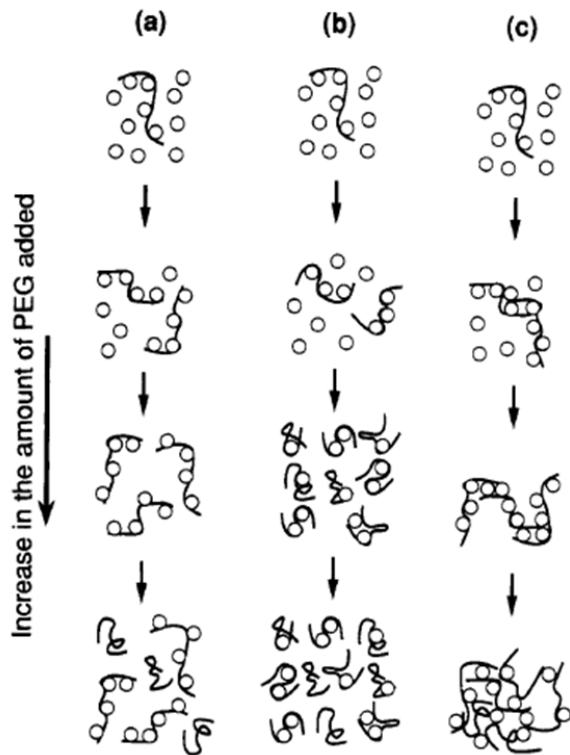


Figure 1.10: Three different types of PEG:pepsin complexes according to Xia et al. (1993)

Further separation of the enzyme-PEG complex by polyelectrolyte precipitation led to the formation of a soluble protein-polyelectrolyte complex from which insoluble macroaggregates were formed as a result of further interactions between macromolecules (Boeris et al., 2008). In previous studies, the water-insoluble complexes were detected by turbidimetric measurements (Boeris et al., 2009; Kokufuta et al., 1982; Kokufuta & Takahashi, 1990; Park et al., 1992).

Subsequent separation of the components, which are predominantly bound together by electrostatic forces, can be performed by the addition of salt (ca. 0.6 M) or a change in pH (Boeris et al., 2009).

The last step in isolating and purifying pepsin is the activation of pepsinogen to pepsin. Under acid conditions (pH 2-3) and at warm temperatures (25-36 °C), pepsinogen is autocatalytically converted to pepsin. For the first purification process (conventional method), this involves a separate purification step, whereas during the second purification process (aqueous two-phase extraction and polyelectrolyte precipitation), the acid environment during the precipitation may be sufficient. Depending on the type of pepsinogen, various activation times and different

methods of activation (direct pathway, sequential pathway) can be expected (Chapter 1.2.5.1) (Bougatef et al., 2008; Wu et al., 2009; Zhou et al., 2008).

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2. Purification and characterization of pepsinogen and pepsin from the stomach of rainbow trout (*Oncorhynchus mykiss*)

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2.1. Abstract

Three pepsinogen isolates (PG-I till III) from the stomach of rainbow trout (*Oncorhynchus mykiss*) were purified by using ammonium sulfate precipitation, ion exchange chromatography and two subsequent gel filtrations containing seven pepsinogen isoforms. SDS-PAGE revealed that pepsinogen isolate I contained two isoforms with molecular masses of 45 kDa (PG-I a) and 44 kDa (PG-I b) and the molecular masses of the PG isolates PG-II and PG-III were 42 kDa, respectively. P-I till P-III converted into the corresponding pepsins (P) at pH 2.0 with molecular masses of 37 kDa (P-I a, b, P-III) and 35 kDa (P-II). The isoelectric points were 4.0 (PG-I a, b), 5.9 (PG-II), and pepsinogen isolate III exhibited four isoforms with isoelectric points of 3.73 (PG-III a), 3.78 (PG-III b), 4.0 (PG-III c) and 4.15 (PG-III d). After conversion from pepsinogen, trout pepsins exhibited optimal activity at pH 3.0 and 40 °C (P-I) and pH 2.5 and 30 °C (P-II, P-III). The N-terminal amino acid sequences of the three pepsin isolates (P-I till III) were determined up to 20 amino acids. P-II showed 100 % identity (ID) to pepsin A of *Oncorhynchus keta*, but P-I and P-III revealed high similarity to chitinases (85 % ID). Catalytic constants K_M and k_{cat} for proteolysis of acid-denatured hemoglobin were determined as $2.8 \cdot 10^{-8}$ M (P-I), $1.3 \cdot 10^{-8}$ M (P-II) and $7.9 \cdot 10^{-9}$ M (P-III), and 5.43-19.1 S^{-1} , respectively.

2.2. Introduction

Pepsins (EC 3.4.23.1-2) are subdivided in pepsin A (EC 3.4.23.1), which preferably exhibited aromatic amino acids at P1 and P1' position and pepsin B (EC 3.4.23.2), both pepsins belonging to the class of aspartic endopeptidases (EC 3.4.23) responsible for digestion of proteins in the stomach of animals [1]. The precursor pepsinogens are synthesized by the gastric mucosa and secreted into the lumen as required. In the acid environment of the stomach, pepsinogens are autocatalytically converted to pepsins by removal of the N-terminal prosegment [2].

Pepsins from the stomach of pigs and calves (chymosins) have been used in the food industry for cheese production for decades [3], and fish pepsins may offer a future alternative as shown by Tavares et al. 1997 [4,5]; further technological applications of pepsins are the digestion of fish fillet to determine the number of nematodes [6], collagen extraction and gelatine preparation [7], fish processing and fish silage as reviewed by Zhao et al. [8].

Wild and farmed fish provide alternative sources of pepsin, which have hardly been used in the food industry up to the present time. In the course of fish processing, most of the guts are either wasted or used for fish meal production [9]. A prerequisite for a better utilization of fish pepsins is the study of their enzymatic properties.

In recent decades, pepsinogens and pepsins, respectively, have been isolated and characterized with regard to their physical and catalytical properties including enzyme stability from a number of marine and freshwater fish species [8], Atlantic cod, *Gadus morhua* [10], Antarctic rock cod, *Trematomus bernacchii* [11], albacore tuna, *Thunnus alalunga* [12] and smooth hound, *Mustelus mustelus* [2].

In contrast to that, only a small number of pepsinogens and pepsins from farmed fish have been isolated and characterized, including the following species: European eel, *Anguilla anguilla* [13], Japanese seabass, *Lateolabrax japonica* [14], giant catfish, *Pangasianodon gigas* [15], and mandarin fish, *Siniperca chuatsi* [16]. The continuous availability and the constant living conditions of farmed fish species are clear advantages for industrial use. Additionally, the treatment (feeding, feeding time, food quantities and living environment) of farmed fishes, which has a great influence on the intestinal digestive activity, can be controlled.

Vannabun et al. [15] and Liaset et al. [17] showed, that pepsin isolated from giant fish and Japanese sea bass exhibited good cleavage activity against animal proteins. Thus fish pepsin may offer a further alternative to microbial enzymes such as Protamex[®] and Alcalase[®] (both proteases from *Bacillus licheniformis*) and Flavourzyme[®] (protease from *Aspergillus oryzae*), which are predominately used for protein hydrolysis.

One of the commercially most important farmed fish species in the EU is rainbow trout (*Oncorhynchus mykiss*), with an annual production of more than 370 000 tons in 2013 [18]. The large quantity of processing waste – 222 000 tons (60 %) – from this species offers a valuable, continuously available source of pepsins and other proteases from the digestive tract of trout [19]. Apart from the work of Twining et al. [20] investigating pepsins and pepsinogens in rainbow trout without separation of isoforms, no further studies on rainbow trout pepsinogens and pepsins have been published.

The aim of the present study is the isolation and characterization of pepsin isoforms derived from the respective pepsinogens from the stomach of rainbow trout. The influence of different ammonium sulphate concentrations and the order of the chromatographic steps (anion-exchange chromatography and gel filtration) were systematically tested to develop a reliable process for the isolation of trout pepsin. Based on the results of this study, possible applications of the isolated enzymes are discussed.

2.3. Materials and methods

2.3.1. Sampling of tissue

Rainbow trout (weight: 250 g, age: 18 months) were provided by a fish farm located in Hessia (Germany). Two weeks before slaughtering the fishes, feeding had been stopped resulting in empty stomachs. After gutting the fishes, stomachs were collected and stored in polyethylene bags at -20 °C, until used for pepsinogen purification.

2.3.2. Reagents

Bovine serum albumin, hemoglobin from bovine blood, Pepstatin A, pepsin from porcine gastric mucosa (3,200-4,500 units/mg protein) (Sigma Aldrich, St. Louis, Missouri, USA); Bradford reagent for protein determination, sodium dihydrogenphosphate, di-sodium hydrogenphosphate (Merck, Darmstadt, Germany); Laemmli sample buffer, β -mercaptoethanol, Tris/glycine/SDS buffer, molecular weight marker (14,4- 97,4 kDa), mini-Protean TGX Precast gel 10 % (BioRad, München, Germany), ammonium sulfate (99,6 %) (Prolabo VWR, Leuven, Belgium); gel filtration calibration kit LMW (GE Healthcare, Buckinghamshire, UK); protease inhibitor panel (Carl Roth, Karlsruhe, Germany); ultrafiltration membrane (Macrosep 3K, Pall, Port Washington, USA); Servalyte Precote 3-10, pI marker 3-10 (SERVA, Heidelberg).

2.3.3. Purification of pepsinogens

Extraction and purification of pepsinogens was performed at 4-6 °C, except for Superdex G75 gel filtration chromatography, which was conducted at room temperature [13,2]. Five to six trout stomachs were rinsed with cold distilled water, cut into small pieces of 1 – 2 cm³ and homogenized for 1 min in 10 volumes of ice-cold 50 mM sodium phosphate buffer, pH 7.0, by means of the Ultra Turrax T 25 running at 20 500 rpm (IKA, Staufen, Germany). Over the whole purification process sodium phosphate buffer was used, because the enzyme was very stable under these solvent and a good separation could be observed [21,22,13]. The homogenate was centrifuged for 30 min at 15 000 g at 4 °C, and the supernatant was collected as raw extract.

Isolation of pepsinogens was carried out based on the method of Bougateg et al. [2] with slightly modifications. The isolation of trout pepsinogen comprised four steps: ammonium sulfate precipitation, anion exchange chromatography, and two subsequent gel filtration chromatography steps. Ammonium sulfate was added to the raw extract to reach 30 % saturation; after stirring the

solution for 1 h at 6 °C, the precipitated proteins were collected by centrifugation for 15 min at 10 000 g at 4 °C. Ammonium sulfate was added to the supernatant to reach 50 % saturation, followed by incubation and centrifugation as described. The precipitate was dissolved in 30 ml of 20 mM sodium phosphate buffer pH 7.0, and dialyzed for 20 h against 3 L of buffer. The dialysate was given onto the weak anion exchange column, packed with DEAE sepharose (1.6 x 10 cm, GE healthcare) previously equilibrated with phosphate buffer. Unbound proteins were removed by washing with buffer until the absorbance at 280 nm reached the baseline. A linear gradient of 0 – 1.0 M buffered NaCl was applied to elute bound protein at a flow rate of 3.5 ml/min, and fractions were tested for pepsin activity. Fractions with enzyme activity eluted in three peaks, which were separately pooled and dialyzed against 20 mM sodium phosphate buffer pH 7.0 for 20 hours. Dialysates were concentrated by ultrafiltration using Macrosep Advance Centrifugal Device. The concentrated pepsinogen isolate I to III were applied to a gel filtration column (HiLoad 16/600, Superdex G75 (GE Healthcare) equilibrated with 20 mM sodium phosphate pH 7.0 in 0.15 M NaCl and eluted at a flow rate of 1 ml/min. Fractions of 5 ml were collected and those with proteolytic activity pooled and concentrated by ultrafiltration as described above. Gel filtration of each of the concentrated fractions was repeated using a flow rate of 1.5 ml/min. Fractions of 2.5 ml were collected, tested for pepsin activity, pooled and concentrated by ultrafiltration as described. The resulting pepsinogen solutions were used for biochemical analysis and pepsin isolation.

2.3.4. Activation of pepsinogen

Pepsinogen preparations I-III were converted to pepsin according to Nalinanon et al. [12]. Pepsins I-III were subjected to gel filtration as described above, and used for enzymatic and biochemical studies.

2.3.5. Determination of protein concentration

Protein concentration was determined by measuring the absorbance at 280 nm or by the Method of Bradford [23] using the protein kit HC309760 from Merck; bovine serum albumin served as protein standard.

2.3.6. Pepsin assay

Pepsin activity was measured by the method of Xu et al. [24] with a slight modification using acid denatured hemoglobin as substrate. The extents of hydrolysis of acid denatured hemoglobin were determined by measuring the concentration of the products that are not precipitated by trichloroacetic acid.

Pepsinogen was rapidly converted to pepsin under the conditions of the assay (pH 2.0, 37°C) [25]. Bovine hemoglobin (0.5 g) was dissolved under constant stirring in distilled water (20 ml) and dialyzed for 24 hours (3 x 2 L). The dialysate was acidified by adding a quarter of volume of 0.3 M HCl, clarified by centrifugation at 6 000 g for 15 min at 6 °C and used as substrate solution.

Substrate solution (200 µl) was heated to 37 °C, mixed with 40 µl of enzyme solution and incubated for 10 min at 37 °C. The reaction was terminated by addition of 1 ml of 4 % (w/v) trichloroacetic acid (TCA). After centrifugation (10 000 g, 15 min) the products, which are soluble in trichloroacetic acid solution were measurement at a wavelength of 280 nm against a blank containing 20 mM sodium phosphate buffer pH 7.0 instead of enzyme solution. One unit of pepsin activity was defined as an absorbance increase of 1.0 at 280 nm within 1 min of incubation. All enzyme assays were run in triplicate.

2.3.7. Time course of pepsinogen activation

To visualize the conversion process of pepsinogen to pepsin the method of Nalinanon et al. [12] with slight modifications were used. 400 µl of pepsinogen solution (0.3 mg/ml) were adjusted to pH 2.0 with 1M HCl and incubated at room temperature for different time intervals. The reaction was stopped by addition of 40 µl Laemmli sample buffer to 40µl enzyme solution; the mixture was neutralized with 0.3 M NaOH, centrifuged at 10 000 g for 5 min and analyzed by SDS-PAGE.

2.3.8. Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE with 10 % (acrylamide concentration) separating gel was performed according to Laemmli [26] for molecular weight determination, evaluation of the purity of enzyme preparations and to follow the conversion of pepsinogen to pepsin. Proteins were visualized by staining with Coomassie Brilliant Blue R-250, the molecular weights of sample proteins were

estimated by using the low molecular weight marker from Bio-Rad. Gel filtration with Hiload 16/600 Superdex G75 column was performed as described above to compare the molecular weight of native and SDS-denatured pepsinogens and pepsins.

2.3.9. Isoelectric focusing (IEF)

The isoelectric point (pI) of pepsinogens and pepsins was determined by IEF using Servalyt Precotes 3-10 according to Altinelataman et al. [27]; proteins were visualized by silver staining [28].

2.3.10. Effect of pH and temperature on pepsin activity

The pH dependence of pepsin activity was studied by adjusting the pH of acid denatured hemoglobin solution to pH 1 – 6 with 1M HCl or 1 M NaOH. To investigate the effect of pH on the activity of pepsin, pepsinogen were first converted to pepsin as described (1.7). Then 40 μ l of pepsin solution (0.1 mg protein/ml) were added to 200 μ l of substrate solution. Thereafter the pH of the solution was checked and adjusted to the required value. The assay was run at 37 °C under gentle mixing (300 rpm, HLC block thermostat MRK 13) and performed as described above.

The effect of temperature on pepsin activity was determined by varying the assay temperature from 20 to 80 °C; reaction time was 20 min [9]. Percentage of enzyme activity was estimated considering 100 % the highest activity detected in the assay.

2.3.11. Effect of inhibitors on proteinase activity

Different kinds of proteinase inhibitors were mixed with pepsin solution (final concentration 0.01 μ M) to obtain the assay concentrations of 50 μ M EDTA, 1 μ M E-64, 1 μ M leupeptin, 50 μ M AEBSF and 0.15 μ M aprotinin. The inhibitory effect of pepstatin A was investigated in detail with concentrations ranging from 0.1 nM to 1 μ M of pepstatin A; 200 μ l of enzyme solution (0.01 μ M) were mixed with the same volume of pepstatin A solution and incubated for 15 min at room temperature [14,22]. Thereafter pepsin activity was measured as described above (chapter 2.3.6).

2.3.12. Enzyme kinetic studies

Kinetic parameters were determined by measuring the activity of purified pepsin isolates at different concentrations (0.1 to 30 mg hemoglobin/ml substrate solution) of acid-denatured hemoglobin [14,9]. Assay temperature was 37 °C, reaction time 20 min and enzyme

concentration 0.1 mg protein/ml enzyme solution (0.01 μM), determined by measuring the absorbance at 280 nm with bovine serum albumin as standard. The determinations were repeated thrice and the respective kinetic parameters including K_M (M) and V_{Max} were evaluated by plotting the data on a Lineweaver Burk double-reciprocal graph ($1/V$ versus $1/S$) [29]. Values of turnover number (k_{cat} (s^{-1})) and catalytic efficiency were calculated from the following euqilibration: $k_{\text{cat}} = V_{\text{Max}} / c_{\text{enzyme}}$ and $k_{\text{eff}} = k_{\text{cat}} / K_M$

where c_{enzyme} is the active enzyme concentration and V_{max} in the maximal velocity. The kinetic data were calculated by the software GraphPad Prism (version 5).

2.3.13. Amino acid sequence determination

Isolated pepsin fractions were separated from potentially contaminating proteins by SDS-PAGE, and transferred onto polyvinylidenedifluoride (PVDF) membrane by tank blotting. The N-terminal amino acid sequence of the proteins eluted from the membrane was analyzed by Edman [30] degradation using the Precise Sequencer Model 492A (Applied Biosystems, Foster City, USA).

2.3.14. Statistical analysis

Data regarding the yield, purification factor and specific activity of the isolated enzyme are presented as mean value \pm standard deviation of triplicate samples. All data were submitted to Analysis of Variance (ANOVA) and mean comparisons were evaluated by Bonferroni test. The data were analyzed using the software GraphPad prism (version 5). Differences were considered significant at $p < 0.05$.

2.4. Results and discussion

2.4.1. Pepsinogen and pepsin purification

From the stomach of rainbow trout, three pepsinogen isolates were purified. Testing several combinations of the purification procedure, the highest purification factor as well as the highest enzyme yield was achieved by ammonium sulfate precipitation, anion exchange chromatography and two subsequent gel filtrations (Table 2.1).

Table 2.1 Several combinations of the purification procedure for the isolation of trout pepsinogen

Purification steps						Purification factor	Enzyme yield
Ammonium sulfate saturation	Chromatography order						
1 50-60 %	Gel filtration	Anion-exchange	Gel filtration			++	++
2 50-60%	Gel filtration	Anion-exchange				+	++
3 50-60 %	Anion-exchange	Gel filtration				+	+++
4 50-60%	Anion-exchange	Gel filtration	Anion-exchange	Anion-exchange		-	-
5 50-60 %	Anion-exchange	Gel filtration	Gel filtration			+++	+++
6 50-60 %	Gel filtration	Anion-exchange	Gel filtration	Anion-exchange		-	-

- no enzyme activity, + low purification factor/enzyme yield, ++ middle purification/enzyme yield, +++ high purification factor/enzyme yield. 1- Klomklao et al. (2007), 2- Bougatef et al. (2008), 3- Cao et al. (2011), 4-Tanji, Kageyama, and Takahashi (1988), 5- similar to Nalinanon et al. (2010) with the exception that ammonium sulfate precipitation was used as the first purification step instead of gel filtration

Different combinations of ammonium sulfate concentrations for the precipitation steps were testing and optimal pepsinogen enrichment (1.8-fold, recovery of enzyme activity 77 %) was achieved for a combination of 30 % and 50 % saturation (supplementary Figure 2.1). Thus, a lower ammonium sulfate concentration (50 %) is sufficient for precipitation of pepsinogens from rainbow trout, compared to mandarin fish pepsinogen (60 % ammonium sulfate saturation, 1.5-fold purification) [16] and smooth hound (70 %, 3.71-fold) [2], corroborating the finding of Twining et al. [20]. The crude extract was then subjected to ion exchange chromatography using

DEAE cellulose. Anion exchange chromatography was used to separate different pepsinogen and pepsin isoforms and to remove containing proteins [12,9,22].

The elution profile obtained by anion exchange chromatography (Figure 2.1) showed three peaks of protease activity to be assigned to pepsinogen isolate I till III (PG). PG-I eluted in the fraction of unbound proteins, whereas PG-II and PG-III were eluted at 0.39 M respectively 0.67 M NaCl; similar results were reported for the pepsinogens from the european eel, *Anguilla anguilla* [13]. The fractions from each peak were separately pooled, dialyzed, concentrated and subjected to gel filtration using a HiLoad 16/600, Superdex G75 column. This procedure was repeated to improve the purity of the pepsinogens (Table 2.2).

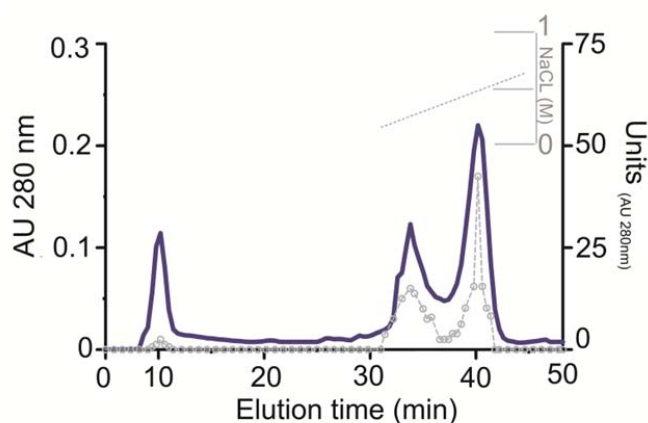


Figure. 2.1: Anion exchange chromatography of trout pepsinogen.

DEAE- cellulose chromatography to separate different pepsinogen isoforms, hemoglobin hydrolyzing activity (grey line), absorbance at 280 nm (black line)

The results from the purification procedure of rainbow trout PG-I to III are summarized in Table 2.2. After the purification process 33 mg pepsin were obtained from 100 g stomach. Similar results were obtained in the investigations of Zhou et al. [22], Zhou et al. [16] and Tanji et al. [21] for mandarin fish pepsin, sea bream pepsin and African coelacanth pepsin with 36.6 mg, 35.5 mg and 37.4 mg, respectively. But also lower amounts for pepsin were found in the stomach of arctic capelin (14.4 mg) [31] and European eel (3.63 mg) [13].

Table 2.2: Summary of purification of pepsinogens from trout stomach

Purification step	Total protein (mg)	activity (units AU _{280nm})	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Crude extract	954	366	0.38 ± 1.8	1	100
Ammonium sulfate precipitation	412	280	0.68	1.8 ± 1.3	77 ± 3.5
Anion-exchange chromatography	34	54	1.6	4.2 ± 1.6	15 ± 1.4
PG-I	4.6	1.1	0.24	0.6 ± 3.3	0.3 ± 5.0
PG-II	39	102	2.6	6.8 ± 1.4	28 ± 3.4
PG-III	22	62	2.8	7.4 ± 1.3	17 ± 0.6
Gel filtration chromatography	8	34	4.3	4.3 ± 1.3	9 ± 1.5
PG-I	0.6	0.9	1.5	4 ± 2.0	0.3 ± 3.1
PG-II	5.71	87	15	40 ± 1.7	24 ± 1.2
PG-III	0.9	18	20	53 ± 0.9	5 ± 0.7
Gel filtration chromatography	2.6	17	6.5	17 ± 1.8	5 ± 1.3
PG-I	0.29	0.8	3.1	8 ± 3.1	0.2 ± 2.9
PG-II	1.88	42	22	58 ± 1.3	6 ± 1.2
PG-III	0.34	13	38	100 ± 4.2	4 ± 3.1

*One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. Pepsin activity was assayed at pH 2.0, 37 °C for 10 min using acid hemoglobin as a substrate. Means ± S.D. (%) from triplicate determinations. Enzyme yield was calculated as a relative percentage of the enzyme activity of fraction obtained by each purification step to the enzyme activity of crude extract. The specific activity, purification factor and the enzyme recovery was calculated as follow:

$$\text{Specific activity} = \frac{\text{activity (Units)}}{\text{total protein (mg)}}; \text{purification factor} = \frac{\text{specific activity}_{\text{sample}}}{\text{specific activity}_{\text{crude extract}}}; \text{recovery (\%)} = \frac{\text{activity}_{\text{sample}}}{\text{activity}_{\text{crude extract}}} * 100$$

Comparison of our results with those for other fish species demonstrates that the degrees of recovery and enrichment are within the same range [13,14,24]. The homogeneity of purified pepsinogen was checked by SDS Page under reducing conditions [22]. As shown in Figure 2.2 all pepsinogen fractions gave single or double bands on SDS-PAGE, which indicates that the extract did not exhibit heavy contamination by other proteins.

2.4.2. Pepsinogen and pepsin characterization

2.4.2.1. Molecular weight

The molecular weights of PG-I till III as determined by SDS page is shown in Figure 2.2. Under reduced conditions (SDS-PAGE); due to the content of anionic detergent sodium dodecyl sulfate (SDS) and the reducing agent β -mercaptoethanol (BME) the separation was only influenced by the molecular size. As shown in Table 2.3 comparable molecular weights could be achieved under native (gel filtration) and reduced conditions. Under reduced conditions PG-I till PG-III indicated molecular weights of 45 kDa (PG- I a), 44 kDa (PG-I b), 42 (PG- II) and 42 kDa (PG- III), whereas those of the corresponding pepsins were 37 (PG-I a,b), 35 (PG-II) and 37 kDa (PG- III).

Table 2.3: Molecular weight of pepsinogen and pepsin

Pepsinogen	native conditions	reduced conditions
PG-I a, b	44 kDa, 40 kDa	45 kDa, 44 kDa
PG-II	45 kDa	42 kDa
PG-III	44 kDa	42 kDa
Pepsin	native conditions	reduced conditions
P-I	35 kDa	37 kDa
P-II	38 kDa	35 kDa
P-III	37 kDa	37 kDa

Pepsinogen isolate I contained two pepsinogen isoforms, PG-I a and PG-I b. After the purification process as shown in Figure 2.2, especially PG-I exhibited a noticeable amount of P-I. Under the purification conditions (pH > 4.0), PG-I may be converted to P-I during the bimolecular activation process as described by Marciniszyn, Huang, Hartsuck, and Tang (1976). During the purification process activated enzyme may denatured as a consequence of the high pH value (7.0), which may explain the low enzyme yield of 0.2 % for P-I (Table 2.2).

The molecular weights of trout pepsins were similar to those from albacore tuna, *Thunnus alalunga*, [12], the African coelacanth, *Latimeria chalumnae*, [21] and smooth hound, *Mustelus*

mustelus, [2]. The pepsins isolated from japanese seabass, *Lateolabrax japonicas* [14], mandarin fish, *Siniperca chuatsi* [16] and keta salmon, *Oncorhynchus keta*, [33] had slightly lower values ranging from 32 to 36 kDa.

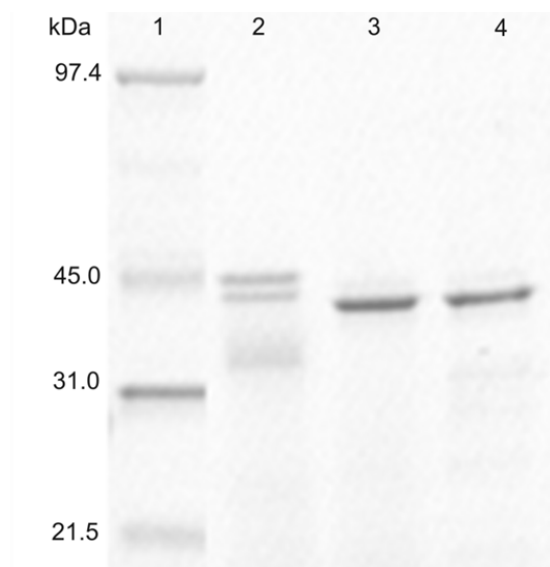


Figure 2.2: SDS Page of purified trout pepsinogens (PGs)

Lane 1, molecular marker; *lane 2*, PG-I; *lane 3*, PG-II; *lane 4*: PG-III, the gel was stained with Coomassie brilliant blue

2.4.2.2. Pepsin formation

The direct conversion of pepsinogen to pepsin under the acidic conditions applied occurred very rapidly for PG-II, but took 1 hour until completion for PG-I (Figure 2.3). No intermediate product could be observed during conversion of PG-I and II into the active enzymes. In contrast, PG-III was activated by a two-step mechanism with the formation of a very stable 39 kDa intermediate. This conforms to a former study of PG-III from *Latimeria chalumnae* which was shown to be converted into an intermediate product being stable for at least 10 h without further conversion into pepsin [21]. In contrast, rapid conversion of pepsinogens to pepsins, either by the one- or two-step mechanism, has been reported for most of the fish species studied [2,16,13].

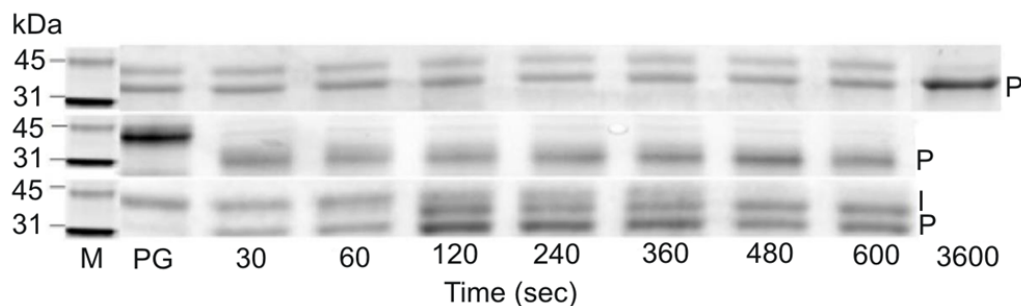


Figure 2.3: Time courses of the activation of trout pepsinogens (PGs) as analyzed by SDS-PAGE

The activation was carried out at pH 2.0 at 25 °C, reaction was stopped at appropriate time intervals as indicated on each lane and subjected to SDS-PAGE, *upper row* PG-I; *middle row* PG-II; *bottom row* PG-III; M: molecular marker; PG: pepsinogen; I: intermediate form; P: pepsin, the gel was stained with Coomassie brilliant blue

2.4.2.3. Isoelectric point (pI)

The pI values of pepsinogen isolates were determined by IEF in polyacrylamide gels (Figure 2.4). The three pepsinogen isolates showed a common strong band at pH 5.2-5.3 and pepsinogen isolate I and III an additional band at pH 3.5, which were a staining artifact. For PG-I a strong band was visible at pH 4.0, for PG-II at pH 5.9, while PG-III contained four pepsinogen isoforms with protein bands at pH 3.73 (PG-III a), 3.78 (PG-III b), 4.0 (PG-III c) and 4.15 (PG-III d). Low pI values were also found for pepsinogen isoforms (I,II a, II b) from orange roughy, *Hoplostethus atlanticus* (5.3, 4.35, 4.4) [24], polar cod, *Boreogadus saida* (3.75 pepsinogen A, 4.75 pepsinogen B) [34], Japanese seabass, *Lateolabrax japonica* (5.3, 5.1, 4.7) [14] and Pacific bluefin tuna, *Thunnus orientalis* (6.06, 4.33, 4.31) [35]..

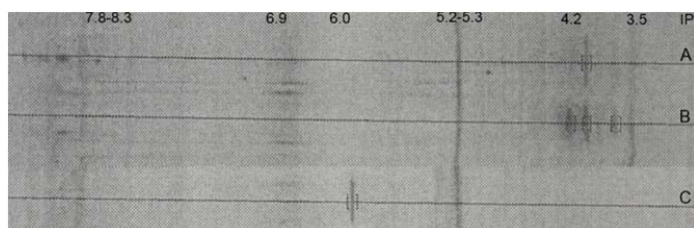


Figure 2.4: Isoelectric points of pepsinogen isolates I-III.

Upper row (1) PG-I; middle row (B) PG-III, bottom row (C) PG-II; analyzed by native IEF with Servalyt Precote 3-6, the gel was stained with Coomassie brilliant blue. The band at pH 5.2 is a staining artefact.

2.4.2.4. pH- and temperature-dependent activity

The activity requirements of trout P-I till III were compared to those of porcine pepsin, using acid denatured hemoglobin as substrate (Figure 2.5 and 2.6).

Trout pepsins exhibited the highest enzyme activity at pH 3.0 (P-I) and 2.5 (P-II and P-III); P-I showed a broad optimum in the pH range from 1.5 to 4.0, while the optima for P-II and III were restricted to the pH range from 2.0 to 3.0. At pH 4.0, P-I had 94 % optimal activity, P-II 72 % and P-III 65 %.

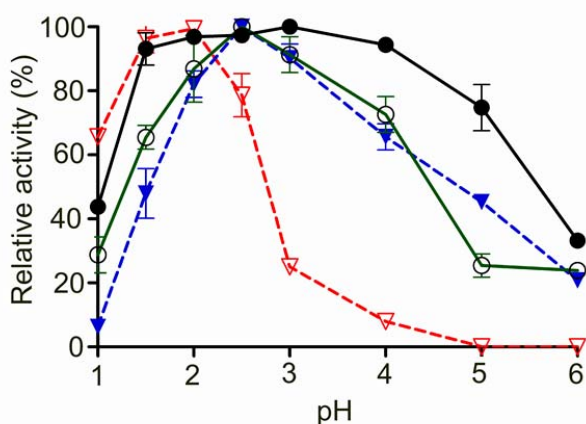


Figure 2.5: Optimal pH of trout pepsins.

Effect of pH on the activities for hemoglobin hydrolysis by purified trout pepsin and porcine pepsin A, the pH-activity curves are shown taking the maximal activity as 100 % for each case, bars represent the standard deviation (n=3), P-I (black circle), P-II (blue triangle), P-III (green circle), porcine pepsin (red triangle).

At pHs higher and lower than the optimal pH the activity of pepsin generally decreased, due to the conformational changes of enzyme under harsh condition [12].

All three isolates showed residual activity of about 20 % at pH 6.0 (Figure 2.5). The pH dependency of trout pepsins resembled the pH profiles reported for pepsins from other fish species such as North Pacific bluefin tuna [36], sea bream [22], pectoral rattail [9], mandarin fish [16] and European eel [13], but lower pH optima have been reported for some fish species polar cod [34], African coelacanth [21], smooth hound [2] and albacore tuna [12]. In their previous study of rainbow trout pepsin, Twining et al. [20] found a pH optimum of 3. In the present study, porcine pepsin behaved differently to trout pepsin; the pH optimum was at 2.0, and at pH 5.0 activity was lost completely.

The optimal pH values of the pepsin isolates (2.5-3.0) were in the same range compared to the pH value of the stomach of rainbow trout (2.5-3.0) [37].

The optimal temperature for P-I was 40 °C, while the activities of P-II and III were highest at 30 °C (Figure 2.6). The optimal temperature were similar to those from smooth hound (40 °C) [2], polar cod (37 °C) [34] and European eel [13] but higher optimal temperatures were reported for pepsins from sea bream (45 °C for P-I) [22], pectoral rattail (45 °C) [9] and albacore tuna [12]. Gildberg [38] reported that the differences in optimal temperature might be associated with the differences in enzyme conformation as intended by habitat, environment and genetics.

In the range of 20 to 30 or 40 °C the activity of trout pepsins increased gradually. Thereafter, activities of P-II and III decreased sharply. In contrast, P-I inactivation occurred between 50 °C and 60 °C, very likely due to thermal denaturation. In comparison, porcine pepsin exhibited optimal activity at 50 to 60 °C, but a relatively low activity at between 20 °C - 30 °C as shown in Figure 2.6.

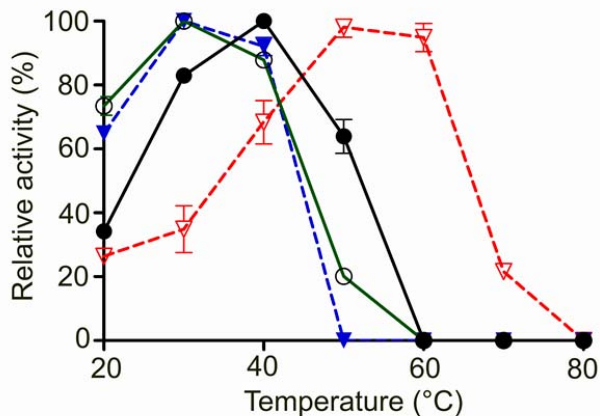


Figure 2.6: Optimal temperature of trout pepsins.

Effects of temperature on the activities for hemoglobin hydrolysis by purified trout pepsin and porcine pepsin A, the temperature activity curves are shown taking the maximal activity as 100 % for each case, bars represent the standard deviation (n=3), P-I (black circle), P-II (blue triangle), P-III (green circle), porcine pepsin (red triangle).

The optimal temperatures determined with hemoglobin were much higher than the living conditions of rainbow trout (10-18°C) [39,37]. These values are only useful to compare the results with other fish species or mammalian one, but they do not represent the natural conditions

as also shown in the study of Brier et al. [40] for pepsins A1 and A2 from the Antarctic rock cod *Trematomus bernacchii*.

2.4.2.5. Enzyme inhibitors

The effect of a number of protease inhibitors was investigated to elucidate the type of protease under study. All three pepsin isolates were inhibited only by pepstatin A, but not by EDTA, E-64, leupeptin, AEBSF or aprotinin. indicating that the isolated trout pepsins belong to the class of aspartic proteases [12]. This means that pepsin exhibited an aspartic acid residue in the active side and displayed maximum activity at acid pH. The high activity at acid pH has the advantage, that during the hydrolysis processes of food proteins the bacterial contamination is reduced [41].

Sensitivity to pepstatin A was quite different for the three pepsin isolates. Like porcine pepsin, P-I was completely inhibited with an equimolar ratio of pepstatin A. In contrast to this, a 10-fold molar excess of pepstatin A was necessary for nearly total inhibition of P-II and III (Figure 2.7). The results obtained for trout pepsins are in accordance with reports for other fish pepsins being completely inhibited by 0.05 μM to 20 μM of pepstatin A (up to 17-fold molar excess) [36,2,16,14].

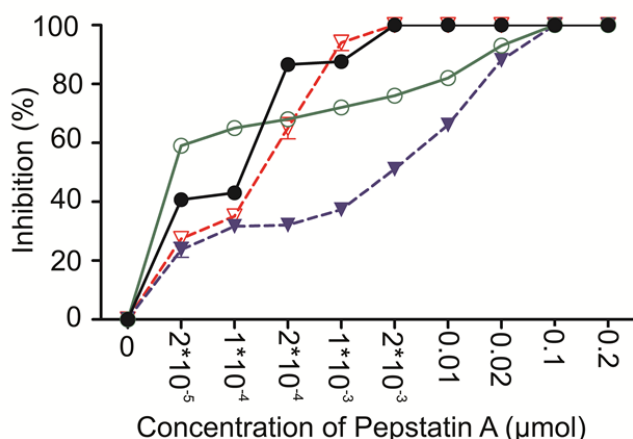


Figure 2.7: Pepstatin A inhibition of pepsin.

Pepsin was assayed in the presence of increasing concentration (x-axis) of pepstatin A using 2% acid hemoglobin as substrate, bars represent the standard deviation (n=3), P-I (black circle), P-II (blue triangle), P-III (green circle), porcine pepsin (red triangle).

2.4.2.6. Enzyme kinetic studies

Catalytic constants K_M and k_{cat} for proteolysis of acid-denatured hemoglobin were determined for each of the three pepsin isolates to evaluate their catalytic efficiency [29,9]. K_M and k_{cat} values of P-I till III were 1.3×10^{-8} - 7.9×10^{-9} M and 5.43 - 19.1 S^{-1} , respectively (Table 2.4). The K_M value of P-III was lower than that of P-II and P-I, suggesting that P-III has a higher affinity to hemoglobin. The K_M values of the three pepsins were similar to those from Sea bream [22], Pectoral rattail [9] and Orange roughy [24], but higher K_M values were reported for pepsin from European eel [13], Japanese seabass [14] and pig [10] (Table 2.4).

Of all the isolated trout pepsins, P-III showed the highest turnover number (k_{cat}) and the highest catalytic efficiency, suggesting that P-III works more efficiently than the other two pepsins. In addition, the catalytic efficiencies of trout P-I and II were 4–12 fold higher than those of pectoral rattail pepsin A [9].

Table 2.4: Kinetic properties of trout pepsins for the hydrolysis of hemoglobin

Fish species	Pepsin fraction	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot S^{-1}$)
<i>Oncorhynchus mykiss</i>	I	2.8×10^{-8}	5.43	1.9×10^8
	II	1.3×10^{-8}	8.37	6.4×10^8
	III	7.9×10^{-9}	19.1	2.4×10^9
<i>Anguilla anguilla</i> ^a	I	8.8×10^{-5}	23.7	2.7×10^5
	II	9.2×10^{-5}	19.4	2.1×10^5
	III	7.0×10^{-5}	34.4	4.9×10^5
<i>Hoplostethus atlanticus</i> ^b	I	1.24×10^{-8}		
	II	5.17×10^{-8}		
<i>Lateolabrax japonicas</i> ^c	I	5.5×10^{-5}	7.34	1.3×10^5
	II	1.2×10^{-5}	4.02	5.9×10^4
	III	3.4×10^{-5}	3.09	8.1×10^4
<i>Sparus latus Houttuyn</i> ^d	I	8.7×10^{-8}	ND	ND
	II	1.0×10^{-7}	ND	ND
	III	8.6×10^{-8}	ND	ND
	IV	7.3×10^{-8}	ND	ND
Porcine Pepsin ^e		2.1×10^{-7}	44	2.0×10^8

The kinetic parameters of trout pepsins were compared with these of *Anguilla Anguilla* [13]^a, *Hoplostethus atlanticus* [24]^b, *Lateolabrax japonicas* [14]^c, *Sparus latus Houttuyn* [22]^d and Porcine Pepsin [10]^e. Reaction was carried out at 37°C using acid denatured hemoglobin as substrate. ND, not determined

2.4.2.7. N-terminal amino acid sequence

The sequence of up to 20 amino acids at the N-terminal end of pepsin preparations I-III was determined by Edman [30] degradation. The sequences are compiled in Table 2.5 and compared to pepsin amino acid sequences from other fish species [36,42,21,14]. The sequences of trout P-I and III showed high similarity to each other, but not to those from other fish species. Protein BLAST revealed high similarity to chitinases for both sequences (P-I: 85 % ID to GenBank accession no CDQ98810.1; P-III: 85 % ID to GenBank accession no BAL40979.1), whereas P-II showed 100 % ID to pepsin A of *Oncorhynchus keta* (GenBank accession no BAO24099.1).

Table 2.5: N-terminal amino acid composition of trout pepsins

Fish species/ Fraction	N-terminal amino acid sequence																							
<i>Oncorhynchus mykiss</i>	P-I	Y	I	L	S	H	Y	/	T	N	W	G	Q	Y	/	P	G	A	G	K	Y			
	P-II					G	D	E	A	M	T	N	D	A	D	L	S	Y	Y	G	V	I	S	I
	P-III	Y	I	L	S	H	Y	F	T	N	W	A	Q	Y	/	P	G	A	A	K	Y			
<i>Oncorhynchus keta</i> ^a	P-A	F	Y	Q	T	G	D	E	A	M	T	N	D	A	D	L	S	Y	Y	G	V	I	S	I
Chitinases ^b (<i>Oncorhynchus mykiss</i>)		Y	I	L	S	C	Y	F	T	N	W	G	Q	Y	R	P	G	A	G	K	Y			
Chitinases ^c (<i>Scomber japonicus</i>)		Y	I	L	S	C	Y	F	T	N	W	A	Q	Y	R	P	G	A	G	K	Y			
<i>Lateolabrax japonica</i> ^d	P-I			A	V	G	D	E	S	M	T	N	D	A	D	L	A	Y	Y	G	V	I		
	P-II	Y	I	N	N	Y	A	D	T	T	Y	F	G	A	I	S	X	G	T					
	P-III		I	Q	S	G	T	E	P	M	T	N	D	A	D	L	D	Y	Y	G	V	V		
<i>Thunnus orientalis</i> ^e	P-I	F	A	/	V	A	G	E	P	M	T	N	D	A	D	L	A	Y	Y	G				
	P-II		Y	Q	D	G	T	E	P	M	T	N	D	A	D	L	S	Y	Y	G	V	V	S	I
	P-III		A	T	A	N	Y	M	Y	I	N	Q	Y	A	D	T	I	Y	Y	G	A	I	S	I
Porcine pepsin ^f	B			A	V	A	Y	E	P	F	T	N	Y	L	D	S	F	Y	F	G	E	I	S	I

Comparison of N-terminal amino acid sequence of purified pepsinogens from trout with other enzymes: Pepsinogen from *Oncorhynchus keta* (Protein Blast dbj. BAO24099.1)^a, *Lateolabrax japonica* [14]^d, *Thunnus orientalis* [36]^e, porcine pepsin B [42]^f and chitinases from *Oncorhynchus mykiss* (Protein Blast BALCDQ98810.1)^b and *Scomber japonicus* (Protein Blast BAL40979.1)^c. Amino acid residues equal to Pepsins from *Oncorhynchus mykiss* are marked with a blue background for P-I and P-III and marked with a grey background for P-II.

2.5. Conclusion

Three pepsinogen isolates from the stomach of rainbow trout were purified containing seven pepsinogen isoforms (P-I a, b, PG-II, PG-III a,b,c,d). The enzyme kinetic properties, the pH- and temperature-dependent activity, their binding properties to the ion-exchanger column DEAE-Sephacel and their sensitivity to pepstatin A are quite similar within the pepsinogen isolates. Minor differences in the molecular mass (PG-I a, PG-I b) and isoelectric points (PG-III a,b,c) were identified.

The biochemical and kinetic properties of the pepsinogen and pepsin isolates are similar to those of pepsinogens and pepsins from other fish species. Trout pepsins are active at low (30-40 °C) temperature and have a higher catalytic efficiency than porcine pepsin. Based on these findings, fish's own pepsin, which is currently not subject to industrial use, could be used as a cost-effective enzyme in hydrolysis processes (1) to produce functional peptides and (2) to reduce the anti-nutritional factor of plant proteins (publications are in preparation). Additionally, trout pepsins could also be used as a biotechnological alternative for food processing when low temperature and acidic pH values are required as in milk clotting, collagen extraction and gelatine preparation. Due to the large quantity (370 000 tons in 2013) of trout farmed and harvested in the EU, continuous production of trout pepsins may be achieved. This is a prerequisite for the use of trout pepsin in the food industry and other fields.

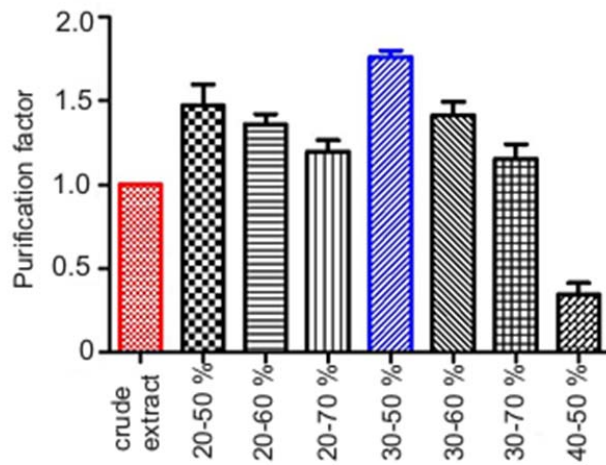
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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

2.6. Supplementary



Supplementary Figure 2-1: Ammonium sulfate saturation

Different combinations of ammonium sulfate concentrations for the precipitation steps for achieve optimal pepsinogen enrichment. X-axis ammonium sulfate concentrations, y-axis purification factor

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3. Isolation of trout pepsinogen by a combination of aqueous two-phase extraction and polyelectrolyte precipitation with pectin

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3.1. Abstract

Trout pepsinogen was isolated from the stomach of rainbow trout by a combination of aqueous two-phase extraction and polyelectrolyte precipitation. The acid environment during the pectin precipitation leads to a partly conversion of pepsinogen into the active enzyme pepsin.

Pectin (anionic natural polyelectrolyte) induced pepsinogen precipitation as an insoluble complex and facilitated an enzymogen yield of approximately 25 % with a 2 % polyelectrolyte solution. Electrophoretic study revealed that the purified enzyme had a major protein band with a molecular weight (MW) of 45 kDa and an additional band at 37 kDa for pepsin. Pepsin was inhibited by pepstatin A with a 10-fold molar excess, but not by other protease inhibitors. Trout pepsin exhibited high cleavage activity on animal proteins and therefore provides an alternative to microbial enzymes or plant enzymes for proteolysis. This fast and cost-effective purification method increases the potential for using fish by-products as an enzyme source.

Keywords: Aqueous two-phase system, pectin precipitation, polyelectrolyte, trout pepsin, digestion of plant and animal proteins

Chemical compounds studied in this article:

Polyethylene glycol (PubChem CID: 174 (Ethylene Glycol)), pectin (PubChem CID: 441476), chitosan (PubChem CID: 71853 (Deacetylchitin)), pepstatin A (PubChem CID: 5478883),

3.2. Introduction

In traditional enzyme isolation methods, the purification process constitutes approximately 50-90 % of the total expenditure (Boeris et al., 2009; Spelzini et al., 2005), usually consisting of a series of chromatographic separations preceded by ammonium sulphate precipitation (Nalinanon et al., 2010; Tanji et al., 2007). Conventional enzyme isolation methods are costly and time-consuming and result in high enzyme purity but low enzyme yields (Spelzini et al., 2005).

Two methods for isolating enzymes with good up-scaling properties that are not costly in terms of time and material expenditure are aqueous two-phase (ATPs) extraction and polyelectrolyte precipitation (Boeris et al., 2009; Nalinanon et al., 2009). The two aqueous phases can be generated by mixing solutions of two immiscible hydrophilic polymers or a polymer and a salt solution. In addition to the molecular weight and concentration of the polymer, the type and concentration of the salt affect phase separation (Nalinanon et al., 2009). A number of other factors such as the molecular weight of the enzyme, the surface texture, the pH and temperature of the surrounding environment also affect the separation (Gautam et al., 2006). The affinity to the PEG-rich (polyethylene glycol) phase increases with increasing negative charge of the enzyme, while there is increased affinity to the salt-rich phase with pH values below the isoelectric point (Boeris et al., 2009; Gautam et al., 2006). Boeris et al. (2009) and Nalinanon et al. (2009) have been able to show high enzyme yields of between 85-95 % using ATPs to isolate pepsin or pepsinogen. However, subsequent separation of the enzyme from the polymer using simple dialysis methods or addition of salts as used by Teotia et al. (2004) for chitinase purification, was very difficult due to the high binding affinity between the enzyme and the PEG (Boeris et al., 2009; Nalinanon et al., 2009). Using polyelectrolyte precipitation with chitosan, Boeris et al. (2009) demonstrated a method whereby up to 40 % of the porcine pepsin could be separated from the polymer. In the first pepsinogen from the abomasum homogenate was activated and after that isolated by a combination of ATPs extraction and polyelectrolyte precipitation with chitosan (Boeris et al., 2009). An interesting alternative could be the negatively charged polysaccharide pectin (Boeris et al., 2009; Gautam et al., 2006). It is therefore of interest to evaluate pectin (negatively charged polysaccharide) in comparison of chitosan (positively charged polysaccharide), because a much lower precipitation pH of the polyelectrolyte-pepsin complex could be expected. In the acidic environment of the pectin precipitation, pepsinogens are autocatalytically converted to pepsin, therefore no additional conversation step should be

necessary. So far pectin was not reported for pepsinogen or pepsin precipitation and only a small number of studies have used pectin for polyelectrolyte precipitation so far (Bigucci et al., 2008; Tsai et al., 2014).

An electrostatic interaction between the protein and the polyelectrolyte under prevailing ambient conditions results in the formation of an initially soluble complex from which insoluble macro-aggregates emerge as a result of further interactions between macromolecules (Boeris et al., 2008). Subsequent separation of the components, which are predominantly hold together by electrostatic forces, can be performed by the addition of salt (ca. 0.6 M) or a change in pH (Boeris et al., 2009).

The development of a cost-reduction method for isolating pepsin from trout stomachs would significantly increase the potential of fish offal as an enzyme source for industrial use. This study investigates the possibility of isolating and purifying pepsin from the stomach of rainbow trout using ATPs with subsequent polyelectrolyte precipitation (pectin vs. chitosan) and determines the characteristics of the enzyme obtained.

A potential area of application for trout pepsin is in the enzymatic cleavage of dietary proteins to receive antioxidative (Barkia et al., 2010) and antibacterial peptides (Wald et al., 2016) or to reduce the anti-nutritive factor of plant feed proteins (Adelizi et al., 1998). Therefore, the use of trout pepsin was examined for the enzymatic digestion of plant proteins (potato and pea proteins) and animal proteins (fish by-products, fishmeal).

3.3. Materials and methods:

3.3.1. Materials:

Pepstatin A (from microbial source), bovine serum albumin, haemoglobin from bovine blood, (Sigma Aldrich, St. Louis, Missouri); a protease inhibitor panel (Carl Roth, Karlsruhe, Germany); Laemmli sample buffer, β -mercaptoethanol, Tris/glycine/SDS buffer, mini-Protean TGX Precast gel 4-15 % (BioRad, München, Germany); PEG 500, 1000, 1500 und 2000, chitosan 85 % deacylated (Alfa Aesar, Karlsruhe, Germany); PEG 20000, Bradford reagent (Merck, Darmstadt, Germany); dialysis tubing (Spectra pore 1), magnesium sulphate ($MgSO_4$), molecular weight marker (14,4- 97,4 kDa) (Prolabo VWR, Leuven, Belgien); Servalyte Precote 3-10, pI marker 3-10 (SERVA, Heidelberg, Germany), pectin classic AF702 (Herbstreith & Fox KG, Werder/Havel, Germany); pea protein isolate (Erdschwalbe, Neu-Ulm, Germany); potato protein isolate (KMC potato protein 121 V2208) (Brande, Denmark); and fish meal (Biomar Inicio plus, Brande, Denmark).

3.3.2. Chemicals

The chitosan solution was prepared by adding 2 g of chitosan to 100 ml 0.1 M acetic acid and stirring it for 3 h at 25 °C. Under gentle mixing, pectin was dissolved in distilled water (63 °C) at a concentration of 2 %.

3.3.3. Preparation of stomach extract

Rainbow trout (weight: 250 g, age: 18 months) were provided by a fish farm located in Hestia (Germany). Two weeks before slaughtering the fishes, feeding had been stopped resulting in empty stomachs. After gutting the fishes, stomachs were collected and stored in polyethylene bags at -20 °C, until used for pepsinogen purification.

The stomachs of 2-3 trout were pooled, rinsed with cold distilled water on the outside and cut into small pieces of 1 – 2 cm³. The material was homogenized for 1 min with 10 volumes of ice-cold 50 mM sodium phosphate buffer, pH 7.0, using an Ultra Turrax T 25 running at 20500 rpm (IKA, Staufen). The homogenate was centrifuged for 30 min at 15000 g at 4 °C. The supernatant was regarded as the stomach extract.

3.3.4. Pepsin assay

Pepsin activity was measured by the method of Xu et al. 1996 with a slight modification using acid denatured hemoglobin as substrate (described in chapter 2.3.6; online version of the paper doi:10.1007/s00217-016-2692-2).

3.3.5. Determination of protein concentration

Protein concentration was measured by the method of Bradford (1976) using a kit (Protein Kit HC309760) from Merck; bovine serum albumin served as the protein standard.

3.3.6. Preparation of ATPs systems

ATPs system were prepared in 50 ml centrifuge tubes by adding the appropriate amounts of PEG, salt and trout stomach extract, according to the methods of Suppasith Klomklao et al. (2005) and Nalinanon et al. (2009), with slight modifications. To study the effect of $MgSO_4$ (concentrations of 15 %, 20 % and 25 % (w/v)) on the partitioning of the stomach protease, salt solutions were mixed with 20 % PEG 1500 (w/w) solution giving a total value of 40 ml. After gentle mixing, 10 ml stomach extract was added. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortexgenie2). Low-speed centrifugation was used to speed up the phase separation (5 min, 3000 rpm, 4 °C). The phases were carefully separated using a Pasteur pipette. The volumes of the separated top and bottom phases were measured. Aliquots from each phase were taken for enzyme assay and protein determination. The specific activity of stomach protease in the ATPs was defined as the ratio of protease activity (unit) to total protein concentration (mg). The purification factor (PF) was defined as the ratio of the specific proteinase activity of each phase to the initial specific proteinase activity of the raw extract. The partition coefficient (K_E or K_P), defined as the ratio of the enzyme activity or protein concentration of the top phase to the value in the bottom phase, was recorded. To determine the amounts of protein in the supernatant and the sediment, the protein concentration was determined using the Bradford method (Bradford, 1976) and multiplied by the extraction volume. The volume ratio (V_R), defined as the ratio of the volume in the top phase to the volume in the bottom phase, was calculated. To study the effect of the concentration (15, 20, 25 % w/w) of PEG 600, PEG 1000, PEG 1500 and PEG 2000 on the partitioning of protease in stomach extract, 20 % $MgSO_4$ was used in the system. Partitioning was performed as described above. The ATPs with the highest specific activity was chosen for polyelectrolyte precipitation.

3.3.7. Pepsinogen precipitation from the ATPs extract

Ten grams of the upper phase after the ATPs extraction (called ATPs extract) were stirred and mixed with chitosan or pectin (2 %) solutions in the ratio 1:1 (w/v). The solubility of ATPs extract in water at pH 2.0 - 6.0 were evaluated and used as blank. The pH was adjusted to pH 5.5 (chitosan) or 2.5 (pectin), which indicates the precipitation pH (section 3.3.8) by adding 0.5 M NaOH or 0.5 HCL. The suspensions were incubated for 30 min under gentle shaking. Then, the precipitated material was separated by centrifugation (3100 rpm, 15 min, 4°C) and subsequently dissolved by adding 10 ml acetate buffer (1 M) pH 4.0 (chitosan) or distilled water (pectin). The different solvents were used because the complex between the enzyme and chitosan or pectin dissociated under the experimental conditions, which was analysed by turbidimetric measurement. Enzyme extract was concentrated by being placed in PEG 20000 using dialysis tubes (spectrapor 1, MWCO 6000-8000 Da) for 6 h at 7 °C. Concurrently, solvent pectin and chitosan molecules were removed based on their low molecular weight of 0.06-0.13 kDa (pectin) and 1.5 kDa (chitosan). The resulting enzyme extract was used for biochemical analysis. The distribution of the protein in ATPs is characterized by the K and the V_R value. To characterize the purification process, the specific enzyme activity, enzyme yield and purification factor were also determined. For enzyme characterization (enzyme inhibition, isoelectric point), the extracts were acidified therefore pepsinogen was completely converted to pepsin. According to the method of Nalinanon et al. (2010) with slight modifications an aliquot of 400 µl of pepsinogen solution (0.3mg/ml) was adjusted to pH 2.0 with 1 M HCL and incubated at 7°C for 60 min.

3.3.8. Determination of precipitation pH

The pH dependent formation of the insoluble polymer-protein complex was followed by turbidimetric titration according to the method of Boeris et al. (2009) and the enzyme recovery was measured in the precipitate by determining the enzyme activity and protein concentration. For this purpose ATPs extract and polysaccharide (pectin or chitosan) solution (0.5 %) was mixed in a 1:1 (v/v) ratio. 0.05 M HCL or 0.05 M NaOH was added to the solution for pH adjustment, and after gentle mixing, the solution absorbance at 420 nm was measured. Aliquots from each phase were taken for enzyme assays and protein determination.

3.3.9. Effect of the concentration of chitosan/pepsin

To study the effect of the concentration of polysaccharide (pectin or chitosan) on the formation of the polymer-protein complex, concentrations of 0.5, 1, 2, and 3 % (w/v) were used in the system. The formation of the insoluble polymer-protein complex was performed as previously described (section 3.3.7).

3.3.10. Isoelectric point of pepsinogen/pepsin

The isoelectric point (pI) of pepsinogen and pepsin was determined by IEF using Servalyt Precotes 3-10 according to Altinelataman et al. (2009); proteins were visualized using Coomassie brilliant blue R-250.

3.3.11. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE with 4 % stacking and 15 % separating gel was performed according to Laemmli (1970) for molecular weight determination and evaluation of the purity of enzyme preparations. Proteins were visualized by staining with Coomassie Brilliant Blue R-250, and the molecular weights of sample proteins were estimated using the low molecular weight marker (14.4-97.4 kDa) from VWR.

3.3.12. Enzyme inhibition

Different types of proteinase inhibitors were mixed with pepsin solution (final concentration 0.01 μM) to obtain assay concentrations of 50 μM EDTA, 1 μM E-64, 1 μM leupeptin, 50 μM AEBSF and 0.15 μM aprotinin. The inhibitory effect of pepstatin A was investigated in detail with concentrations ranging from 1×10^{-4} μM to 1 μM of pepstatin A; 200 μl of enzyme solution (approximate 0.01 μM) was mixed with the same volume of pepstatin A solution and incubated for 15 min at room temperature (Cao et al., 2011; Zhou et al., 2007). Thereafter, pepsin activity was measured as described above (section 3.3.4).

3.3.13. Uses of the trout enzyme

The enzymatic cleavage efficiency of the trout enzyme was compared for potato and pea protein substrates, fish offal and commercial fish feed. For this purpose, the hydrolysis method modified in accordance with Barkia et al. (2010) was used. First, 0.5 g of raw protein was filled into a 50 ml centrifuge tube and suspended in 20 ml of distilled water. The protein concentration of the fish offal and fish feed was determined by the Bradford method (Bradford, 1976), and the amounts of

protein per gram of sample weight in potato and pea proteins were taken from the specification. The protein solutions were then homogenised for 30 sec using an ultra Turrax at 13500 rpm and heated to 80 °C for 10 min. After cooling to 37 °C, the pH value was adjusted using 0.5 M HCl to the enzyme activity optimum of 3.0. The addition of the enzyme in a ratio of 0.04 U/mg of substrate protein started the enzymatic cleavage. The degree of hydrolysis (DH %) was determined using the pH stat method. DH (%) was defined as the percentage of peptide bonds cleaved (h) in relation to the total amount of bonds per unit of weight. The calculation was performed based on the Adler-Nissen (1986) method as modified by Diermayr and Dehne (1990), using the following correction factors ($\alpha_{pH\ 3,0} = 0.443$, $h_{tot} = 8$, $F_{pH} = 1.80$) (P. Diermayr et al., 1990). As the average molecular weight of the amino acids amounts to 125 g/mol, h_{tot} was indicated with 8 g/kg protein (Nielsen et al., 2001). B stands for the amount of HCl used to maintain the pH value during hydrolysis. Nb represents the normality of the acid and MP the protein concentration. The DH (%) values were calculated as follows:
$$DH (\%) = h/h_{tot} \times 100 = ((B \times Nb) / MP) \times 1 / \alpha \times 1 / h_{tot} \times 100$$

3.3.14. Statistical analysis

All experiments were run in triplicate. All data were submitted to Analysis of Variance (ANOVA) and mean comparisons were evaluated by Bonferroni test. The data were analyzed using the software GraphPad prism (version 5). Differences were considered significant at $p < 0.05$.

3.4. Results:

3.4.1. Purification process

The isolation of trout pepsinogen by ATPs using PEG with different molecular weights and concentrations (15-25 %) was investigated in the presence of 15 % or 20 % MgSO₄. Under the experimental conditions (pH 6-7) pepsinogen indicated a high affinity to PEG. To separate the pepsinogen from the PEG (ATPs-extract), pectin was tested as an alternative polyelectrolyte and compared to chitosan at different polyelectrolyte concentrations. During the precipitation with pectin pepsinogen partly converted to pepsin due to the acidic conditions (pH 2.5) (Figure 3.3).

3.4.2. Influence of PEG molecular weight and salt concentration on phase separation

The two-phase systems comprising PEG (15-25 %) and MgSO₄ (15-20 %) formed 3 phases after separation: the PEG-rich upper phase, the salt-rich lower phase and an intermediate phase. Due to the high binding affinity, pepsinogen transferred, with the exception of the intermediate layer, into the PEG-rich phase. However, no phase separation could be achieved in the system containing 15-25 % PEG 600 and 15 % and 20 % MgSO₄. Phase combinations with PEG 1000 and 15 % MgSO₄ only led to phase separation at high PEG concentrations (25 %) (supplementary material, Table 3-1). The results presented in Table 3.1 show that the variation in salinity significantly affects the V_R, the K_p value and the enzyme yield. The V_R value of all tested systems decreased significantly with increasing salt concentration. In addition, increasing the salt concentration from 15 % to 20 % led to a reduction in K_p values, lower protein concentrations in the supernatant and higher specific enzyme activities. Table 3.1 also shows that an increase in salt concentration led to lower enzyme yields. Determination of the K_E value was not possible, as the protease was almost completely transferred into the upper phase.

Table 3.1: Effect of salt concentration in a PEG- MgSO₄ ATPs on partitioning of stomach protease of trout

Phase composition (% w/w)	V _R	K _p	SA	PF	Yield (%)
15 % PEG 2000 + 15 % MgSO ₄	39 ± 2.0	0.65 ± 0.24	1.04 ± 1.1.6	1.4 ± 0.6	91 ± 4
15 % PEG 2000 + 20 % MgSO ₄	0.40 ± 0.07	0.14 ± 0.02	14.1 ± 1.0	18.7 ± 1.3	69 ± 2
20 % PEG 1500 + 15 % MgSO ₄	9.3 ± 1.0	0.17 ± 0.01	1.1 ± 1.1	1.5 ± 0.8	92 ± 3
20 % PEG 1500 + 20 % MgSO ₄	0.62 ± 0.08	0.12 ± 0.03	20.0 ± 0.9	25.3 ± 1.0	87 ± 4
20 % PEG 2000 + 15 % MgSO ₄	1.53 ± 0.5	0.16 ± 0.01	9.4 ± 2.0	14.0 ± 1.0	92 ± 6

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20 % PEG 2000 + 20 % MgSO ₄	0.49 ± 0.3	0.11 ± 0.01	16.1 ± 2.8	21.4 ± 3.2	68 ± 4
25 % PEG 1000 + 15 % MgSO ₄	6.85 ± 0.55	0.42 ± 0.11	1.3 ± 1.1	1.7 ± 0.6	99 ± 2
25 % PEG 1000 + 20 % MgSO ₄	0.77 ± 0.13	0.12 ± 0.01	14.7 ± 1.2	19.6 ± 1.5	98 ± 4
25 % PEG 1500 + 15 % MgSO ₄	1.62 ± 0.25	0.17 ± 0.01	9.1 ± 0.7	12.2 ± 1.4	93 ± 4
25 % PEG 1500 + 20 % MgSO ₄	0.95 ± 0.15	0.11 ± 0.01	14.5 ± 0.7	19.3 ± 0.88	87 ± 4
25 % PEG 2000 + 15 % MgSO ₄	1.31 ± 0.09	0.14 ± 0.02	8.6 ± 1.2	11.5 ± 1.58	91 ± 4
25 % PEG 2000 + 20 % MgSO ₄	0.82 ± 0.08	0.10 ± 0.02	14.2 ± 0.7	19.5 ± 1.5	85 ± 4

V_R=volume ratio (upper/lower); K_p: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF purification factor; Yield: activity recovery, Means ± S.D. (%) from triplicate determinations.

Phase combinations consisting of PEG and 20 % MgSO₄ led to the highest specific enzyme activities. This salt concentration was therefore used to investigate the effect of PEG concentration and PEG molecular weight on proteinase distribution (Table 3.2).

Table 3.2: Effect of PEG molecular mass and concentration in a PEG-MgSO₄ system on the partitioning of the stomach protease

Phase composition (% v/w)	V _R	K _p	SA	PF	Yield (%)
15 % PEG 1000 + 20 % MgSO ₄	n.s.				
15 % PEG 1500 + 20 % MgSO ₄	0.52 ± 0.05	0.16 ± 0.05	17.7 ± 2.5	24.3 ± 1.08	93 ± 3
15 % PEG 2000 + 20 % MgSO ₄	0.40 ± 0.07	0.14 ± 0.02	14.1 ± 1.0	18.7 ± 1.3	69 ± 2
20 % PEG 1000 + 20 % MgSO ₄	0.82 ± 0.09	0.12 ± 0.03	13.0 ± 2.0	16.7 ± 2.0	99 ± 3
20 % PEG 1500 + 20 % MgSO ₄	0.62 ± 0.08	0.12 ± 0.03	20.0 ± 0.9	25.3 ± 1.0	87 ± 4
20 % PEG 2000 + 20 % MgSO ₄	0.49 ± 0.3	0.11 ± 0.01	16.1 ± 2.8	21.4 ± 3.2	68 ± 4
25 % PEG 1000 + 20 % MgSO ₄	0.77 ± 0.13	0.12 ± 0.01	14.7 ± 1.2	19.6 ± 1.5	98 ± 4
25 % PEG 1500 + 20 % MgSO ₄	0.95 ± 0.15	0.11 ± 0.01	14.5 ± 0.7	19.3 ± 0.88	87 ± 4
25 % PEG 2000 + 20 % MgSO ₄	0.82 ± 0.08	0.10 ± 0.02	14.2 ± 0.7	19.5 ± 1.5	85 ± 4

V_R=volume ratio (upper/lower); K_p: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF purification factor; Yield: activity recovery, Means ± S.D. (%) from triplicate determinations.

By varying the PEG molecular weight (1000-2000), as indicated in Table 3.2, the K_p values of all investigated system combinations were only slightly affected. All phase combinations indicated a K_p value below 1, suggesting good separation of undesirable proteins. PEG molecular weights had a significantly greater impact on specific enzyme activities, V_R values and enzyme yields.

With increasing molecular weight, reductions in specific enzyme activity, V_R value and enzyme yield could be demonstrated.

However, with PEG 2000, increasing the PEG concentration to 25 % led to significantly higher enzyme yields and to an increase in protein concentration in the supernatant (20 % PEG-25 % PEG). Finally, it could be demonstrated that PEG molecular weights of 1000 and 1500 Da were particularly suitable for pepsinogen distribution, as they resulted in high specific enzyme activities and PF values.

The phase combination of 20 % PEG 1500 + 20 % $MgSO_4$ led to the highest specific enzyme activities, while the phase combination 25 % PEG 1000 + 20 % $MgSO_4$ showed the highest enzyme yield with good specific enzyme activity. The use of PEG 2000 often led to low enzyme yields. Furthermore, a reduction in specific enzyme activity could be identified in phase combinations with PEG 2000 compared to PEG 1500.

3.4.3. Factors influencing polyelectrolyte precipitation

As interaction between the polyelectrolyte and the protein is mainly based on electrostatic forces (Boeris et al., 2009), the isoelectric point of the trout pepsinogen and pepsin was determined by IEF to measure the pH range in which the proteins exhibited a positive or negative charge. The complex formation between polyelectrolyte and the enzyme was mainly influenced by pH and polyelectrolyte concentration. To separate the enzyme from the polymer, a polyelectrolyte precipitation with pectin was conducted and compared to the results from chitosan precipitation. In addition to turbidimetric measurement, enzyme activity in the supernatant and the sediment was measured to determine the optimum precipitation pH. In the case of trout pepsinogen, in addition to several other bands, a strong protein band at pH 4.2 was detected, and a similar band for pepsin was detected at 3.5 (Figure 3-1, supplementary material). To indicate the pepsin and pepsinogen bands, a further purification of the extracted enzyme by anion-exchange chromatography and gel filtration was carried out and the isoelectric points after IEF has been compared (data not shown). By varying the pH value when using the positively charged polysaccharide (pH 4.5-6.5) chitosan and the negatively charged polysaccharide pectin (1.0-3.0), it was possible to achieve enzyme precipitation (Figure 3.1a and 3.1b). The acid environment during the pectin precipitation leads to a partly conversion of pepsinogen into the active enzyme pepsin as indicated in Figure 3.3, lane 3 with an additional band by 37 kDa. The conversion of

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pepsinogen to pepsin under the acidic conditions took 1 hour until completion. To achieve a complete conversion the incubation time during the precipitation with pectin must be increased from 30 min to 60 min. Due to complex formation between pectin and the enzyme, a significant increase in viscosity could be observed visually. As a result of further interactions between these macromolecules insoluble macro-aggregated emerge, which can analysed by turbidimetric measurement (Xia et al., 1993). It was observed that a pH reduction (1-3 units) below the IP of the enzyme led to increased enzyme activity in the sediment as well as higher turbidity (Figure 3.1b). This study showed that the pH-dependent solubility of chitosan was significantly modified as a result of the protein binding. The complexes precipitate after a pH of 6.0, whereas the pure chitosan solution precipitates at pH 8.0 (Figure 3.1a).

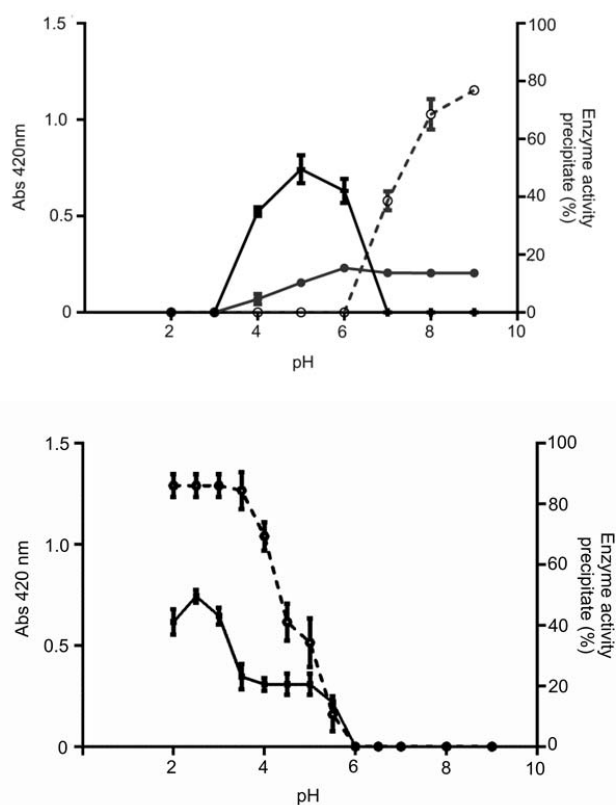


Figure 3.1: Solubility curve of pectin and chitosan with and without the addition of ATPs extract at different pH values.

a) Absorbance at 420 nm of chitosan (chi) solution vs. medium pH. b) Absorbance at 420 nm of pectin solution vs. medium pH. The medium pH was varied by adding increasing amounts of NaOH or HCl. Chi and pectin concentration: 0.5 %, Pepsin (P) activity in the precipitate (■), Abs_{420nm} Chi solution and pectin solution (----), Abs_{420nm} Chi+ P (●). Bars represent the standard deviation (n= 3).

To investigate the influence of the polyelectrolyte concentration on enzyme separation, the enzyme yield was examined in the sediment with polyelectrolyte concentrations between 0.001 g and 0.009 g (300 μ l). Figure 3.2 shows that an increasing polyelectrolyte concentration initially resulted in a linear increase in the enzyme yield in the sediment.

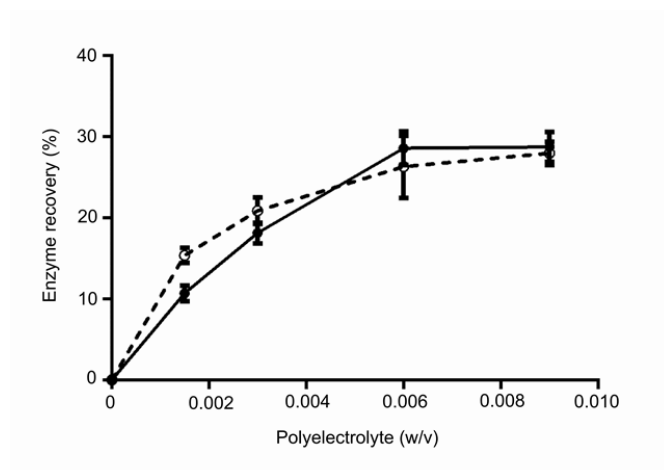


Figure 3.2: Turbidimetric titration of ATPs extract with increasing pectin or chitosan concentrations.

Enzyme recovery in the precipitate (y-axis) vs. polyelectrolyte concentration (x-axis). Precipitation with chi (●) and pectin (○). Bars represent the standard deviation (n=3).

It was possible to achieve maximum yields of 27.4 % with pectin and 29.9 % with chitosan. With a 2 % (0.006 g) solution, chitosan already showed the maximum possible enzyme yield. However, in the case of pectin, it was possible to show a further increase in the enzyme yield at 0.006 - 0.009 g. Pectin concentrations about 0.009 g resulted in a high viscosity of the solution, in which case a further separation was difficult.

To separate the polyelectrolyte from the protein, the precipitate was re-suspended in 0.1 M acetic acid (chitosan) or distilled water (pectin) and poured into a dialysis tube, embedded in polyethylene glycol 20000 and concentrated for 6 hours at 7°C. The enzyme-containing supernatant could then be separated from the polyelectrolyte-containing sediment by centrifugation. After the purification process, enzyme yields of 27 % (chitosan) and 23 % (pectin) could be obtained (Table 3.3).

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Table 3.3: Recovery and purification factor obtained from partition in ATPs and Chi/pectin precipitation of a trout homogenate.

Purification step	Purification fold	Recovery (%)
Crude extract	1	100
Partition in ATPs (PEG 1500 and 20 % MgSO ₄)	20±2.5	87 ± 4
Partition in ATPs ((PEG 1500+ 20 % MgSO ₄) + 2 % chitosan)	25±5	23 ± 4
Partition in ATPs ((PEG 1500+ 20 % MgSO ₄) + 2 % pectin)	23±5	27 ± 7

± S.D. (%) from triplicate determinations

3.4.4. Enzyme characterisation

Following purification, SDS electrophoresis was performed to determine the molecular weight of the enzyme and the degree of purification, as well as enzyme inhibition with protease inhibitors pepstatin A, EDTA, 4-benzenesulfonyl fluoride, E-64, leupeptin and aprotinin.

3.4.4.1. Protein pattern of the stomach protease after the two-stage precipitation

The degree of purification after two-stage precipitation was analysed using SDS-PAGE. As shown in Figure 3.3, the crude extract comprised a range of proteins with different molecular weights. After ATPs, a large proportion of unwanted proteins were removed, as a result of which strong protein bands at 45 kDa and 40 kDa were visible after Coomassie staining. After polyelectrolyte precipitation, the protein band was also clearly detectable at 45 kDa for the precipitation with chitosan (lane 4) and pectin (lane 3). An additional protein band at 37 kDa (Figure 3.3, lane 3) was detectable for the extract after extraction with pectin. A number of faint protein bands were present in the range between 20 and 66 kDa.

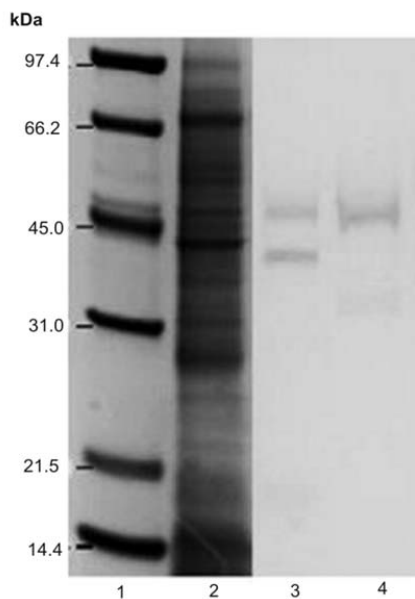


Figure 3.3: SDS-Page of trout stomach extract and purified enzyme extract.

Lane 1, molecular marker; *lane 2*, stomach extract; *lane 3*, purified pepsin extract (pectin precipitation); *lane 4*, purified pepsin extract (chitosan precipitation). The gel was stained with Coomassie brilliant blue.

3.4.4.2. Inhibition of enzyme activity

The influence of different inhibitors (pepstatin A, EDTA, 4-benzenesulfonyl fluoride, E-64, leupeptin, aprotinin) on enzyme activity at high inhibitor concentrations was also studied. Only pepstatin A inhibited the trout pepsin (Figure 3-2 supplementary material). After extraction with pectin and with chitosan, the overall enzyme activity could be inhibited by the use of a molar mass ratio of 1/10 (enzyme/pepstatin A). The inhibition of the trout pepsin by pepstatin A confirms that the isolated enzyme belongs to the group of acidic aspartic proteases.

3.4.5. Effect of the isolated enzyme on protein hydrolysis

After complete enzyme activation, trout pepsin was used for the hydrolysis of pea and potato proteins and fish by-products, and the degree of hydrolysis obtained was compared after 3.5-hour cleavage with the degree of hydrolysis of commercial fish feed. The enzymatic cleavage was conducted at an E/S ratio of 0.04/1 (U/mg of protein) and with an enzyme activity optimum of pH 3 and 37 °C. Enzymatic hydrolysis could be observed for all proteins using trout pepsin (Figure 3-3 supplementary material).

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After 60 min protein cleavage, the final degree of hydrolysis of 10.6 % was already achieved for the pea protein and 12.6 % for the potato protein. The fish by-products and the commercial fish food led to degrees of hydrolysis of 18.4 % and 13.5 %, respectively after 3.5 hours.

3.5. Discussion:

3.5.1. Purification process

A combination of ATPs with subsequent polyelectrolyte precipitation led to the isolation of pepsinogen and pepsin (as indicated by the inhibition with pepstatin A) from the stomach of rainbow trout. The purification process leads to enzyme yields of 25-30 %, which were lower compared to the investigation of Boeris et al. (2009), who reported enzyme yields of 40 %. Polyelectrolyte precipitation mainly based on electrostatic forces, thus the isoelectric point of pepsinogen and pepsin is of high importance. Based on previous studies trout pepsinogen consists of seven different pepsinogen isoforms which exhibited different isoelectric points (PG I a,b 4.0, PG II 5.9, PG III a-d 3.8-4.2). Under the experimental conditions (precipitation at pH 2.5) only PG I and III, with a molecular weight of 37 kDa were isolated. In contrast to that, pepsinogen II indicated a molecular weight of 35 kDa, which was analysed in a previous investigation. The two pepsinogen isoforms (PG I and III) represented 40 % of the total amount of pepsinogen.

3.5.1.1. ATPs extraction

After the ATPs extraction the resulting enzyme, however, contained impurities, as previously reported by Yang et al. (2008) and Gautam et al. (2006). The degree of purification of the protease and the separation of other proteins were subject to various factors such as PEG molecular mass, PEG concentration and salt concentration.

Using PEG 600 and 15-20 % MgSO₄, no phase separation could be generated in this study (Table 3-1 supplementary material). The concentration of MgSO₄ exhibited a significant impact on the destabilisation of the thermodynamic system and the phase separation (Lee et al., 1981). Lee et al. (1981) observed that with increasing PEG molecular weight, a lower concentration of MgSO₄ was necessary to produce phase separation with lysozyme. In addition, higher PEG molecular weights led to phase separation with lower PEG concentrations. This effect has been previously described by Raja et al. (2011) and could also be observed in this study (Table 3.1).

The enzyme yields obtained using the ATPs method are generally very high. Due to the very high binding affinity of pepsinogen to PEG (80-95 %) under the experimental conditions (pH 6-7) (Boeris et al., 2009; Nalinanon et al., 2009), higher enzyme yields could be observed compared to other enzymes, such as lysozyme (Su & Chiang, 2006) and tuna spleen protease, a trypsin-like

serine protease (Klomklao et al., 2005). Due to the high binding affinity, pepsinogen transferred with the exception of the intermediate layer, which Rito-Palomares (2004) attributes to the macromolecular-macromolecular interaction, into the PEG-rich phase, and therefore, no K_E value could be determined in this study by the approach of Nalinanon et al. (2009). Generally, low K (<1) values showed increased migration of unwanted proteins into the lower phase (Nalinanon et al., 2009).

Due to an increase in the salt concentration, Nalinanon et al. (2009) could also observe a reduction in the V_R value, which was ascribed to the rearrangement of the water molecules around the PEG molecule (Table 3.1). The formation of an aqueous layer around the cations creates a more compact molecular structure with minimal PEG volume (Farruggia et al., 2004; Nalinanon et al., 2009). An increase in the salt content also led to a reduction in the enzyme yield. According to Nalinanon et al. (2009) and Antov et al. (2006), enzyme activity losses could be attributed to the denaturation of the protease caused by the salting-out effect. This behaviour could also be observed in the studies conducted by Klomklao et al. (2005). In addition to the salt concentration, Nalinanon et al. (2009) and Klomklao et al. (2005) were able to show that the type of salt has a significant effect on the partition coefficient, specific enzyme activity and enzyme yields. Based on these findings, in this study, only $MgSO_4$ was used as the second phase of the bi-phase system. The enzyme yield increased with decreasing PEG molecular weight (Table 3.2). This result can be related to the “excluded value effect”, which means that with increasing molecular weight, there is a more compact PEG molecular structure resulting in weaker interactions with the protein (Arakawa et al., 1985; Spelzini et al., 2005; Yang et al., 2008). At high PEG concentrations, a reduction of this effect could be determined, which Spelzini et al. (2005) attribute to the penetration of PEG into the hydration shell of the protein, allowing interaction between the PEG and the protein’s hydrophobic regions (Lee et al., 1981; Spelzini et al., 2005). Kokufuta et al. (1991) demonstrated that pepsin and PEG form a soluble complex under the pH condition of 1-5.

3.5.1.2. Polyelectrolyte precipitation

The high binding affinity of pepsinogen and pepsin to the PEG often hampers subsequent separation by simple dialysis methods as described by Boeris et al. (2009). By polyelectrolyte precipitation with pectin (pH 2.5) a part of trout pepsinogen was separated from the soluble ATPs

extract and activated during the acid conditions of the precipitation. The low precipitation pH exhibited some advantages, under acid conditions (pH 3.0) PEG seem to be less strongly associated with pepsin through hydrogen bonding of pepsin carboxyl groups with the ether groups of PEG under acid conditions compared to neutral pH (Kokufuta et al., 1991). Moreover pepsin is very stable under acid conditions. The achieved enzyme yield was in the same range compared to the precipitation with chitosan, but much lower compared to the findings of Boeris et al. (2009), who separated pepsin (from abomasum homogenate) from ATPs extract using a 2 % chitosan solution. In contrast to the study of Boeris et al. (2009), the purification factor was 2.5 times higher. Due to the fact, that the precipitation is affected by the isoelectric point and molecular weight of the enzyme, as well as the ionic strength and the pH of the surrounding environment, further information about the enzyme would be helpful. After the complex formation between pectin and ATPs extract, a significant increase in viscosity could be observed visually. This behaviour can be attributed to the interaction between the amino groups of pectin and the positively charged protein groups. Compared to the findings of Boeris et al. (2009), the precipitation pH for chitosan was lower by approximately 0.5 in this study. The lower pH value can presumably be attributed to the higher pI of the trout pepsinogen compared (3.5) to the porcine pepsin (2.2-3.0 Sigma Aldrich, cat.: P6887). The determination of all factors influencing the phase separation and polyelectrolyte precipitation led to the conclusion that the highest specific enzyme activity could be shown in the combination of 20 % MgSO₄ and 20 % PEG 1500. Subsequent separation of the PEG-pepsinogen complex by polyelectrolyte precipitation using a 2 % pectin or chitosan solution led to enzyme yields of 25 % and 27 % and to specific enzyme activities of 18 or 17 U/mg of protein (Table 3.3). Compared with the chromatography separation method, with enzyme yields of approximately 0.5-7.5 % (Cao et al., 2011; Nalinanon et al., 2010; Tanji et al., 2007), higher enzyme yields can be preserved by the demonstrated separation method (Klomklao et al., 2005; Nalinanon et al., 2009).

3.5.2. Enzyme characterisation

At the end of the purification process, other protein bands between 20-62 kDa could be detected alongside the enzyme by SDS-PAGE, as reported, for example, by Nalinanon et al. (2009), but with a significantly lower concentration compared to the desired enzyme. Subsequent studies on enzyme inhibition showed that the isolated enzyme is one of the acidic aspartic proteases, as it was only inhibited by pepstatin A. In previous studies where enzyme isolation was conducted

using the conventional isolation method, comparable inhibitor/enzyme ratios led to complete enzyme inhibition. Pepsin I and II (a) isolated from the stomach of *Siniperca chuatsi* and pepsin isolated from the stomach of *Mustelus mustelus* were inhibited by pepstatin A with a 10 or 16-fold molar excess of pepstatin A to pepsin (Twining et al., 1983; Zhou et al., 2008).

A potential area of application for this enzyme is in the enzymatic cleavage of different dietary proteins to receive antioxidative peptides (Barkia et al., 2010) or to reduce the anti-nutritive factor of plant feed proteins (Adelizi et al., 1998). It could be shown that, compared to trypsin, the use of trout pepsin could produce comparable degrees of hydrolysis in the digestion of pea proteins under optimal cleavage conditions (Karamać et al. 2002). Trout pepsin, like giant fish pepsin and Japanese sea bass pepsin, is particularly suitable for the enzymatic cleavage of animal proteins (Liaset et al., 2000; Vannabun et al., 2014). Comparable degrees of hydrolysis in fish by-products could be achieved in the enzymatic hydrolysis of Atlantic salmon, *Salmo salar* and Atlantic cod, *Gadus morhua* by-products, with 13.4 % and 14.1% hydrolysis after a 60 min cleavage and 14.5 % and 15.6 % after a 120 min cleavage (Liaset et al., 2000).

3.6. Conclusion:

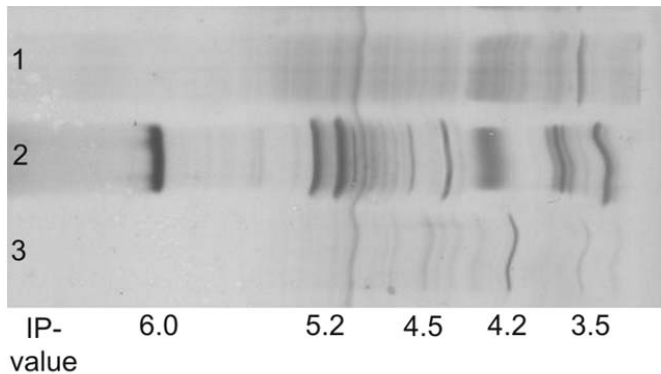
Trout pepsinogen could be isolated from the stomach of rainbow trout by a combination of aqueous 2-phase extraction and polyelectrolyte precipitation and partly activated during the precipitation with pectin. This study was able to show that pectin was an alternative to chitosan for polyelectrolyte precipitation. In conclusion it can be shown that both pectin and chitosan facilitated an enzyme yield of about 25% with a 2% polyelectrolyte solution.

Compared with the chromatography isolation method, a significantly higher enzyme yield (23 % vs. 0.5-7.5 %) could be achieved but with lower enzyme purity (Cao et al., 2011; Nalinanon et al., 2010; Tanji et al., 2007). The isolated enzyme had high cleavage activity on animal proteins, and therefore provides an alternative to microbial enzymes, such as alcalase and flavourzyme and plant enzymes such as papain, as cleavage enzyme in enzymatic hydrolyses (Chalamaiah et al., 2010; Dong et al., 2008). This fast and cost-effective purification method increases the potential for using fish by-products as an enzyme source and for using this enzyme, for example, for biotechnological purposes.

Conflict of interest:

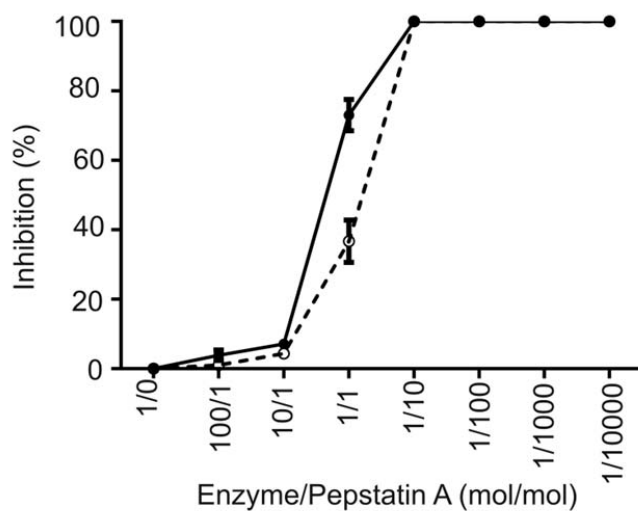
The authors declare that they have no conflict of interest.

3.7. Supplementary



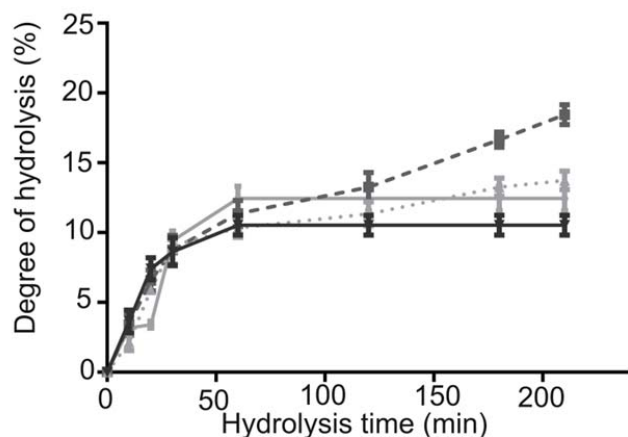
Supplementary Figure 3-1: Isoelectric points of pepsinogen and pepsin.

Analysed by native IEF with Servalyt Precote 3-6. The band at pH 5.2 is a staining artefact. Lane 1, trout pepsin; lane 2, IP marker; lane 3: trout pepsinogen. The gel was stained with Coomassie brilliant blue.



Supplementary Figure 3-2: Pepstatin A inhibition of pepsin

PEP was assayed in the presence of increasing concentrations (x-axis) of pepstatin A using 2% acid haemoglobin as substrate. Bars represent the standard deviation (n=3). Chi/Pepsin (●), pectin/Pepsin (----).



Supplementary Figure 3-3: Effect of acid protease on protein degradation.

X-axis: hydrolysis time, y-axis: degree of hydrolysis. Bars represent the standard deviation (n=3). Potato protein (●), pea protein (▼), fish by-products (·Δ·), fish meal (-■-).

Supplementary Table 3-1: Effect of PEG molecular mass and concentration on partitioning of the two-phase system

Phase composition (% w/w)	Separation
15 % PEG 600 + 15 % MgSO ₄	n.s.
15 % PEG 1000 + 15 % MgSO ₄	n.s.
15 % PEG 1500 + 15 % MgSO ₄	n.s.
15 % PEG 2000 + 15 % MgSO ₄	p.s.
20 % PEG 600 + 15 % MgSO ₄	n.s.
20 % PEG 1000 + 15 % MgSO ₄	n.s.
20 % PEG 1500 + 15 % MgSO ₄	p.s.
20 % PEG 2000 + 15 % MgSO ₄	p.s.
25 % PEG 600 + 15 % MgSO ₄	n.s.
25 % PEG 1000 + 15 % MgSO ₄	p.s.
25 % PEG 1500 + 15 % MgSO ₄	p.s.
25 % PEG 2000 + 15 % MgSO ₄	p.s.

n.s. = no separation, p.s. = phase separation

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4. Detection of antibacterial activity of an enzymatic hydrolysate generated by processing rainbow trout by-products with trout pepsin

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4.1. Abstract

Trout by-product hydrolysates, generated using trout pepsin, were characterized and studied in terms of their antibacterial effects against food contaminants and fish farming pathogens. After a hydrolysis time of 25 min, the hydrolysates demonstrated inhibitory activity against several gram-positive and gram-negative bacteria. The degree of hydrolysis (DH) was found to exert a considerable influence on antibacterial activity, with a significant increase in the observed inhibitory effect at the beginning of hydrolysis. The highest antibacterial activity was obtained at a DH of 30 % (enzyme/ protein ratio 0.04 U/mg of protein, enzyme activity 6.5 U/mg protein, hydrolysis conditions 37°C, pH 3.0). The highest antibacterial activity detected was against the fish farming bacteria *Flavobacterium psychrophilum* and *Renibacterium salmoninarum*, with minimal inhibition concentrations of 2 mg/ml and 5 mg/ml, respectively. The amino acid determination of the hydrolysate (DH 30%) revealed that lysine, leucine, alanine, arginine, glycine, aspartic acid and glutamic acid residues represented the major amino acids.

Keywords:

Antibacterial peptides, Trout by-product hydrolysate, Trout pepsin, Food spoilage, Fish diseases

4.2. Introduction

The widespread use of antibiotics, in recent years, has led to a rapid increase in antibiotic-resistant bacteria. In recent decades, this development, and the desire of consumers to buy food products with natural ingredients, have resulted in research into novel antimicrobial agents (Beaulieu, Thibodeau, Bonnet, Bryl, & Carbonneau, 2013; Lin, Hui, Chen, & Wu, 2013).

For example, in addition to immune system proteins (Khoo, Robinette, & Noga, 1999), food proteins have been found to be natural sources of antimicrobial agents containing antimicrobial peptide sequences. Numerous studies have already proven that the enzymatic cleavage of milk or egg proteins can release antibacterial peptides (Bellamy et al., 1992; Ibrahim, Sugimoto, & Aoki, 2000). However, only few data is available on antibacterial peptides derived from meat sources (Beaulieu et al., 2013; Robert et al., 2015; Sila et al., 2014); only one study by Jang, Jo, Kang, and Lee (2008) was carried out to evaluate the antibacterial effect of peptides isolated from mammalian meat (beef sarcoplasmic proteins). Beaulieu et al. (2013), Robert et al. (2015) and Sila et al. (2014) showed that in addition to a number of animal proteins, fish by products may represent sources from which antibacterial peptides can be isolated. The enzymatic hydrolysis of these muscle proteins was mainly performed using microbial enzymes such as Protamex and Alcalase (both proteases from *Bacillus licheniformis*) and Flavourzyme (protease from *Aspergillus oryzae*), which renders the hydrolysis process cost-intensive (Benhabiles et al., 2012). The use of the fish's own pepsin enzymes offers an alternative to the aforementioned approach because of to its cleavage specificity for proteins and polypeptides and the ability to reduce the risk of bacterial contamination during the hydrolysis process due the acidic hydrolytic pH (Benhabiles et al., 2012; Bougatef et al., 2009; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011).

The biological activity of the peptides released is dependent on the protein and enzyme used, and is significantly influenced by the hydrolysis conditions, as described by Adler-Nissen (1986) and Kristinsson and Rasco (2000). Hydrophobicity, molecular weight and the proportion of polar groups determine the antibacterial activity of the peptides and can differ significantly depending on the degree of hydrolysis (Cheng, Tang, Wang, & Mao, 2013). Cheng et al. (2013) and Pathak et al. (1995) were unable to demonstrate a linear correlation between peptide hydrophobicity and antibacterial effects. Furthermore, as was evidenced by the studies carried out by Cheng et al.

(2013) and Sila et al. (2014), hydrolysates exhibit antibacterial effects at certain degrees of hydrolysis, and the strength of these effects varies significantly depending on the degree of hydrolysis. Cheng et al. (2013) attributed this relationship to a hydrophobicity threshold, with hydrophobicities above, and below, this threshold value exerting a negative effect on antibacterial activity. The enzymatic cleavage of the crude protein may lead to an increase, or reduction, in hydrophobicity, depending on the protein, molecular weight and size of the peptides produced (de la Barca, Ruiz-Salazar, & Jara-Marini, 2000). Sufficient peptide solubility is also crucial for the ability of antimicrobial peptides to function at bacterial growth sites in an aqueous phase (Branen, 1993). Dong et al. (2008) and Klompong, Benjakul, Kantachote, and Shahidi (2007) demonstrated that the solubility of hydrolysates is significantly increased, compared to the crude protein, due to the reduction in size that occurs when proteins are converted into peptides.

The exact mechanism of action of antibacterial peptides is not yet fully understood. The interaction of these molecules with the bacterial membrane could potentially lead to the leakage of cell constituents due to the formation of pores or blockage of membrane ion gradients. Furthermore, a variety of peptides have been identified that can generate bacterial death without detectable cell lysis. This finding is indicative of substantial impairment of the cellular metabolism. Generally, antibacterial peptides that interact with the bacterial membrane, nonspecifically, exhibit the broadest spectrum of activity (Branen, 1993; Moll, Konings, & Driessen, 1999).

The AMPs (antimicrobial peptides) that have been produced from fish proteins, to date, have demonstrated growth-inhibiting properties towards a number of food-spoiling bacteria, including *Escherichia coli*, *Enterococcus* sp., *Micrococcus luteus*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Listeria monocytogenes* and towards fish pathogenic bacteria such as *Aeromonas* sp., *Yersinia ruckeri* and *Edwardsiella tarda* (Beaulieu et al., 2013; Robert et al., 2015; Sila et al., 2014).

The aim of this study was to generate antibacterial peptides from trout by-products, using trout pepsin, and to evaluate the antibacterial activity of these molecules, based of their degree on hydrolysis.

4.3. Materials and Methods

4.3.1. Materials

Piscidin-1 (Bio world), R₂A bouillon/agar, (Carl Roth, Karlsruhe), Müller Hinton bouillon/agar, ringers solution tablets (Sigma Aldrich, St Louis), MRS bouillon/agar (Carl Roth, Karlsruhe), sodium dihydrogen phosphate, disodium hydrogen phosphate (Merck, Darmstadt), NaCl (99%), HCl, NaOH (VWR, Prolabo, Darmstadt), bacitracin, insulin, aprotinin (Sigma Aldrich, St. Louis), Asp-Phe-MeOH (Merck, Darmstadt), HCl, NaOH (VWR, Prolabo, Darmstadt), petroleum ether, and Bradford reagent (kit HC309760) (Merck, Darmstadt) were purchased.

4.3.2. Bacteria

Aeromonas media (ATCC 33907 fish farm effluent), *Aeromonas salmonicida* (ATCC 33659, trout), *Flavobacterium psychrophilum* (ATCC 49511, kidney of diseased rainbow trout fry), *Flavobacterium araucanum* (LM-20 FP, external lesion of *Salmo salar* from a fish farm), *Renibacterium salmoninarum* (ATCC 33209, yearling chinook salmon), *Weissella minor* (ATCC 35412, milk machine slime), *Weissella paramesenteroides* (ATCC 33313), *Citrobacter freundii* (ATCC 8090), *Pseudomonas fluorescens* (ATCC 13525), *Proteus mirabilis* (ATC 29906), *Escherichia coli* (K12 E851), *Micrococcus luteus* (ATC 4698), *Bacillus cereus* (ATC12826), and *Enterococcus faecalis* (ATCC 51299) were used in this study.

4.3.3. Trout pepsin

Pepsin was isolated from the stomach of rainbow trout by a 4-step procedure, including ammonium sulfate precipitation, anion exchange chromatography and two subsequent gel filtration steps (online version of the paper doi:10.1007/s00217-016-2692-2). Very similar purification processes were also described for pepsins obtained from albacore tuna (*Thunnus alalunga*) and smooth hound (*Mustelus mustelus*) (Bougatef, Balti, Zaied, Souissi & Nasri, 2008; Nalinanon, Benjakul & Kishimura, 2010). The related enzyme exhibited a specific enzyme activity of 6.5 U/mg protein, as measured by the method described by Ryle (1984), using acid denatured hemoglobin as a substrate. The trout pepsin exhibited optimal enzyme activity at pH 3.0 and 37 °C using hemoglobin as substrate.

4.3.4. Trout viscera

Rainbow trout (weight: 250 g, age: 18 months) were provided by a fish farm located in Hesse, Germany. Two weeks before the fish were sacrificed, feeding was stopped, which resulted in empty viscera. After fish were gutted, the viscera (throat, stomach and intestines) were collected, ground by a mincer and stored in polyethylene bags at -20 °C, until they were used for digestion by pepsin.

4.3.5. Production of protein hydrolysates

For the enzymatic cleavage of trout viscera by pepsin, the hydrolysis method described by Barkia, Bougatef, Khaled, and Nasri (2010) was used with modifications. Five hundred grams of trout viscera were minced with 500 ml of distilled water using an Ultra Turrax (13500 rpm) for 30 s (Barkia et al., 2010). Aliquot from the homogenate was centrifuged (6000g, 15 min, 7°C) and the supernatant was used for the determination of the protein content, according to the method of Bradford (1976) using bovine serum albumin as standard. The protein concentration of the supernatant was 6.3 mg/ml. Thereafter the homogenate was heated to 80 °C for 10 min to inactivate the endogenous enzymes. The cooked by-products were then homogenized again as described above. After cooling to 37 °C, the pH value was measurement and adjusted to the initial pH of 3.0 for trout pepsin and 2.0 for porcine pepsin using 0.5 M HCl. The proteins of the acidified homogenates were digested by the trout/porcine pepsin at a ratio of 0.04 U/mg of protein. Due to the hydrolysis process, the pH value of the hydrolysate increased continuously as described by Diermayr and Dehne. (1990). Therefore, the pH of the mixture was maintained by repeatedly adding 1 M HCl solution. After the required digestion time, the reaction was stopped by heating the solution to 80 °C for 10 min to inactivate the enzymes. The hydrolysates were then centrifuged at 10,000g for 10 min to remove the insoluble fraction, and the supernatants were freeze-dried (Christ ALPA 1-2). The protein powders were then stored at 7 °C as hydrolysed by-products (hydrolysates). The hydrolysis process was repeated three times.

The degree of hydrolysis (DH) in % was determined using the pH-stat method according to Adler-Nissen (1986). The DH was defined as the percentage of peptide bonds cleaved (h) in relation to the total amount of bonds per unit of weight. The calculation was made based on the Adler-Nissen method (Adler-Nissen, 1986) and the modification by Diermayr and Dehne (1990) using the correction factor $\alpha=0.443$ ($pK_A= 3.1$). As the average molecular weight of the amino

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acids amounts to 125 g/mol, h_{tot} was set at 8.3 g per kg protein (Nielsen, Petersen, & Dambmann, 2001). B stands for the amount of hydrochloric acid used to maintain the pH value during hydrolysis, Nb stands for the normality of the acid, and MP for the protein concentration. The degree of hydrolysis was calculated as follows:

$$DH (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100 \quad \alpha = \frac{10^{pH-pK_A}}{1+10^{pH-pK_A}}$$

4.3.6. Proximate analysis and characterization of hydrolysates

4.3.6.1. Protein solubility

Protein solubility was determined according to the method of Klompong et al. (2007), with slight modifications (triple determination). Hydrolysate (200 mg) or undigested viscera was dispersed in 20 ml of deionized water, and the pH of the mixture was adjusted to 3, 4, 5, 6, 7, 8 or 9 with 0.5 M HCl and 0.5 M NaOH. The mixture was stirred for 30 min at room temperature and centrifuged at 4500g for 30 min. The protein content in the supernatant and total protein content in the original samples was determined using the Bradford method (Bradford, 1976), as described below. The experiment was repeated thrice. The protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

4.3.6.2. Moisture content

The moisture content was measured with a MA 40 moisture analyzer (triple determination) (Sartorius).

4.3.6.3. Determination of protein concentration

The protein content of the homogenate (4.3.5) was measured by the Bradford method (1976) using the protein kit HC309760 from Merck; bovine serum albumin served as the protein standard (triple determination) (Bradford, 1976). Fifty microliters of the homogenate (supernatant) was mixed with 2500 μ l of Bradford reagent and the absorbance of the reference and sample were measured at 595 nm in a 10 mm cuvette after a reaction time of 15 min. The protein concentration of the unknown sample solution was determined by using the standard curve created with bovine serum albumin. The experiment was repeated twice. The reference

contained distilled water instead of protein solution. The Bradford assay was used instead of the Lowry assay (Liu et al., 2014; Nalinanon et al., 2011), because this assay is less susceptible to interference caused by lipids and sugar (Lucarini & Kilikian, 1999).

The peptide concentration of the hydrolysate (absorbance at 214 nm) was determined by chromatography (HILoad Superdex 30 pg; GE Healthcare) (triple determination). The lyophilized hydrolysates (0.5 g), obtained by treatment with trout pepsin were suspended in 1 ml of distilled water and centrifuged at 10,000g for 10 min, then loaded onto a Superdex Hiloal 30 pg (GE Healthcare) gel filtration column (600 mm x 16 mm) pre-equilibrated and eluted with 20 mM sodium phosphate buffer, pH 7.0, with 0.15 M NaCl at room temperature. The flow rate was 0.6 ml/min, the detection wavelength 214 nm, the injection value 10 µl and the sample loop size 250 µl. To determine the peptide content of the sample, the area under the curve of the sample was compared against the reference standard (Bacitracin A) (Vemuri, 2005). Bacitracin was taken as standard, because this 1.4 kDa protein reflected the average molecular mass of the hydrolysate. The liquid chromatography system consisted of a Waters alliance 2690 and a Waters 996 photodiode array detector was used. Millennium software was used to plot, acquire and analyze chromatographic data.

4.3.6.4. Fat content

The Soxhlet method was used to determine the fat content (triple determination). After acid extraction (method according to Weibull-Stoldt), the dried powder (2–5 g viscera /20–40 g protein hydrolysate) was packed in an extraction thimble, and the lipids were extracted with petroleum ether (boiling point 40–60 °C) for 6 h. Upon completion of the extraction, the extract was concentrated using a vacuum rotary evaporator, and the remaining residues were weighed (Matissek & Setiner, 2006).

4.3.6.5. Determination of the molar mass of peptides

The hydrolysates were subjected to gel filtration chromatography after sample preparation as described under section 4.3.6.3. The hydrolysates (100 µl) were loaded onto a Superdex 30 gel filtration column (600 mm x 16 mm). The elution was carried out with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The 5-ml fractions were collected and their absorbance was monitored at 214 and 280 nm. The molecular mass distribution was determined from the retention time of selected fraction, by the comparison with the reference proteins lysozyme (14

kDa), insulin (3.4 kDa), bacitracin (1.2 kDa) and the peptide Asp-Phe-MeOH (0.294 kDa) at a concentration of 1 mg/ml. The measurement was repeated three times. The liquid chromatography system and analysis software described under 4.3.6.3 was used. The molecular mass distribution of antibacterial and antioxidative peptides were also analyzed by gel filtration as described by Nalinanon et al. (2011), Robert et al. (2015) and Sila et al. (2014).

4.3.6.6. Amino acid analysis

The amino acid composition of the trout protein hydrolysate (H₄) was analyzed according to the method of Spackman, Stein, and Moore (1958), with slight modifications (double determination). The lyophilized hydrolysate (H₄), with a concentration of 0.45 mg/ml was incubated with 6 M HCl for 60 h at 110 °C. After the hydrolysis, the sample was dried (vacuum centrifuge) and resolved in 500 µl sodium-acetate buffer pH 2.2. The amino acids were separated by ion exchange chromatography, therefore 20 µl of the sample was loaded onto an amino acid analyzer LC 3000 (Eppendorf-Biotronik) equipped with a polymer cation exchange column (125 x 4 mm), followed by a post-column derivatization with ninhydrin at 125 °C. The detection was carried out at 570 nm and 440 nm for proline. The chromatographic data were acquired and analyzed using the software ChromStar 6.0. This method has been described by Robert et al. (2015) to determine the amino acid content of tilapia protein hydrolysate.

4.3.7. Antibacterial effect of fish protein hydrolysate

The antibacterial effects of the fish protein hydrolysate, with degrees of hydrolysis of 10%, 20%, 25% and 30%, were determined using the minimum inhibitory concentration (MIC) method described by Zhang, Guan, Huang, and Xiong (2013). The hydrolysate with the highest antibacterial effect was compared to the antibiotics erythromycin and oxytetracycline and the antibacterial peptides piscidin-1 and nisin, using a disk diffusion test based on the method described by Tao, Zhao, and Wen (2014). Piscidin-1 was also used as a comparator peptide to determine the MIC of the hydrolysate. In the test series, 24-h cultures were used, with the exception of *Flavobacterium*, for which a 48 h culture was used.

To determine the influence of enzymatic cleavage, the antibacterial effect of the crude extract was also studied prior to enzymatic cleavage. MIC values of the hydrolysates with varying degrees of hydrolysis and the crude extract in the concentration range of 47-2 mg/ml were calculated to assess the relationship between the hydrolysis time and the associated size reduction

and antibacterial effect. The total bacterial count, as determined by counting in a Thoma chamber, amounted to a bacterial suspension of $1-2 \times 10^6$ cfu/ml (cfu: colony forming units).

4.3.7.1. Minimum inhibitory concentration (MIC)

The antimicrobial activity of the hydrolysates was determined using a liquid growth inhibition assay according to the procedures of Zhang et al. (2013). Twenty microliters of a hydrolysate solution was mixed with 20 μ l of a bacterial suspension. After a 3-h incubation period under optimum conditions, 50 μ l of the culture media was added into each well for further incubation under optimal conditions. The number of bacteria was determined by measuring the change in the OD_{600 nm} value on a microtiter plate reader (Bioscreen C, Sartorius). The experiment was repeated three times. For the sample blank, 20 μ l of hydrolysate solution was mixed with 20 μ l of physiological saline solution (ringers solution); for the bacterial blank, 40 μ l of physiological saline solution was used; and for the bacterial positive control, 20 μ l of physiological saline solution was mixed with 20 μ l of bacterial suspension.

4.3.7.2. Agar well diffusion assay

One hundred microliters of each culture was mixed with 10 ml of agar medium and poured into a 90 mm x 15 mm petri dish. After gelation, wells were made using a sterilized punch. Twenty microliters of hydrolysate solution or antibiotics was added and incubated under optimal conditions for 24 or 48 h. The diameters of the zones of growth inhibition around each well were measured to the nearest millimeter by viewing the plate with reflected light above a black nonreflecting background. Then, the zone of inhibition was determinate by mechanical calipers (Tao et al., 2014). The data preserved are based on triplicates.

4.3.8. Statistical analysis

All data were submitted to Analysis of Variance (ANOVA) and differences between means were evaluated by Bonferroni test. The data were analyzed using the software GraphPad Prism (version 5). Differences were considered significant at $p < 0.05$.

4.4. Results and Discussion

4.4.1. Hydrolysis of trout viscera

The hydrolysis of trout by-products with trout and porcine pepsin was carried out using the pH-stat method. Pepsin was chosen for the hydrolysis because this enzyme demonstrates good specificity for the hydrolysis of polypeptides and because bacterial contamination is limited under acidic pH conditions (Benhabiles et al., 2012). In addition, autopeptic hydrolysis, as described in studies carried out by Benhabiles et al. (2012), is possible using the fish's own pepsin which in turn optimizes the hydrolysis process.

Using the same amount of enzyme trout pepsin exhibited a significantly higher degree of hydrolysis compared to porcine pepsin (Figure 4.1). The higher DH% illustrates the greater proteolytic activity of the trout pepsin relative to the substrate. This finding is in accordance with those of Kristinsson and Rasco (2000) who reported that the degree of hydrolysis is influenced by the enzyme used.

In the initial phase, both enzymes generated rapid protein cleavage and therefore a rapid increase in the degree of hydrolysis (Figure 4.1). The rate of hydrolysis was reduced after 60 min with trout pepsin and after only 10 min with porcine pepsin, reaching the stationary phase after 210 and 240 min (trout-porcine pepsin).

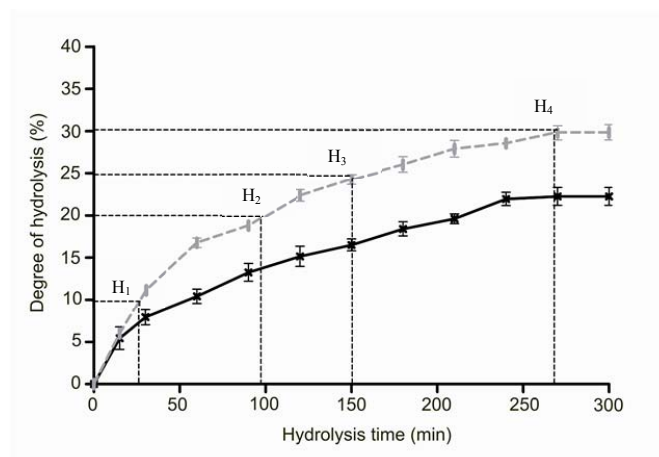


Figure 4.1: Hydrolysis of trout viscera by trout pepsin.

The enzymatic cleavage by trout pepsin (grey line) was compared to that by porcine pepsin (black line). The enzyme/substrate ratio was 0.04 U/mg protein. The x-axis shows the hydrolysis time, and the y-axis the degree of hydrolysis (DH%). H₁–H₄ illustrate 10 %, 20 %, 25 % and 30 % hydrolysis, respectively, obtained using trout pepsin.

Similar courses of protein hydrolysis using different enzymes have been described by Klompong et al. (2007) (*Selaroides leptolepis*), Benjakul and Morrissey (1997) (*Merluccius productus*), Bougatef et al. (2009) (*M. mustelus*) and Sila et al. (2014) (*Barbus callensis*). In the hydrolysis of the muscle proteins of *Nemipterus hexodon*, tuna fish pepsin (isolated from *Katsuwonus pelamis*) also resulted in a DH of 30% (Nalinanon et al., 2011). In contrast to this study, the hydrolysis of *M. mustelus* muscle proteins using the fish's own pepsin resulted in a significantly lower DH of 9.2 % by Bougatef et al. (2009).

4.4.2. Characterization of hydrolysates

For hydrolysates used in human and animal nutrition, their chemical composition and solubility at different pH values, is of crucial importance (Klompong et al., 2007; Robert et al., 2015). A marked change in chemical composition occurred as a result of hydrolysis. By separating the undissolved, uncleaved substances after hydrolysis, we were able to minimize the fat content (55-0.74%) and increase the protein content (32-68%) in the hydrolysate compared to the raw substrate. The observed increase in solubility can be attributed to the increase protein content as a result of the hydrolysis process (15–30%) (Chalamaiah, Rao, Rao & Jyothirmayi, 2010; Yarnpakdee, Benjakul, Penjamras, & Kristinsson, 2014).

The trout hydrolysate possessed lower fat content compared to that of *Oreochromis niloticus* (1.3–1.7%) and *M. mustelus* (3%) (Bougatef et al., 2009; Yarnpakdee et al., 2014). The trout hydrolysate protein content matched the protein content of *O. niloticus* (62.7%) (Robert et al., 2015) but was lower than that in *M. productus* hydrolysate (82.25%) (Benjakul & Morrissey, 1997).

The trout hydrolysate was more than 92 % soluble over a wide pH range (3–9), whereas the raw substrate exhibited significantly lower solubility (27– 82 %) in the same pH range, with optimal solubility at pH 9 (82 %). Dong et al. (2008) and Klompong et al. (2007) demonstrated that solubility increased as a result of the reduction in size that occurs when proteins are converted into peptides. Gbogouri, Linder, Fanni, and Parmentier (2004) attributed this effect to the fact that hydrolysates have proportionally more polar amino acids, which thereby allows the formation of hydrogen bonds with water and increased solubility. Comparable solubility of hydrolysates with a high degree of hydrolysis has been reported in the fish species of shellfish silver carp (85–95 %) (Dong et al., 2008) and yellow stripe (> 90 %) (Klompong et al., 2007)

generated using alcalase and flavourzyme, as well as in meriga egg (> 80 %) using alcalase and papain (Chalamaiah et al., 2010).

The amino acid profile of the trout protein hydrolysate (H₄) was determined, as shown in Table 4.1. The highest concentrations were found for leucine (4.63 %), lysine (5.11 %), alanine (4.35 %), arginine (4.80 %), aspartic acid (5.84 %), glutamic acid (8.71 %) and glycine (5.34 %). During acid hydrolysis, asparagine is converted to aspartic acid and glutamine to glutamic acid; thus the amount of these detected amino acids represents the total quantity of glutamic acid and glutamine and of aspartic acid and asparagine (Beaulieu, Thibodeau, Bryl & Carbonneau, 2009). Similar to this study, the major amino acids present in tilapia by-product hydrolysate (*O. niloticus*) (Robert et al., 2015), k-casein hydrolysate (Cheng et al., 2013) and snow crab hydrolysate (*Chionoecetes opilio*) (Beaulieu et al., 2009) were aspartic and/or glutamic acid. The percentage of trout protein hydrolysate residues with hydrophobic side chains was higher (22.74%) than that of other amino acid residues with a polar side chain (12.90%). This result was in accordance with those of Cheng et al. (2013) and Robert et al. (2015), who reported a hydrophobic amino acid contents of 27.44% and 14.92% and polar amino acid quantities of 10.48% and 6.05%, respectively (Table. 4.1). Most antibacterial peptides possess of a high content of hydrophobic residues (up to 50 %) (Cheng et al., 2013; Yeaman and Yount, 2003). Increasing hydrophobicities, which are determined by the percentage of hydrophobic amino acids, leads to increased membrane permeability by peptides as demonstrated via model peptides for target membranes (Yeaman & Yount, 2003). Additionally, Wieprecht et al. (1997) observed that increasing peptide hydrophobicity resulted in a higher permeabilization of phosphatidylcholine (PC) and phosphatidylglycerol (PG) membranes. Moreover Cheng et al. (2013), reported, that the interaction between antibacterial peptides and the bacterial membrane may be associated with the hydrophobic and basic amino acids due to the formation of hydrophobic bounds.

Taurine, an amino acid that is not utilized in protein synthesis but is rather found free in solution or in simple peptides, was found the trout protein hydrolysate (3.2 %), and has also been observed in snow crab and tilapia by-product hydrolysates, although at a much lower concentrations (1.76 % and 0.79 %, respectively) (Beaulieu et al., 2013; Robert et al., 2015).

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Table 4.1. Amino acid content of trout protein hydrolysate (H₄) produced with trout pepsin, compared to k-casein hydrolysate and nisin

Amino acid	Amino acid content (mg g ⁻¹ protein)		
	DH 30 % (H ₄)	k-casein ^{*1} hydrolysate ^{*1}	nisin ^{*2}
hydrophobic amino acids			
Isoleucine	52.1 ± 0.4	59.6	168.5
Leucine	93.0 ± 0.3	91.0	
Methionine	35.6 ± 2.4	21.1	64.1
Phenvalanine	47.2 ± 0.9	50.0	-
Valine	65.5 ± 0.9	56.1	50.7
Alanine	87.5 ± 0.3	48.8	47.6
Proline	76.3 ± 0.6	98.7	38.6
Total	457.3 ± 0.9	425.4	201
basic amino acids			
Lysine	102.8 ± 0.6	73.9	189.5
Arginine	96.6 ± 0.4	35.8	-
Histidine	37.3 ± 1.3	27.4	71.7
Total	236.6 ± 0.2	137.0	261.2
polar amino acids			
Tyrosine	45.1 ± 3.6	53.2	-
Threonine	56.8 ± 3.3	42.3	-
Glycine	107.4 ± 0.3	20.0	66.2
Serine	47.4 ± 0.5	46.9	15.3
Cysteine	3.2 ± 0.8	-	-
Total	259.2 ± 1.7	162.4	81.5
Aspartic acid	117.5 ± 0.3	72.8	54.6
Glutamic acid	175.1 ± 1.9	164.9	-
Taurine	48.7 ± 1.3	-	-
β-methyl-lanthionine			233.0
lanthionine			64.6
Total amino acid	2010.9 ± 0.8	1550.4	1346.9
Hydrophobic amino acids (%)	22.7 ± 0.9	27.4	14.9
Basic amino acids (%)	11.8 ± 0.2	8.8	19.4
Amino acids with polar residues (%)	12.9 ± 1.7	10.5	6.1

^{*1} (Cheng et al., 2013), ^{*2} (Cheeseman et al., 1959), Means ± S.D. (%) from duplicate determinations.

4.4.3. Antibacterial activity of trout protein hydrolysate depends on the degree of hydrolysis (DH %)

The biological activity of hydrolysates is largely affected by their molecular structure (amino acid composition and sequence) and peptide length, which in turn depends on the degree of hydrolysis. The DH is an important factor for generating reproducible hydrolysates with consistent biological activity (Sila et al., 2014). The hydrolysis was terminated at different points of times with the aim of producing a maximum level of peptides <10 kDa, as peptides of this size possess improved antibacterial activity (Beaulieu et al., 2013).

After a period of hydrolysis of 25 min (DH 10 %), the hydrolysate contained a large proportion of peptides under 3 kDa (> 50 %). No significant difference in the molar mass distribution of the hydrolysates could be detected between H₃ and H₄. The hydrolysates with DH 10 % (H₁), 20 % (H₂), 25 % (H₃) and 30 % (H₄) were studied for their inhibitory effects on 14 different bacteria consisting mainly of food contaminants and fish farming pathogens.

To enable quantitative and qualitative analysis of the effect of the degree of hydrolysis on hydrolysate antibacterial activity, the minimum inhibitory concentration (MIC) was determined using a microdilution test. The MIC value defines the lowest concentration of an antibacterial agent that has a 100% inhibitory effect on bacterial growth.

At the start of hydrolysis, the antibacterial effect of the hydrolysates increased significantly with an increasing degree of hydrolysis (Table 4.2). After a period of hydrolysis of 150 minutes (H₃=DH 25 %), the hydrolysates demonstrated no significant difference in their antibacterial activity ($P > 0.005$) or molecular weight distribution (data not shown). Cheng et al. (2013) also observe similar results in their studies on k-casein hydrolyzate, as shown in Table 4.2. However, Sila et al. (2014) reported that hydrolysates with a low DH (2.8%) or with high DH (15.54 % and 16.2 %) were unable to inhibit bacterial growth. Hydrolysates, with an average DH of 6.6 % demonstrated an antibacterial effect against a broad spectrum of bacteria. Cheng et al. (2013) attributed this result to peptide hydrophobicity. The hydrophobicity of antibacterial peptides has been described as a major factor in peptide interactions with bacterial cytoplasmic membranes.

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Table 4.2: Effect of hydrolysis time on the antibacterial rate of trout protein hydrolyzate obtain with pepsin compared to k-casein hydrolyzate obtained with trypsin against *E. coli*

DH	Antibacterial rate	
	Trout protein hydrolyzate * ¹	k-casein hydrolyzate * ²
0 %	-	++
0-10 %	-	+++
11-20 %	++++	++++
21-25 %	+++++	++++
26-30 %	+++++	

Antibacterial rate: - (no activity), ++ > 20 % inhibition, +++ > 30 % inhibition, ++++ > 40 % inhibition, +++++ > 50 % inhibition, *¹ concentration 20 mg/ml, *² concentration 0.5 mg/ml (Cheng et al., 2013),

In studies carried out by Cheng et al. (2013) and Liu, Kong, Xiong, and Xia (2010), the hydrolysis of k-casein using trypsin and of porcine blood plasma proteins using alcalase resulted in reduced hydrophobicity of the proteins/peptides. Apart from hydrophobicity needed for interaction with the hydrophobic bacterial membrane, peptides must also possess hydrophilic properties to ensure that the antibacterial agent can be dissolved in the aqueous phase in which bacterial growth takes place (Branen, 1993; Cheng et al., 2013).

Despite lower hydrophobicity, the hydrolysates produced by Cheng et al. (2013) and Liu et al. (2010) demonstrated significantly higher antibacterial activity compared to their crude proteins. Cheng et al. (2013) attributed this difference to the existence of a certain hydrophobicity threshold at which the protein/peptide exhibits the highest antibacterial effect. However, hydrophobicities beyond this threshold favor enhanced protein dimerization in solution, which in turn leads to reduced antibacterial effects (Cheng et al., 2013). This result is because protein dimers are unable to pass through the cell wall, which prevents interactions with the cell membrane (Y. Chen et al., 2007).

No antibacterial effects were observed for trout protein in this study, as shown in Tables 4.3 and 4.4. Similar results were also reported for *B. callensis* muscle protein (Sila et al., 2014) and caprine whey protein (Cheng et al., 2013).

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Table 4.3. Antibacterial activity of trout hydrolysates H₁–H₄ obtained by treatment with trout pepsin, compared to undigested trout viscera

Bacterial strain	Gram	Undigested viscera	Trout by-product hydrolysate			
			H ₁	H ₂	H ₃	H ₄
<i>A. media</i>	-	-	+	+	+	++
<i>A. salmonicida</i>	-	-	+	+	++	++
<i>F. araucanatum</i>	-	-	+++	++++	++++	++++
<i>F. psychrophilum</i>	-	-	++++	+++++	+++++	+++++
<i>R. salmoninarum</i>	+	-	+++	+++	++++	++++
<i>W. minor</i>	+	-	-	+	+	+
<i>W. paramesentoides</i>	+	-	+	+	++	++
<i>M. luteus</i>	+	-	+	+	++	++
<i>B. cereus</i>	+	-	+	++	++	++
<i>C. freundii</i>	-	-	+	+	+	+
<i>E. coli</i>	-	-	+	+	+	+
<i>Ent. faecalis</i>	+	-	+	++	++	++
<i>Pro. mirabilis</i>	-	-	++	+++	+++	+++
<i>P. fluorescens</i>	-	-	+	++	++	++

MIC: + 47 mg/ml, ++ 20 mg/ml, +++ 15 mg/ml, ++++ 5 mg/ml +++++ 2 mg/ml, - no activity; degree of hydrolysis (%): H₁ (10 %), H₂ (20 %), H₃ (25 %), H₄ (30 %)

Table 4.4: Antibacterial activity of trout protein hydrolysate (H₄) compared to nisin, piscidin-1 and oxytetracycline

Bacterial strain	Antibacterial agent (mg/20 µl)				
	trout hydrolysate (H ₄)		nisin	piscidin-1	oxytetracycline
	15 mg	1.5 mg	1.5 mg	0.02 mg	0.002 mg
<i>A. media</i>	+++	+	+	+	+++
<i>A. salmonicida</i>	++++	-	+	-	+++++
<i>F. araucanatum</i>	+++++	+++	-	-	+++
<i>F. psychrophilum</i>	+++++	++++	-	-	++
<i>R. salmoninarum</i>	+++++	+++	-	++	+++
<i>W. minor</i>	++++	-	+	+	++++
<i>W. paramesentoides</i>	++	-	+	+	+++
<i>M. luteus</i>	++++	++	+	++	+++
<i>B. cereus</i>	++	+	-	+	+++
<i>C. freundii</i>	++	+	-	+	+++
<i>E. coli</i>	++	+	-	+	+++
<i>Ent. faecalis</i>	++	+	-	+	++++
<i>Pro. mirabilis</i>	++	+	-	+	++
<i>P. fluorescens</i>	+++	+	-	+	++

Inhibition zone: +++++ >3.0 cm, ++++>2.5 cm, +++ > 2.0 cm, ++>1.5 cm, + 0.8–1.5 cm

The trout hydrolysates demonstrated a broad spectrum of activity and inhibited the growth of both gram-positive and gram-negative bacteria (Table 4.3). Depending on the bacteria, a peptide quantity of 47-2 mg/ml resulted in complete growth inhibition with an incubation time of 24 h and a bacterial concentration of $1-2 \times 10^6$ cfu/ml. For complete inhibition of bacterial growth, higher peptide concentrations were often necessary for hydrolysates of DH 10%, compared to hydrolysates with a higher degree of hydrolysis. Similarly, hydrolysates from *B. callensis* (Sila et al., 2014) and *Cancer irroratus* (Beaulieu et al., 2013) also exhibited a broad spectrum of activity against gram-positive and gram-negative bacteria.

The antibacterial peptides inhibited bacterial growth in the first few hours, as shown in Figure 4.2. This effect could also be observed at levels below the MIC. The positive controls (a: *E. coli*, b: *M. luteus*) exhibited a typical bacterial growth curve; after an incubation time of 24 h, bacterial concentrations of $1-2 \times 10^6$ cfu/ml and 1×10^7 cfu/ml were achieved for *E. coli* and *M. luteus*, respectively. As Figure 4.2 shows, both bacteria underwent a short lag phase, which was significantly prolonged by the presence of the antibacterial peptides. Depending on the degree of hydrolysis of the hydrolysates used, different exponential growth phase gradients were generated. After an incubation time of 24 h, the hydrolysate with DH 10 % (H_1) and DH 20 % (H_2) resulted in bacterial concentrations of $2-3 \times 10^7$ cfu/ml and $1-5 \times 10^7$ cfu/ml for *E.coli* and *M. luteus*, respectively.

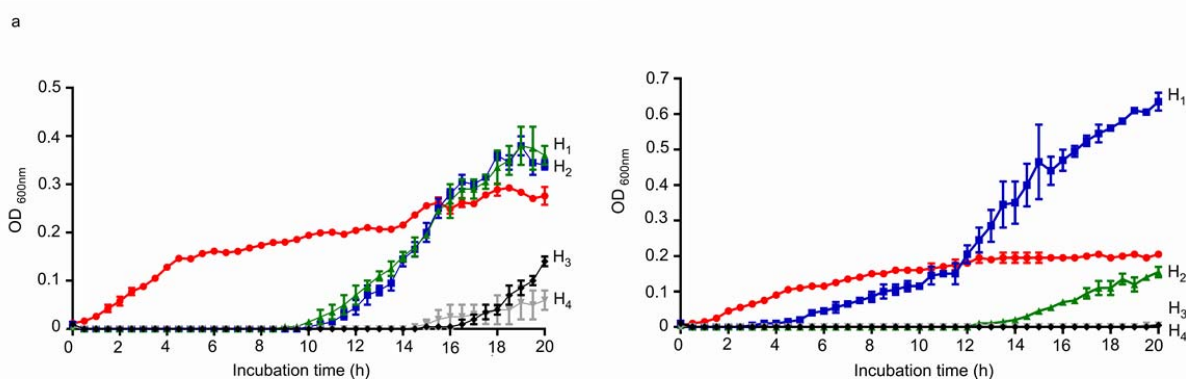


Figure 4.2. Inhibitory effect of the trout protein hydrolysates with different DH % (H_1 – H_4) on bacterial growth.

(a) represents the growth of *E. coli*, and (b) of *M. luteus*. The x-axis indicates the incubation time (h), and the y-axis the optical density (OD) measured at 600 nm. The initial bacterial concentration of $1-2 \times 10^6$ cfu/ml was determined by counting using a Thoma chamber, as well as the bacterial concentration at the end of the incubation period. The positive control (red line) shows bacterial growth without the addition of hydrolysates. H_1 – H_4 illustrates degrees of hydrolysis of 10 % (H_1), 20 % (H_2), 25 % (H_3) and 30 % (H_4), respectively. The error bars represent standard deviations from triplicate determinations.

A growth-promoting effect on the bacteria was observed for the raw substrate and hydrolysates with low degrees of hydrolysis (Figure 4.2). After an incubation period of 24 h, depending on the bacterium, the bacterial concentration increased up to ten-fold in comparison with the positive control. For example, Dufosse, De La Broise, and Guerard. (2001) demonstrated that fish protein hydrolysates may exert a growth-promoting effect on bacteria by serving as a source of nitrogen. Increased bacterial growth was also observed in the disk diffusion tests around the well (undigested extract), as well as in with decreasing peptide concentrations, in the form of an additional circle around the inhibition zone (data not shown).

The hydrolysates exhibited the greatest antibacterial effect and therefore the lowest MIC values for *Flavobacterium* sp. and *R. salmoninarum*, at 2 mg/ml and 5 mg/ml, respectively.

To compare the antibacterial activity of the hydrolysates with various antibiotics and antibacterial peptides, a plate diffusion test was carried out, and the inhibition zone diameters were then compared between treatments.

The antibiotics, oxytetracycline and erythromycin, which are often used in fish farming (Harnisz, Korzeniewska, & Gołaś, 2015; Moore et al., 2014), and the antibacterial peptides, piscidin-1 and nisin, which inhibit bacterial growth through a mechanism similar to that of most antibacterial protein hydrolysates, due to their interaction with anionic lipids in the bacterial membrane, were used as comparative samples (Moll et al., 1999). In contrast to antibacterial protein hydrolysates and piscidin-1, nisin consists of special amino acids with intermolecular thioether rings, such as lanthionine and β -methyl-lanthionine (Cheeseman et al., 1959) (Table 4.1).

The results of the microdiffusion test could be confirmed by the plate diffusion test (Table 4.4). Thereby, the hydrolysate also generated large inhibition zone diameters for the bacteria at low MIC values. The greatest growth inhibition by the hydrolysate was also observed for *Flavobacterium* sp. and *R. salmoninarum*. The effect of the hydrolysate on *Flavobacterium* sp. was significantly higher compared to the effect of nisin and matched that of oxytetracycline at a concentration that was 75 times greater. *R. salmoninarum* was inhibited by hydrolysate H₄ and by oxytetracycline and erythromycin at a concentration that was 750 times greater. The trout protein hydrolysate (H₄) exhibited up to 75-fold lower antibacterial activity compared to piscidin-1 (Table 4.3). In general, the antibacterial effect on food-borne microorganisms was lower, compared to the antibacterial effect on fish farming pathogens.

4.5. Conclusion

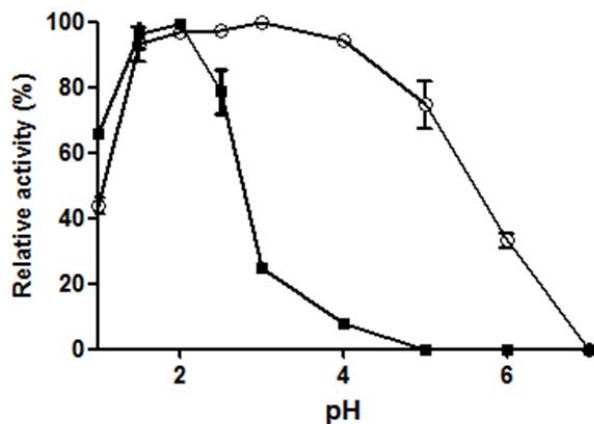
Enzymatic hydrolysis by trout pepsin was used to generate antibacterial hydrolysates from fish by-products. The degree of hydrolysis (DH%) was found to exert a considerable influence on antibacterial activity, with a significant increase in the observed inhibitory effect against food contaminants and fish farming bacteria at the beginning of hydrolysis.

The peptides predominantly prolonged the lag phase of bacterial growth. In addition, the broad spectrum of action, shows the high suitability of this hydrolysate as a preservative in food processing or to protect fish health in fish farming.

Conflict of interest:

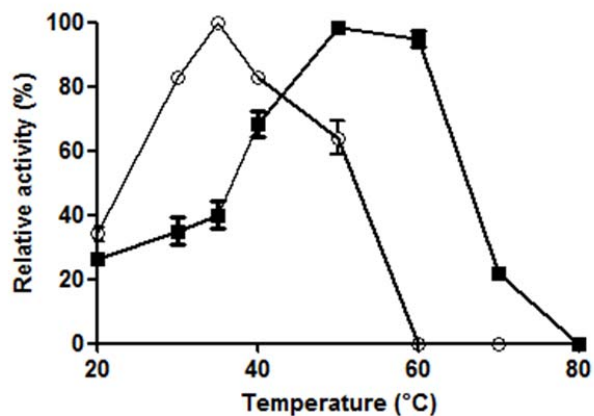
The authors declare that they have no conflict of interest.

4.6. Supplementary material



Supplementary Figure 4-1: Optimal pH of trout pepsins

Effect of pH on the activities for hemoglobin hydrolysis by purified trout pepsin and porcine pepsin A, the pH-activity curves are shown taking the maximal activity as 100% for each case, bars represent the standard deviation (n=3), trout pepsin (O), porcine pepsin (▲)



Supplementary Figure 4-2: Optimal temperature of trout pepsins

Effects of temperature on the activities for hemoglobin hydrolysis by purified trout pepsin and porcine pepsin, the temperature-activity curves are shown taking the maximal activity as 100 % for each case, bars represent the standard deviation (n=3), trout pepsin (O), porcine pepsin (▲)

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5. Antibacterial peptides isolated from pepsin catalyzed trout protein by-product hydrolysate via ion-exchange chromatography and gel filtration

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5.1. Abstract

Anionic and cationic antibacterial peptides were isolated via ion-exchange chromatography and gel filtration from trout protein hydrolyzate. The isolated peptides achieved an approximately nine-fold higher activity against the examined bacteria than the hydrolyzate. Cationic peptides with molecular weights below 1 kDa showed the broadest spectrum of activity. These peptides had the highest content of amino acids with hydrophobic side chains (66%). The peptides predominantly exhibited a prolongation effect during the lag phase of bacterial growth. Depending on the bacteria, peptide concentrations ranging from 1 mg/ml to 5 mg/ml resulted in a complete growth inhibition of the bacteria with a concentration of $1-2 \times 10^6$ cfu/ml (cfu: colony-forming units).

Keywords:

Cationic and anionic antibacterial peptides, trout protein hydrolyzate, ion-exchange chromatography, gel filtration

5.2. Introduction

The increased amount of antibiotic-resistant bacteria and a growing interest in the use of natural substances for food preservation have in recent years triggered great interest in the study of antimicrobial peptides (AMPs) [1,2].

Research conducted by Sila et al. [3], Beaulieu et al. [1], Doyen et al. [4] and Robert et al. [5] showed that in addition to a number of animal protein hydrolyzates from casein [6], ovotransferrin [7] and lactoferrin [8], fish protein hydrolyzates may represent sources from which AMPs can be isolated. The isolated peptides showed a low molecular weight that was < 10 kDa. They had a predominantly amphiphilic structure, a high content of hydrophobic amino acids, mostly cationic properties at physiological pH, and the major amino acids often include asparagine, glutamine, lysine, leucine, glycine, phenylalanine, and proline [1,5,9,10].

The efficacy of AMPs is affected by peptide hydrophobicity, their charge, and the peptide size [10]. Accordingly, Beaulieu et al. [11] and Cheng et al. [9] isolated AMPs from protein hydrolyzates using ion-exchange chromatography, hydrophobic interaction chromatography or gel filtration. In addition to chromatography methods, Doyen et al. [4] showed another method of separation, (electrodialysis), which enabled the simultaneous separation of the peptide mixture according to charge and size. This method increased purity by a factor 10 [4].

The molecular weights of the active fractions of fish and/or crustacean protein hydrolyzate (*Chionoectes opilio*, *Nile tilapia*, *Barbus callensis*, and *Cancer irroratus*) were between 200-3.624 Da [5,1,3,4]. Based on the average weight of an amino acid of approximately 110 Da [1], the peptides isolated by Robert et al. [5], Beaulieu et al. [1], Sila et al. [3] and Doyen et al. [4] can be assumed to have contained approximately 2-33 amino acids. In addition to cationic peptides, anionic antimicrobial peptides were also isolated from fish protein hydrolyzates (*Cancer irroratus* and *Chionoectes opilio*) [1,12].

AMPs exert their activity in a two-step process by (1) binding on the bacterial membrane and (2) disturbing the lipid matrix. The mechanism for the antibacterial effect and cell lysis is often attributed to the formation of pores in the membrane or via a blockage of the ion gradient; however, none of the mechanisms alone cause an antibacterial effect [10,13]. The cationic properties of the peptides are undoubtedly important for the initial electrostatic attraction of antibacterial peptides to negatively charged phospholipid membranes of bacteria. There is a

strong correlation between peptide cationicity and antibacterial activity; however, this relationship is not strictly linear. At a certain level, the antibacterial activity no longer increased according to an increasing positive charge of the peptides [10]. Dathe et al. [13], for example, indicated that the limit value for magainin is a net charge of + 6 to + 7. Generally, extremes of certain properties, such as hydrophobicity, amphipathicity and peptide charge, discourage the antibacterial activity of peptides [10]. The high diversity of AMPs and thus the differential antimicrobial activity versus the target cells may be a strategy to prevent or delay the tendency of the bacteria to become resistant against these AMPs [10].

The aim of this study was the isolation of active peptides from the trout protein hydrolyzate using a combination of ion-exchange chromatography and gel filtration. The effectiveness of the isolated peptides against pathogens affecting fish farming and those that induce food spoilage was studied and compared with the efficacy of the crude hydrolyzate. The effectiveness of the peptide in relation to its charge (cationic / anionic), size (> 3 kDa, $1-3$ kDa < 1 kDa) and amino acid composition was illustrated. For comparison, the peptide Piscidin- 1, isolated from the tissues of hybrid stripe bass (*Morone saxatilis*), was used. Piscidin is the first antibacterial amphipathic cationic peptide family identified in fish, whereas piscidin- 1 indicated the highest antibacterial activity [14].

5.3. Materials and Methods

5.3.1. Materials:

Piscidin-1 (Bio World, Dublin USA), R₂A Bouillon (Carl Roth, Karlsruhe), Müller Hinton broth (Sigma Aldrich, St Louis), MRS Bouillon (Carl Roth, Karlsruhe), sodium dihydrogen phosphate, di-sodium hydrogen phosphate (Merck, Darmstadt, Germany), NaCl (99 %) (VWR, Prolabo, Darmstadt), gel filtration calibration proteins and peptides: lysozyme (14 kDa), insulin (3.4 kDa), bacitracin (1.2 kDa), Asp-Phe-MeOH (0.294 kDa) (Sigma Aldrich, St. Louis).

5.3.2. Bacteria stains:

Aeromonas media (ATCC 33907 fish farm effluent), *Aeromonas salmonicida* (ATCC 33659, trout), *Flavobacterium psychrophilum* (ATCC 49511, kidney of diseased rainbow trout fry), *Flavobacterium araucanum* (LM-20 FP, external lesion of *Salmo salar* from a fish farm), *Renibacterium salmoninarum* (ATCC 33209, yearling Chinook salmon), *Weissella minor* (ATCC 35412, milk machine slime), *Weissella paramesenteroides* (ATCC 33313), *Citrobacter freundii* (ATCC 8090), *Pseudomonas fluorescens* (ATCC 13525), *Proteus mirabilis* (ATC 29906), *Escherichia coli* (K12 E851), *Micrococcus luteus* (ATC 4698), *Bacillus cereus* (ATC12826), and *Enterococcus faecalis* (ATCC 51299) were used in this study. All bacteria were purchased from Leibniz Institute DSMZ- German Collection of Microorganism and Cell Culture.

5.3.3. Trout pepsin

Pepsin was isolated from the stomach of rainbow trout by a 4-step procedure including ammonium sulfate precipitation, anion exchange chromatography and two subsequent gel filtration steps (online version of the paper doi:10.1007/s00217-016-2692-2). The related enzyme exhibited a specific enzyme activity of 6.5 U/mg protein, as measured by the method described by Ryle [15] using acid denatured hemoglobin as a substrate. The trout pepsin exhibited optimal enzyme activity at pH 3.0 and 37 °C using hemoglobin as substrate.

5.3.4. Trout viscera

Rainbow trout (weight: 250 g, age: 18 months) were provided by a fish farm located in Hesse, Germany. Two weeks before the fish were sacrificed, feeding was stopped, which resulted in empty viscera. After the fish were gutted, the viscera (throat, stomach and intestines) were

collected, ground using a mincer and stored in polyethylene bags at -20 °C until they were used digested using pepsin.

5.3.5. Enzymatic hydrolysis of trout by-products

For the enzymatic cleavage of trout viscera via pepsin, the hydrolysis was produced according to procedure described previously [16].

5.3.6. Isolation of antibacterial fractions

For this purpose, the freeze-dried hydrolyzate with 30 % hydrolysis ($c = 1.5$ mg/ml) was dissolved in 20 mM sodium-phosphate buffer at pH 7.0. The suspension was centrifuged for 10 min at 10000 g to separate undissolved ingredients, and the supernatant was collected and used for further purification. The supernatant was put onto a DEAE anion-exchange column (1.6 x 10 cm, GE healthcare) that was previously equilibrated with phosphate buffer (20 mM, pH 7.0); the flow rate was 5 ml/min, the fraction volume 10 ml, the injection value 1 ml and the sample loop size 2 ml and the detection wavelength 214 nm. Cationic peptides were removed by washing with buffer until the absorbance at 214 nm reached baseline. A linear gradient of 0–1.0 M buffered NaCl was applied to elute anionic peptides at a flow rate of 5 ml/min. The liquid chromatography system Äkta plus (GE healthcare) was used. PrimeView 5 was used to plot, acquire and analyze chromatographic data.

The fractions were collected, freeze-dried for 24 hours at 1 mbar and -85 °C (Christ Alpha 2-4 LPC) and separated via gel filtration (HiLoad 16/600, Superdex 30 pg, GE Healthcare).

A total of 0.4 g of the freeze-dried sample was dissolved in 2 ml of distilled water and centrifuged under the previously described conditions. The cationic and anionic fractions were applied to a gel filtration column (HiLoad 16/600, Superdex G75, GE Healthcare) equilibrated with 20 mM sodium-phosphate pH 7.0 in 0.15 M NaCl and eluted at a flow rate of 0.6 ml/min. The fraction volume was 10 ml, the injection value 1 ml, the sample loop size 2 ml and detection occurred at 214 nm. The samples were then freeze-dried under the conditions described above and stored at 7 °C for further analysis of their antibacterial activity and characterization of the cationic and anionic fractions.

5.3.7. Peptide characterization

5.3.7.1. Determination and the molecular mass of cationic and anionic peptides

Gel filtration was performed on a Varian system (Waters Alliance 2690) equipped with a UV (214 nm wavelength) detector. Elution was carried out on a gel filtration column (HiLoad Superdex 30 pg) with 20 mM sodium-phosphate buffer (pH 7.0) containing 0.15 M NaCl. The cationic and anionic peptides were dissolved in the same buffer, and 50 μ l was injected into the instrument. The gel filtration system was calibrated with four molecular mass markers: lysozyme (14 kDa), insulin (3.4 kDa), bacitracin (1.2 kDa) and Asp-Phe-MeOH (0.294 kDa) with a concentration of 1 mg/ml. The measurement was repeated three times. The liquid chromatography system consisted of a Waters alliance 2690 and a Waters 996 photodiode array detector was used. Millennium software was used to plot, acquire and analyse chromatographic data. The molecular mass distribution of antibacterial and antioxidative peptides were also analyzed by gel filtration as described by Sila et al. [3], Robert et al. [5] and Nalinanon et al. [17].

5.3.7.2. Amino acid analysis

The amino acid composition of the trout protein hydrolysate (H_4) and cationic and anionic antibacterial peptides were analyzed according to the method of Spackman et al. [18], with slight modifications (double determination). The lyophilized hydrolysate (H_4) and the cationic and anionic peptides with a concentration of 0.45 mg/ml were incubated with 6 M HCl for 60 h at 110 °C. After the hydrolysis, the sample was dried (vacuum centrifuge) and resolved in 500 μ l sodium-acetate buffer pH 2.2. The amino acids were separated by ion-exchange chromatography, therefore 20 μ l of the sample was loaded onto an amino acid analyzer LC 3000 (Eppendorf-Biotronik) equipped with a polymer cation-exchange column (125 x 4 mm), followed by a post-column derivatization with ninhydrin at 125 °C. The detection was carried out at 570 nm and 440 nm for proline. The chromatographic data were stored on the software ChromStar 6.0. This method was already described by Robert et al. [5] to determine the amino acid content of tilapia protein hydrolysate.

5.3.7.3. Determination of peptide concentration

0.5 g of cationic and anionic peptides were suspended in 1 ml of distilled water and centrifuged at 10000 x g for 10 min, then separated onto a Superdex Hiload 30 pg (GE Healthcare) gel filtration

column (600 mm x 16 mm) pre-equilibrated and eluted with 20 mM sodium-phosphate buffer, pH 7.0, with 0.15 M NaCl at room temperature. The flow rate was 0.6 ml/min, the detection wavelength 214 nm, the injection value 10 μ l and the sample loop size 250 μ l. To determine the peptide content of the sample the area under the curve of the sample was compared against the reference standard (Bacitracin A) [19]. Bacitracin was taken as standard, because this 1.4 kDa protein reflected the average molecular mass of the hydrolysate. The liquid chromatography system consisted of a Waters alliance 2690 and a Waters 996 photodiode array detector was used. Millennium software was used to plot, acquire and analyze chromatographic data. The measurement was repeated three times.

5.3.8. Antibacterial effect of cationic and anionic peptides obtained from fish by-product hydrolyzate (DH 30 %)

The antibacterial effect of the anionic and cationic peptides with molecular weights of > 3 kDa, >1 kDa and < 1 kDa (Fr. I-III) were determined using the minimum inhibitory concentration (MIC) method described by Zhang et al. [20].

The antibacterial activity was compared with the effect of the antibacterial peptide Piscidin-1. In the test series, 24 h cultures were used, with the exception of *Flavobacterium*, for which a 48 h culture was used. The total bacterial count, as determined via counting in a Thoma chamber, amounted to a bacterial suspension of $1-2 \times 10^6$ cfu/ml (cfu: colony-forming units).

Twenty microliters of the peptide solutions with a concentration of 10 mg/ml - 1 mg/ml were mixed with 20 μ l of the bacterial suspension. After a 3 h incubation period under optimum conditions, 50 μ l of culture media was added to each well for further incubation. The number of bacteria was determined by measuring the change in the optical density (OD) at 600 nm value on a microliter plate reader (Bioscreen C, Sartorius). The experiment was repeated thrice. For the sample blank, 20 μ l of hydrolyzate solution was mixed with 20 μ l of physiological saline solution (Ringer's solution). For the bacterial blank, 40 μ l of physiological saline solution was used. For the bacterial positive control, 20 μ l of physiological saline solution was mixed with 20 μ l of bacterial suspension.

5.3.9. Statistical analysis

All data were submitted to Analysis of Variance (ANOVA) and differences between means were evaluated by Bonferroni test. The data were analyzed using the software GraphPad Prism (version 5). Differences were considered significant at $p < 0.05$.

5.4. Results and Discussions

The hydrolysis of trout by-products with trout pepsin was carried out using the pH stat method. A final level of 30 % hydrolysis was achieved. This was in accordance with the study by Nalinanon et al. [17], who reported 30 % hydrolysis of muscle proteins (*Nemipterus hexodon*) with *tuna pepsin* (isolated from *Katsuwonus pelamis*). In contrast to this study, the hydrolysis of muscle proteins of *Mustelus mustelus* resulted in a significantly lower DH (9.2 %) using the fish's own pepsin [21].

5.4.1. Peptide characterization

To characterize the purification process of the antibacterial peptides obtained from trout protein hydrolyzate, the peptide contents (at 214 nm) were determined after each purification step. Of 100 % hydrolyzate (378 mg), 63 % (238 mg) cationic and 29 % anionic peptides (109 mg) were generated, resulting in an overall yield of 92 %. All peptides that did not bind to the column resin group (DEAE 1.6x10 cm, GE Healthcare) during anion-exchange chromatography were defined as cationic peptides (I); those that did bind to the eluent (gradient 1 M NaCl) and were thus separated by the column resin were defined as anionic peptides (II) (Figure 5.1). The enzyme yields in the present study were comparable to those by Beaulieu et al. [11] who used cation-exchange chromatography to generate a total of 88 % peptides.

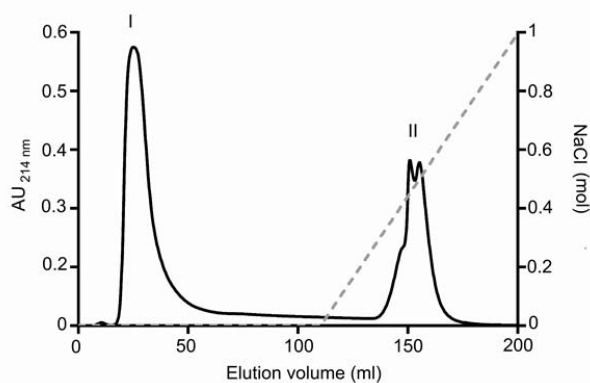


Figure 5.1: Separation of trout protein hydrolyzate via anion-exchange chromatography.

Cationic peptides were demonstrated via I and anionic peptides after elution with NaCl with II, respectively. DEAE cellulose column 16/600, flow rate: 5 ml/min, detection 214 nm, elution with a linear gradient of 1 M NaCl in the elution buffer.

Chapter 5. Antibacterial peptides isolated from pepsin catalyzed trout protein by-product hydrolyzate via ion-exchange chromatography and gel filtration

The cationic and anionic peptides were then separated via gel filtration into the following peptide clusters > 3 kDa, 1 kDa - 3 kDa and < 1 kDa. Due to the high degree of hydrolysis (30 %), most peptides were di- and tripeptides with a molecular weight of less than 1 kDa (53.4 % / 41.3 %) and oligopeptides with molecular weights between 1 and 3 kDa (37.1 % / 57.4 %); there was only a small fraction of polypeptides > 3 kDa (9.6 % / 1.3 %) (Figure 5.2 a and b). Based on the average weight of amino acids [1], most peptides could be concluded to contain less than 15 amino acids.

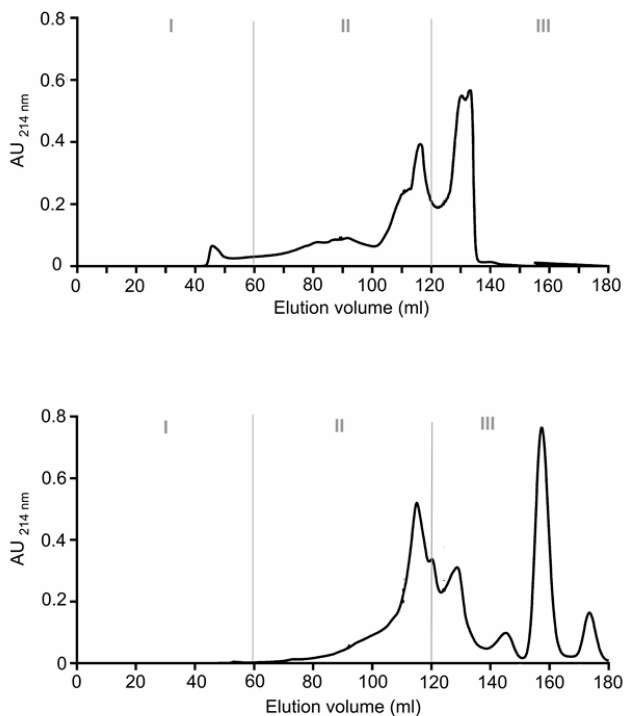


Figure 5.2: Molecular weight distribution of anionic and cationic peptides.

Anionic peptides (Figure 5.2 a) and cationic peptides (Figure 5.2 b). Peptide separation into three peptide fractions as illustrated by I (> 3 kDa), II ($1 - 3$ kDa), III (< 1 kDa). HiLoad Superdex gel filtration column: 30 μ g, flow rate 0.6 ml/min, detection 214 nm, 20 mM sodium phosphate buffer pH 7.0.

5.4.1.1. Amino acid analysis

The amino acid composition of trout protein hydrolyzate and antimicrobial cationic and anionic peptides with molecular weights > 3 kDa (Fr. I), > 1 kDa (Fr. II) and <1 kDa (Fr. III) are shown in Table 5.1.

The cationic and anionic fractions showed significant differences regarding their amino acid composition. These fractions exhibited a balanced ratio between amino acids with hydrophobic and hydrophilic properties, with the exception of Fr. III of the cationic peptides, which exhibited a higher proportion of amino acids with hydrophobic properties (correlation factor 2/1). The correlation factors were in the same range (1/1.4 and 1/1.3) in the studies by Cheng et al. [9] and Robert et al. [5].

The amphipathic structures of peptides significantly impact antibacterial activity, which depends on the hydrophobic moment (M_H) containing the hydrophobicities of the amino acid residues and their orientation and distribution in the protein structure [22,23]. Increasing hydrophobicities, which is determined by the percentage of hydrophobic amino acids, leads to increased membrane permeability by peptides as demonstrated via model peptides against target membranes [10]. Additionally, Wieprecht et al. [24] observed that increasing peptide hydrophobicity resulted in a higher permeabilization of phosphatidylcholine (PC) and phosphatidylglycerol (PG) membranes.

In addition to the amphipathic structures, the membrane interactions (especially partitioning into the lipid bilayer and the specificity of the antimicrobial peptides) were affected by peptide hydrophobicity [10]. The highest proportion of hydrophobic amino acids were found for Fr. II and III of the cationic peptides (50.95 % and 65.95 %, respectively) (Table 5.1), which is in accordance with the finding that most antibacterial peptides consist of approximately 50 % hydrophobic residues [10,9]. Additionally, Chen et al. [25] demonstrated that increasing peptide hydrophobicity resulted in higher antibacterial activity up to a certain threshold. Hydrophobicity beyond this threshold could lead to a higher self-association of the peptides, which prevents the peptides from passing through the cell wall in prokaryotic cells. However, increasing peptide hydrophobicity results in a lower antibacterial specificity [25].

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Table 5.1: Amino acid content (%) and hydrophobic and hydrophilic properties of fish protein hydrolyzate and cationic and anionic peptides

Amino acids (aa)	Amino acid content (%)					Tilapia ^{*1} hydrolyzate	k-casein ^{*2} hydrolyzate
	DH 30 %	Fr. I (cp.)	Fr. II (cp.)	Fr. III (cp.)	Fr. II (ap.)		
hydrophobic amino acids							
isoleucine	2.59	3.29	5.92	1.63	3.14	2.66	6.19
phenylalanine	2.35	1.69	7.91	1.74	3.62	2.64	5.20
leucine	4.63	4.84	13.04	1.90	5.65	4.68	9.45
valine	3.26	4.92	5.16	1.83	3.86	3.14	5.83
tryptophan	0.81	1.68	0.03	32.55	0.00	0.00	0.00
methionine	1.77	2.17	5.01	0.00	2.40	1.71	2.20
alanine	4.35	8.38	5.22	2.99	3.75	5.06	5.07
glycine	5.34	15.09	3.92	18.73	9.57	7.00	2.28
cysteine	0.16	0.27	0.19	0.75	0.28	0.53	0.00
tyrosine	2.24	0.84	4.55	3.83	4.29	1.94	4.95
Total	27.50	43.17	50.95	65.95	36.56	29.36	41.55
hydrophilic amino acids							
proline	3.80	9.55	3.07	1.50	3.20	4.82	10.25
threonine	2.79	4.98	3.39	2.31	4.09	3.03	4.40
serine	2.36	4.69	3.05	5.39	3.76	3.01	4.87
histidine	1.85	2.30	2.80	4.96	2.68	1.53	2.84
glutamic acid	8.71	14.15	10.02	10.88	24.89	9.57	17.13
aspartic acid	5.84	10.45	5.39	4.21	18.49	6.26	7.57
lysine	5.11	6.65	7.14	2.35	2.89	5.09	7.68
arginine	4.80	4.01	8.42	1.80	2.01	4.91	3.71
Total	35.26	56.78	43.28	31.90	62.01	38.22	58.45
Cf hydrophobic aa/hydrophilic aa	1/1.3	1/1.3	1.2/1	2/1	1/1.7	1/1.3	1/1.4
Hydrophobicity (%)	28	43	51	66	37	29	42

cp. cationic peptides, ap. anionic peptides, Robert et al. [5] ^{*1}, Cheng et al. [9] ^{*2}

As indicated in Table 5.2, antibacterial peptides showed a high content of amino acids with positive net charges (between 7.9-20.1 %) under the assay conditions (pH 7.0). Cationic peptide fraction II indicates a high concentration of lysine (7.1 %) and arginine (8.42 %); these amino acids are well known to undergo a strong interaction with phosphate groups in lipid bilayers via electrostatic force [26]. Comparable concentrations of positively charged amino acids were found for k-casein hydrolyzate (16.9 %) and tilapia protein hydrolyzate (11.9 %) [9,5].

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Table 5.2: Proportion of positively charged amino acids and negatively charged amino acids of fish protein hydrolyzate and cationic and anionic peptides

Amino acids	pK	net charge	Amino acid content (%)				
			cationic peptides			anionic peptides	
			DH 30 %	Fr. I	Fr. II	Fr. III	Fr. II
positively charged							
lysine	10.80	+ 1	5.11	6.65	7.14	2.35	2.89
arginine	12.50	+ 1	4.80	4.01	8.42	1.80	2.01
tyrosine	10.90	+ 1	2.24	0.84	4.55	3.83	4.29
Total			12.15	11.50	20.11	7.98	9.19
negatively charged							
aspartic acid	4.10	-1	5.84	10.45	5.39	4.21	18.49
glutamic acid	4.10	-1	8.71	14.15	10.02	10.88	24.89
Total			14.55	24.60	15.41	15.09	43.38

Bessalle et al. [27] demonstrated that the addition of 10 or 20 lysine residues enhanced the antibacterial effect of magainin. Additionally, Yeaman, Yount [10] reported that many antibacterial peptides display net positive charges ranging from + 2 to + 9, Piscidin-1, for example indicates a net charges of + 7 (datasheet, Bio World, Dublin USA). The minimum threshold for antibacterial activity and selective toxicity is approximately + 2. The relationship between cationicity and antibacterial activity is not entirely linear. As described by Dathe et al. [28], net charges beyond + 5 resulted in an increase in the undesired hemolytic effect and a loss of antimicrobial selectivity.

Furthermore, peptide Fr. II of the cationic peptides demonstrated a particularly high content of taurine (6.05 %). However, Beaulieu et al. [1] and Robert et al. [5] reported substantially lower concentrations (1.76 % and 0.79 %) in snow crab and tilapia hydrolyzate by-product..

5.4.2. Antibacterial effect of cationic and anionic peptides after purification

The antibacterial effect of the cationic and anionic peptide fractions with molecular weights of > 3 kDa, > 1 kDa and < 1 kDa were determined using the liquid grow inhibition assay [20]. To quantitate the antibacterial activity, the minimum inhibitory concentration (MIC) was determined at a concentration ranging between 10 mg/ml and 1 mg/ml. The MIC value indicates the lowest concentration of an antibacterial agent that has a 100 % inhibitory effect on bacterial growth [29].

The purification process led to a significant reduction in the minimum concentration required for inhibiting bacteria growth. The peptide concentration necessary to achieve a complete inhibition of the bacterial growth of *A. media*, *A. salmonicida*, *W. paramesenteroides*, *M. luteus* and *B. cereus* in 24 hours was reduced compared with that for the trout protein hydrolyzate, (from 20 mg/ml to 5 mg/ml). For stopping the growth of *W. minor*, *C. freundii* and *E. coli*, the required peptide concentration was more than 9-fold lower (47 mg/ml to 5 mg/ml) compared to the unpurified hydrolyzate. Using an antibacterial peptide derived from *Chionoecetes opilio* hydrolyzate via electroseparation, Doyen et al. [4] achieved a comparable degree of purification (10-fold) with a MIC value of 50 mg/ml after purification. Thus, the efficacy of purified *Chionoecetes opilio* hydrolyzate fraction KC11 (14 V/cm 50 kDa) against *E. coli* (10^7 CFU / ml) should be regarded as significantly lower than that for the purified trout peptides (approximately 5 mg/ml).

For *F. psychrophilum*, *F. araucanum* and *R. salmoninarum*, the purification process resulted in reductions in MIC values (from 2 and 5 mg/ml, respectively) to 1 mg/ml. After separating the polypeptide mixture according to charge and size, only cationic peptides exhibited antibacterial activity against Flavobacteria and *R. salmoninarum*. However, the antimicrobial effect could not be attributed to individual peptides because peptides > 3 kDa and < 1 kDa exhibited antibacterial activity against *F. psychrophilum* and *F. araucanum*. *R. salmoninarum* could even be inhibited by all cationic peptide fractions (Table 5.3). The purification process in some cases only led to a slight reduction in the MIC value; this could be attributed to synergistic effects between the peptides and between the peptides and non-peptides present in the mixture. Synergistic effects between various antibiotic agents were described by Rushing, Senn [30] (in discussions of the combination of sorbic acid and benzoic acid) and by Lin et al. [2] (in analyses of the combination

of peptide and non-peptide antibiotics). Additionally, cationic and non-cationic peptides appeared to demonstrate a synergistic effect in improving the immune response [31,1].

Concentrations below the MIC value led to a significant reduction in bacterial growth, as demonstrated in the cases of *A. salmonicida* and *B. cereus* (Figure 5.3 a and b). Regarding *A. salmonicida* and *B. cereus*, a peptide concentration of 1 mg/ml resulted in a significant prolongation of the lag phase. At the end of the incubation period, the bacteria content was only approximately 50 % that of the positive control.

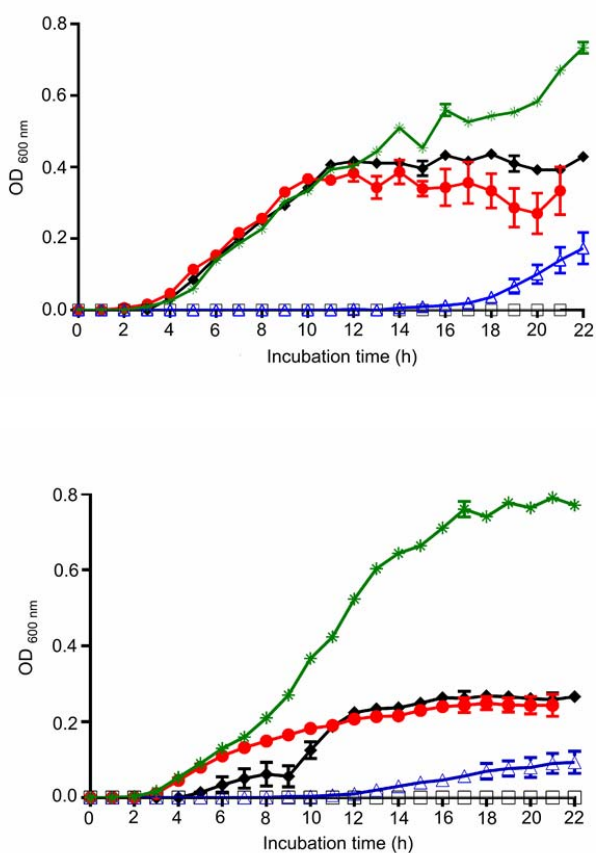


Figure 5.3: Inhibitory effect of the cationic peptide Fr. III (Figure 5.3 a) and anionic peptide Fr. II (Figure 5.3 b) on bacterial growth at various peptide concentrations.

Figure 5.3 a: *A. salmonicida* and 5.3 b: *B. cereus*, incubation time (24 h), initial bacterial concentration of $1-2 \times 10^6$ cfu/ml. x-axis: incubation time (h) and y-axis: optical density (OD) at 600 nm. The positive control (●) shows bacterial growth without the addition of peptides, (◆) indicated the inhibitory effect of Piscidin-1 (0.01 mg/ml) and the inhibitory effect of the peptides at various concentrations were indicated as follow: 5 mg/ml (□), 1 mg/ml (Δ) and 0.5 mg/ml (*), respectively. The error bars represent standard deviations from triplicate determinations.

Chapter 5. Antibacterial peptides isolated from pepsin catalyzed trout protein by-product hydrolyzate via ion-exchange chromatography and gel filtration

After the purification process, predominantly cationic peptides were effective against a variety of bacteria. Peptides with a molecular weight below 1 kDa inhibited the growth of 11 of the studied bacteria. Only 3 types of bacteria were not inhibited by these peptides (Table 5.3). In addition to a low molecular weight, these peptides tended to display a high proportion (66 %) of amino acids with hydrophobic side chains (Table 5.1).

Piscidin-1 exhibited an antibacterial activity against all of the studied bacteria (Table 5.3). As shown in Figure 5.3 b, piscidin-1 caused a small prolongation effect for the lag phase of the growth of *B. cereus* under the MIC (0.01 mg/ml) value. A concentration of 0.1 mg/ml leads to a total inhibition of *A. salmonicida* and *B. cereus*, whereas Piscidin-1 exhibited no effect of the growth of *A. salmonicida* under the MIC value (Figure 5.3 a). Campagna et al. [32] attributed the antibacterial activity of Piscidin-1 to the high content of positively charged residues (two arginines, one lysine and four histidines) and the ability to form an amphipathic helical structure in membrane mimicking environments.

Table 5.3: Antibacterial activity of anionic and cationic peptides compared with Piscidin-1

Bacteria	cationic peptides			anionic peptides			Piscidin-1 2.5 kDa
	Fr. I	Fr. II	Fr. III	Fr. I	Fr. II	Fr. III	
<i>A. media</i>	+	-	-	-	-	-	+
<i>A. salmonicida</i>	-	-	+	-	-	-	+
<i>F. araucanum</i>	+	-	+	-	-	-	+
<i>F. psychrophilum</i>	+	-	+	-	-	-	+
<i>R. salmoninarum</i>	+	+	+	-	-	-	+
<i>W. minor</i>	+	+	+	-	+	-	+
<i>W. paramesenteroides</i>	+	+	-	-	-	-	+
<i>M. luteus</i>	+	+	+	-	+	-	+
<i>B. cereus</i>	-	-	+	-	+	-	+
<i>C. freundii</i>	-	-	+	-	+	-	+
<i>E. coli</i>	-	-	+	-	+	-	+
<i>Ent. faecalis</i>	-	-	+	-	+	-	+
<i>Pro. mirabilis</i>	-	-	-	-	+	-	+
<i>P. fluorescens</i>	-	-	+	-	+	-	+

c = 5 mg/ml

Chapter 5. Antibacterial peptides isolated from pepsin catalyzed trout protein by-product hydrolyzate via ion-exchange chromatography and gel filtration

This is in accordance with the fact that peptide cationicity is important for antibacterial activity because cationicity is a prerequisite for the initial electrostatic attraction of antibacterial peptides to the negatively charged bacteria membrane impacted by lipopolysaccharides and teichoic acids [27]. A strong correlation between peptide cationicity and antibacterial activity was demonstrated by Bessalle et al. [27]. Additionally, the high specificity of AMPs towards bacteria is often attributed to the described electrostatic attraction [33,10].

In several of the tested bacteria, especially in those causing food spoilage, it was also possible to achieve complete growth inhibition using anionic peptides with a molecular weight between 1 and 3 kDa. Anionic antibacterial peptides with antimicrobial activity against *E. coli*, *L. innocua*, *Aeromonas sp.*, *Listeria monocytogenes*, *Morganella morganii*, *Shewanella putrefaciens*, *Staphylococcus sp.* and *Vibrio vulnificus* were isolated from fish protein hydrolyzates derived from *Chionoectes opilio* [4] and *Cancer irroratus* [1]. As indicated by Brogden et al. [34], x-ray spectral microanalysis did not prove an interaction between the antibacterial anionic peptide "SAAP" and the bacterial membrane. Therefore, Brogden et al. [34] attributed the antibacterial effectiveness either to the pH of the surfactant (which contained anionic peptides) or to an effect exerted on bacterial metabolism.

5.5. Conclusion

Trout protein hydrolyzate contained both cationic and anionic antibacterial peptides. Following purification via ion-exchange chromatography and gel filtration, the microbial activity of the peptides against many of the bacteria used in this study was increased (3- to 9-fold). Anionic peptides primarily exhibited an antibacterial activity against bacteria causing food spoilage. The peptides especially influenced the lag phase of bacterial growth; bacterial proliferation was thereby significantly delayed. This and the broad spectrum of activity demonstrate the suitability of peptides for various purposes: they can be used as a preservative in food processing and for reducing bacteria in fish farming.

Conflict of interest:

The authors declare that they have no conflict of interest.

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6. General Discussion

In this thesis, it was shown that trout pepsin can be used to generate antibacterial hydrolysates from trout by-products (viscera). Two isolation and purification processes for pepsin from the stomach of rainbow trout were applied. The first isolation method permits detailed enzyme characterization and achieves a high purification factor. For industrial applications, the high enzyme yields and low purification cost of method 2 are very attractive. Hydrolysis with the fish's own pepsin, which is currently not used in industrial applications, resulted in high degrees of hydrolysis and yielded peptides with high efficacy against gram-positive and gram-negative food contaminants and pathogens in fish farming. Figure 6.1 gives an overview of the isolation processes of pepsin and the use of this enzyme for the production of antibacterial peptides from fish by-products. Antibacterial peptides were further purified via ion-exchange chromatography and gel filtration. Thereby, it was proven that predominantly cationic peptides exhibit high activity against these bacteria. More detailed information explaining the purification and isolation process of pepsin and antibacterial peptides is given below.

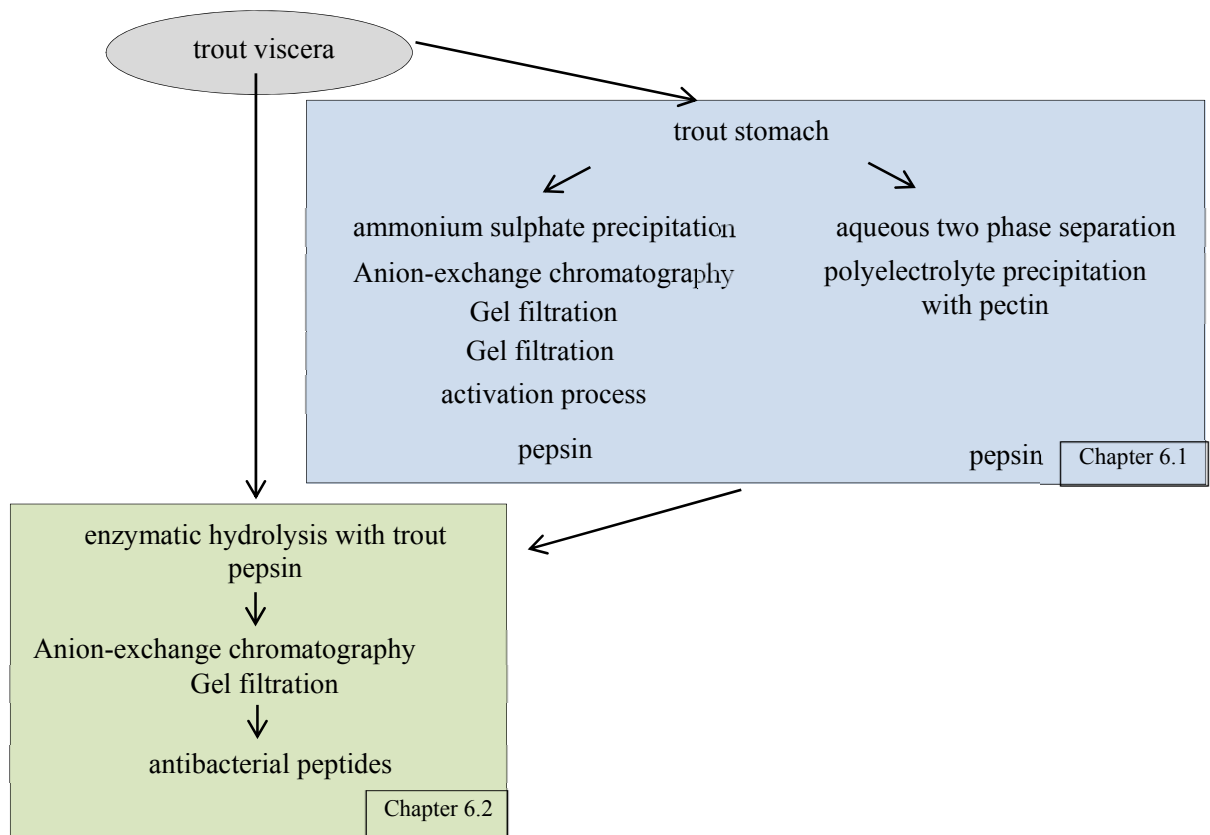


Figure 6.1: Overview of the isolation and purification of pepsin and antibacterial peptides from fish by-products

6.1. Isolation of pepsinogen from the stomach of *Oncorhynchus mykiss*

6.1.1. Conventional Method

6.1.1.1. Purification process:

From the stomach of rainbow trout, three pepsinogen isolates containing seven pepsinogen isoforms were purified by ammonium sulphate precipitation, anion exchange chromatography and gel filtration chromatography. Via ammonium sulphate precipitation, an enrichment of pepsinogen with a purification factor of 1.8 and an enzyme yield of 77 % were achieved using a combination of 30 % and 50 % ammonium sulphate saturation. The required ammonium sulphate concentration for precipitation of pepsinogens from rainbow trout was lower than for mandarin fish pepsinogen (60 % ammonium sulphate saturation, 1.5-fold purification) (Zhou et al., 2008) and smooth hound (70 %, 3.71-fold) (Bougatef et al. 2008), corroborating the findings of Twining et al. (1983).

To separate the various pepsinogen isoforms, ion-exchange chromatography (DEAE sepharose, 1.6 x 10 cm, GE Healthcare, Little Chalfont, United Kingdom) was used. In the elution profile (280 nm), three peaks with protease activity were identified and assigned to pepsinogen isolate (PG) I-III according to their elution time. PG I eluted in the fraction of unbound proteins, whereas PG II and III eluted at 0.39 M and 0.67 M NaCl, respectively; similar results were reported for pepsinogens from the European eel, *Anguilla anguilla* (Wu et al., 2009). The fractions from each peak were pooled, dialysed, concentrated and subjected to gel filtration using a HiLoad 16/600 Superdex G75 column to remove contaminating proteins. This procedure was repeated to improve the purity of the pepsinogens, as shown in Figure 6.2. After enzyme activation in an acid environment (pH 2.0, 60 min), the purity and molecular weights of the pepsins (P) were determined by gel filtration, as shown in Figure 6.2. Under native conditions, pepsins show the following molecular weights: 35 kDa (P-I), 38 kDa (P-II), and 37 kDa (P-III). The purification process leads to specific enzyme activities ($U_{AU\ 280nm} / mg\ protein$) of 2.1 (P I), 22 (P II) and 38 (P III), enzyme recoveries of 0.2 (P I), 6 (P II) and 4 (P III) and purification factors of 8 (P I), 58 (P II) and 100 (P III) compared to the crude extract. These results demonstrate that P II represented the major pepsin group. Comparison of the results with those for other fish species demonstrates that the degrees of recovery and enrichment are within the same range (Table 6.1).

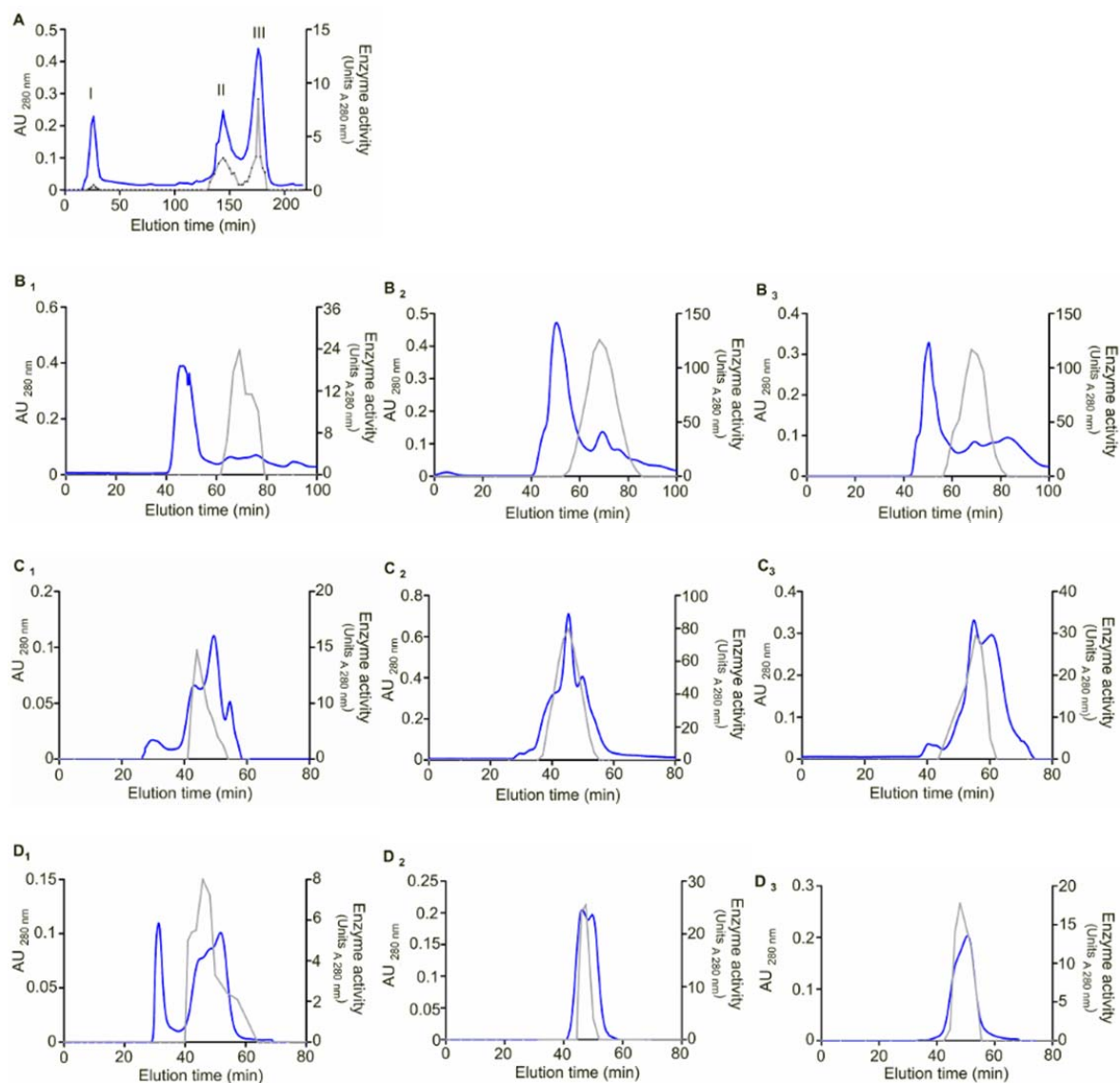


Figure 6.2: Purification of trout pepsinogen by column chromatography.

(A) DEAE cellulose chromatography, (B) Superdex gel filtration purification of pepsinogen isolates I-III (c) second gel filtration step (Superdex G 75) and (D) gel filtration after the activation process. Left y-axis Δ 280 nm and right y-axis enzyme activity (units) at each elution time (x-axis)

Table 6.1: Comparison of purification of trout pepsin with the results reported for other fish species

Fish species	Pepsin fractions	Purification fold
<i>Oncorhynchus mykiss</i>	I	8
	II	58
	III	100
<i>Anguilla anguilla</i> (Wu et al., 2009)	I	28
	II	36
	III	64
<i>Hoplostethus atlanticus</i> (Xu et al., 1996)	I	74
	II	174
<i>Lateolabrax japonicas</i> (Cao et al., 2011)	I	7.9
	II	6.6
	III	12.5

6.1.1.2. Enzyme characterization

The pepsinogen isolates exhibited major differences in their N-terminal amino acid sequences (up to 20 amino acids), kinetic properties, isoelectric points, enzyme activation processes from pepsinogen to pepsin, and sensitivity to pepstatin A. Overviews of the major differences are discussed below and shown in Table 6.2. In addition, electrophoretic tests indicated that pepsinogen I contains two pepsinogen isoforms with different molecular weights (45 kDa (PG-I a) and 44 kDa (PG-I b)) and pepsinogen III contains four isoforms with different isoelectric points (3.73 (PG-III a), 3.78 (PG-III b), 4.0 (PG-III c) and 4.15 (PG-III d)).

Table 6.2: Overview of the enzymatic characteristics of trout pepsin isolates I-III

	PG I/ P I	PG II/ P II	PG III/ P III
N- terminal amino acid composition	Similar to P III 85 % ID to chitinases	100 % ID to pepsin A <i>Oncorhynchus keta</i>	Similar to P I 85 % ID to chitinases
Kinetic properties	K_M $2.8 \cdot 10^{-8}$ M K_{cat} 5.43 s^{-1}	K_M $1.3 \cdot 10^{-8}$ M K_{cat} 8.37 s^{-1}	K_M $7.9 \cdot 10^{-9}$ M K_{cat} 19.1 s^{-1}
Isoelectric point	4.0 (PG I)	5.9 (PG II)	3.73 - 4.15 (PG III)
Activation process	one-step mechanism	one-step mechanism	two-step mechanism
Enzyme inhibition (pepsin/ pepstatin A mol/mol)	1:1	1:10	1:10

The n-terminal amino acid sequences of pepsin I and III were quite different from those of other fish pepsins (Cao et al., 2011; Tanji et al., 1988; Xu et al., 1996), whereas pepsin II showed high similarity to pepsin A of *Oncorhynchus keta* (dbj BAL40979.1) (ID 100 %), to pepsin I (ID 88 %) and P III (ID 76 %) of *Lateolabrax japonica* and to P I (ID 69 %) and P II (ID 74 %) of *Thunnus orientalis* (Cao et al., 2011; Tanji et al., 1988). Apart from that, P I and P III showed high similarity to chitinases (pepsin I: 85 % ID to emb CDQ98810.1 and pepsin III: 85 % ID to BAL40979.1). Additionally, a low similarity to porcine pepsin (Hartsuck et al., 1992) was identified for all trout pepsin isoforms, consistent with published findings for other fish species (Cao et al., 2011; Tanji et al., 1988; Tanji et al., 2007; Xu et al., 1996).

The enzymatic kinetic studies showed that pepsin III displays the highest catalytic efficiency and the highest affinity for haemoglobin of the isolated trout pepsins. The Michaelis constant (K_M) values of the three pepsins were in the same range as those from sea bream (Zhou et al., 2007), pectoral rattail (Klomklao et al., 2007) and orange roughy (Xu et al., 1996), but higher K_M values were reported for pepsin from European eel (Wu et al., 2009) and Japanese seabass (Cao et al., 2011). Compared to pig pepsin, all isoforms exhibited a higher affinity for haemoglobin (Gildberg et al., 1990).

The isoelectric points of the pepsinogen isoforms were determined by IEF (isoelectric focusing) in polyacrylamide gels (Figure 2.4.). Apart from common strong bands at pH 5.2-5.3 and 3.5, which represent a staining artefact, PG I yielded a strong band at pH 4.0 and PG II gave a strong band at 5.9, whereas PG III showed 4 bands in the acid range at pH 3.73, 3.78, 4.0 and 4.15. Low pI values were also found for pepsinogen isoforms I, II a, and II b from orange roughy, *Hoplostethus atlanticus* (5.3, 4.35, 4.4) (Xu et al., 1996), polar cod, *Boreogadus saida* (3.75 pepsinogen A, 4.75 pepsinogen B) (Arunchalam et al., 1985), Japanese seabass, *Lateolabrax japonica* (5.3, 5.1, 4.7) (Cao et al., 2011) and Pacific bluefin tuna, *Thunnus orientalis* (6.06, 4.33, 4.31) (Tanji et al., 2009).

In acid environments (pH 2.0), pepsinogens were converted to pepsins autocatalytically by two different pathways, a one-step pathway that released the activation segment immediately and a stepwise pathway that involved an intermediate product (pseudopepsins) (Kageyama, 2002). PG I and PG II were converted directly to pepsin; this occurred very rapidly for PG II, but complete conversion of PG I required 1 h. Complete activation of smooth hound pepsinogen was carried

out in 30 min, whereas mandarin fish pepsinogens converted in 10 min for PG I and in less than 1 min for PG II, PG III (a) and PG III (b) (Bougatef et al., 2008; Zhou et al., 2008). In contrast, PG III was activated by a two-step mechanism that involved the formation of a very stable 39 kDa intermediate. The presence of a very stable intermediate was also shown by Tanji et al. (2007) for PG III from African coelacanth, *Latimeria chalumnae*; PG III of that species was converted into an intermediate product that was stable for at least 10 h without further conversion into pepsin. Such a two-step conversion mechanism was also reported for two pepsinogens from African coelacanth (Tanji et al., 2007), pepsinogen from bluefin tuna (Tanji et al., 1988) and pepsinogen from sea bream (Zhou et al., 2007).

All the isolated pepsin isoforms were inhibited only by pepstatin A, a pentapeptide from *Streptomyces* that specifically inhibits aspartic proteases (Cao et al., 2011; Nalinanon et al., 2010). The pepsins exhibited different sensitivities to pepstatin A. Like porcine pepsin, P I was completely inhibited at an equimolar ratio of pepstatin A to pepsin. In contrast to this, a 10-fold molar excess of pepstatin A was required to achieve nearly complete inhibition of P II and P III. Cao et al. (2011) and Tanji et al. (2007) reported that a lower susceptibility of isolated pepsin isoforms to pepstatin A could be an indication that they belong to the pepsinogen C group (see the discussion of pepsinogen classification in Chapter 1). The results obtained for trout pepsins are in accordance with reports for other fish pepsins, which have been shown to be completely inhibited by 0.05 μ M to 20 μ M pepstatin A (up to 17-fold molar excess) (Tanji et al. 1988, Bougatef et al. 2008, Zhou et al. 2008, Cao et al. 2011).

Apart from this, slight differences in the molecular weights and the pH and temperature optima of the isolated pepsin isolates were indicated. The molecular weights of PG I-III under reducing conditions were 45 kDa for PG I a, 44 kDa for PG I b and 42 kDa for PG-II and PG-III, whereas those of the corresponding pepsins were 37 kDa (P-I a, b, P-III) and 35 kDa (P-II). The molecular weights of trout pepsins were similar to those from albacore tuna, *Thunnus alalunga* (Nalinanon et al., 2010), the African coelacanth, *Latimeria chalumnae* (Tanji et al., 2007), and the smooth hound, *Mustelus mustelus* (Bougatef et al., 2008). The pepsins isolated from Japanese seabass, *Lateolabrax japonicus* (Cao et al., 2011), mandarin fish, *Siniperca chuatsi* (Zhou et al., 2008) and keta salmon, *Oncorhynchus keta* (Sanchez-Chiang et al., 1987) had slightly lower molecular weights ranging from 32 to 36 kDa.

Trout pepsins exhibited optimal activity at pH 3.0 (P I) and pH 2.5 (P II and P III); P I showed a broad pH optimum between 1.5 and 4.0, whereas the optimal pH range for P II and III was narrower, from 2.0 to 3.0. All trout pepsin isoforms exhibited higher enzyme activity than porcine pepsin over a broad spectrum of pH and a lower temperature (50 °C) for enzyme inactivation. Furthermore, higher enzyme activities were achieved at lower temperatures (up to 75 % at 20 °C). The pH and temperature dependence of trout pepsins are similar to the results reported for other fish species (Zhao et al., 2011).

6.1.2. Alternative method: Aqueous two-phase extraction and polyelectrolyte precipitation with pectin

6.1.2.1. Purification process:

A combination of ATPs (aqueous two-phase separation) and subsequent polyelectrolyte precipitation led to the isolation of pepsin from the stomach of the rainbow trout. Under the experimental conditions employed (pH value of 5.5-6.5), the pepsinogen possessed a negative electrical charge and therefore displayed high affinity for the PEG-rich phase (Nalinanon et al., 2009). Compared to the chromatographic method, the amount of time required for the isolation was significantly less, but the resulting enzyme contained impurities at significantly lower concentrations than that of the desired enzyme. The enzyme preparation prepared by the ATPs method in the investigations of Yang et al. (2008) and Gautam et al. (2006) also contained impurities.

observed, and above this concentration a significant reduction in enzyme yield occurred. Nalinanon et al. (2009) and Antov et al. (2006) attributed the enzyme activity losses under these conditions to denaturation of the protease caused by the salting-out effect. This effect can also be observed in the studies conducted by Klomklao et al. (2005). A reduction in the V_R value due to an increase in the salt concentration was identified in the study of Nalinanon et al. (2009). Nalinanon et al. (2009) ascribed this to rearrangement of the water molecules around the PEG molecule. The formation of an aqueous layer around the cations creates a more compact molecular structure with minimal PEG volume (Nalinanon et al., 2009; Twining et al., 1983). In addition, an increase in salt concentration from 15 % to 20 % led to a reduction in K_p values, lower protein concentration in the supernatant and higher specific enzyme activity, as was also shown by Klomklao et al. (2005).

In addition to salt concentration, Nalinanon et al. (2009) and Klomklao et al. (2005) were able to show that the **type of salt** has a significant effect on the partition coefficient, specific enzyme activity and enzyme yield. Based on these findings, in this study only $MgSO_4$ was used as the second phase of the biphasic system.

Generally, phase separation was more affected by variation in the salt concentration than by changes in PEG molecular weight or PEG concentration. The high affinity of binding of pepsinogen to PEG results in high enzyme yield but often hampers subsequent separation (Boeris et al., 2009). Under the experimental conditions used (pH 5-6), the pepsinogen-PEG complex exhibited a higher solubility compared to the salt phase; therefore, the enzyme could be separated from the undesired components using the ATPs extract, as previously shown in the study of Nalinanon et al. (2009) for albacore tuna (*Thunnus alalunga*) pepsinogen and pepsin. By means of polyelectrolyte precipitation, it was shown that up to 30 % of the pepsinogen/pepsin could be separated from PEG by using pectin or chitosan as the polyelectrolyte.

In the acid environment (pH 2.5) used for pectin precipitation, pepsinogen was partially converted to pepsin. To achieve complete conversion, an incubation time of approximately 60 min should be necessary, as shown by the results obtained using the conventional enzyme isolation method described in Chapter 2. Pectin offers the advantage of precipitating at a low pH; under these conditions, according to Kokufuta et al. (1991), PEG seems to be loosely associated

with the enzyme through hydrogen bonding of pepsin-COOH groups with the ether groups of PEG. In addition, pepsin is very stable in acid environments.

Compared with the chromatographic separation method, 20 % higher enzyme yields could be obtained by the ATPs method (Chapter 3). The enzyme yield achieved in this study was much lower than that reported by Boeris et al. (2009), who separated pepsin obtained from abomasum homogenate in an ATPs extract using a 2 % chitosan solution. However, the purification factor was 2.5 times higher in this study than in the study of Boeris et al. (2009). Due to the fact that the precipitation is affected by the specific properties of the enzyme, especially its isoelectric point, molecular weight and charge, as well as the ionic strength and the pH of the surrounding environment, further information about the enzyme would be helpful, especially in the presence of enzyme isoforms. As indicated, after the conventional isolation method, seven trout pepsinogen isoforms were obtained from the stomach extract. The isoelectric point of pepsinogen isolate II differs significantly that of from the other isolates (4.0 for PG I, 5.9 for PG II and 3.8-4.2 for PG III c-f). Thus, it may be that under the experimental conditions used (precipitation at pH 2.5), only PG I and PG III were isolated; these two pepsinogen isolates represent only 40 % of the total amount of pepsinogen.

Increasing the polyelectrolyte concentration resulted in a linear increase in the enzyme yield in the precipitate up to a certain threshold. The highest enzyme recovery was achieved with 2 % chitosan and 3 % pectin. Because the high viscosity of 3 % pectin solution hampers subsequent separation, only a 2 % solution was used.

After formation of a complex between pectin and the ATPs extract, a significant increase in viscosity could be observed visually. This behaviour can be attributed to the interaction between the amino groups of pectin and the positively charged groups on the protein. The complex between pectin and pepsin was separated by resuspension in distilled water and embedded in polyethylene glycol 20000 using dialysis tubing (Spectrapor 1, MWCO 6000-8000 Da) for 6 h at 7 °C to concentrate the enzyme solution and remove the solvent pectin molecules based on their low molecular weight of 0.06-0.13 kDa. The enzyme-containing supernatant was then separated from the polyelectrolyte-containing sediment by centrifugation.

6.1.2.2. Enzyme characterization

At the end of the purification process, in addition to the enzyme (45 kDa), other protein bands with apparent molecular weights between 20-62 kDa could be detected on SDS page, as also reported, for example, by Nalinanon et al. (2009). However, these proteins were present at significantly lower concentrations than the desired enzyme. Comparable results were obtained for albacore tuna, *Thunnus alalunga* pepsinogen, which has a molecular weight of 40 kDa (Nalinanon et al., 2009).

Subsequent studies of enzyme inhibition showed that the isolated enzyme is an acidic aspartic protease because it was only inhibited by pepstatin A. In previous studies in which enzyme isolation was carried out using the chromatographic isolation method, comparable inhibitor/enzyme ratios produced complete enzyme inhibition. Pepsin I and II (a) isolated from the stomach of *Siniperca chuatsi* and pepsin isolated from the stomach of *Mustelus mustelus* were inhibited by pepstatin A at a 10- or 16-fold molar excess of pepstatin A (Twining et al., 1983; Zhou et al., 2008)

6.1.2.3. Enzyme application

Potential areas of application of pepsin are the enzymatic cleavage of various dietary proteins to expose antioxidative (Barkia et al., 2010) or antibacterial (Robert et al., 2015) peptides and the reduction of the amount of anti-nutritive factors present in plant feed proteins (Adelizi et al., 1998). As shown by enzymatic hydrolysis, the isolated enzyme was suitable for the hydrolysis of animal (fish viscera, conventional fish feed) and plant proteins (pea and potato protein). It was shown that the use of trout pepsin achieved degrees of hydrolysis comparable to those produced by trypsin (10.6 % (trout pepsin) vs. 10.4 % (trypsin)) in the digestion of pea proteins under optimal cleavage conditions (trout pepsin pH 3.0, 37°C; trypsin pH 8.0, 50°C) (Karamać et al., 2002). Trout pepsin, like giant fish pepsin and Japanese seabass pepsin, is particularly suitable for the enzymatic cleavage of animal proteins (Liaset et al., 2000; Vannabun et al., 2014).

6.2. Antibacterial peptides obtained from the processing of by-products of rainbow trout with trout pepsin

The hydrolysis of fish by-products using the fish's own pepsin can provide material for the isolation of antibacterial peptides. The antibacterial peptides so produced display high effectiveness against a wide range of gram-positive and gram-negative bacteria. This was shown by the investigations of Sila et al. (2014) and Beaulieu et al. (2013), who reported a broad spectrum of activity for *Barbus callensis* hydrolysate and *Cancer irroratus* hydrolysate. Depending on the bacterial species, peptide concentrations of 47 mg/ml to 2 mg/ml resulted in complete growth inhibition with an incubation time of 24 h and a bacterial concentration of $1-2 \times 10^6$ cfu/ml. Peptides isolated from the hydrolysis mixture using ion-exchange chromatography and gel filtration displayed up to 9-fold greater effectiveness against the bacteria. In general, the antibacterial effect on food-borne microorganisms was lower than the antibacterial effect on fish farming pathogens. The antibacterial peptides isolated from rainbow trout by-products predominantly affected the lag phase of bacterial growth; thus, a delay in the proliferation of the bacteria was detected. The effectiveness of the hydrolysate was affected by a variety of factors; these are shown in Figure 6.4 and described in detail below.

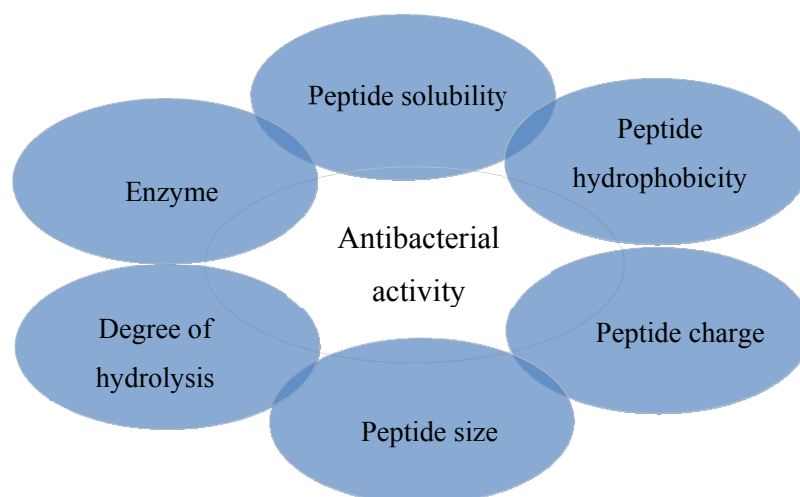


Figure 6.4: Factors that affect the activity of antibacterial peptides

The antibacterial activity of trout protein hydrolysate was significantly influenced by the **degree of hydrolysis**, with an increase in the observed inhibitory effect against food contaminants and fish farming bacteria at the beginning of hydrolysis. During the hydrolysis process, the fish's own pepsin exhibited high cleavage activity against trout proteins. The degree of hydrolysis was 10 %

higher than that achieved using porcine pepsin (DH 31 % vs. DH 21 %). This finding is in accordance with the results of Kristinsson et al. (2000), who reported that the degree of hydrolysis is predominantly influenced by the **enzyme** used.

At the start of hydrolysis, the antibacterial effect of the hydrolysates increased significantly with an increasing degree of hydrolysis (reducing **peptide size**). After a period of hydrolysis of 150 min, the hydrolysates demonstrated no significant differences in their antibacterial activity ($P > 0.005$) or molecular weight distributions (Chapter 4). In the investigations of Cheng et al. (2013), a similar relationship between antibacterial activity and degree of hydrolysis was observed for k-casein hydrolysate. In contrast, Sila et al. (2014) reported that the inhibitory effect of barbel muscle protein hydrolysates only occurs at certain degrees of hydrolysis (DH 6.6 %). These results can be attributed to **peptide hydrophobicity**, which has been described as a major factor in peptide interactions with bacterial cytoplasmic membranes (chapter 1, section 2.5.4). Cheng et al. (2013) attributed the enhanced antibacterial activity obtained at certain degrees of hydrolysis to the existence of a hydrophobicity threshold at which the protein/peptide exhibits the highest antibacterial effect. However, hydrophobicities beyond this threshold favour enhanced protein dimerization in solution, which in turn leads to reduced antibacterial activity (Cheng et al., 2013). This is because protein dimers are unable to pass through the cell wall, which prevents their interaction with the cell membrane (Chen et al., 2007).

The undigested fish proteins and hydrolysates with low degrees of hydrolysis exhibited no antibacterial effects; in contrast, they showed a growth-promoting effect on the bacteria. After an incubation period of 24 h, depending on the bacterial species, the bacterial concentration increased up to ten-fold in comparison with the positive control. Dufosse et al. (2001) demonstrated that fish protein hydrolysates may exert a growth-promoting effect on bacteria by serving as a source of nitrogen. Figure 6.5 shows increasing bacterial growth in the presence of undigested proteins and hydrolysates with low degrees of hydrolysis as well as under conditions of decreasing peptide concentration. A growth-promotion effect could be observed around the well containing decreasing peptide concentrations, in the form of an additional circle around the inhibition zone.



Figure 6.5: Growth promotion effect of undigested fish proteins and inhibitory effect of protein hydrolysate against *citro. freundii*.

1. undigested protein, 2. trout protein hydrolysate (DH 30 %:H₄) (1.5 mg) 3: trout protein hydrolysate (DH 30 %:H₄) (15 mg)

These results are consistent with the observation that small peptides display the strongest antibacterial effects. Similar results were also reported for *Barbus callensis* muscle protein (Sila et al., 2014) and caprine whey protein (Cheng et al., 2013). Apart from the hydrophobicity needed for interaction with the hydrophobic bacterial membrane, the peptides must also possess hydrophilic properties that permit them to dissolve in the aqueous phase in which bacterial growth takes place (Branen, 1993; Cheng et al., 2013).

During the hydrolysis process, as shown in this investigation and also in the studies of Dong et al. (2008) and Klompong et al. (2007), the **solubility of the hydrolysate** increased as a result of the reduction in size that occurs when proteins are converted into peptides.

After the separation of antibacterial peptides according to their charge and size using ion-exchange chromatography and gel filtration, as described in detail below, it was possible to show that the active fractions exhibited **a high content of peptides with positive charge** (the content of amino acids with net positive charge was between 7.9 and 20.1 %). Additionally, the cationic peptide fraction II (molecular weight between 1 and 3 kDa) possesses a high concentration of lysine (7.1 %) and arginine (8.42 %); these amino acids are well known to interact strongly with phosphate groups in lipid bilayers via electrostatic forces (Mavri et al., 1996). Comparable concentrations of positively charged amino acids were found for k-casein hydrolysate (16.9 %) and tilapia protein hydrolysate (11.9 %) (Cheng et al., 2013; Robert et al., 2015). The relationship between cationicity and antibacterial activity is not strictly linear. As described by M. Dathe et al. (2001), a peptide net charge greater than + 5 resulted in an increase in undesired haemolytic effects and a loss of antimicrobial selectivity of the antibacterial peptide magainin.

The amino acid analysis of the active peptide fraction also showed that peptides with a high content of **hydrophobic amino acids** exhibited the strongest inhibitory effects on bacterial growth. This relationship between peptide hydrophobicity and antibacterial activity is also supported by the data showing how the degree of hydrolysis affects the antibacterial activity.

The cationic peptide fractions II and III, which contained 51 % and 66 % hydrophobic amino acids, respectively, showed a broad spectrum of activity and had a significantly higher inhibitory effect on bacterial growth than the hydrolysate (23 % hydrophobic amino acids), fraction I of the cationic peptides (43 % hydrophobic amino acids) and fraction II of the anionic peptides (37 % hydrophobic amino acids). This is in accordance with the finding that most antibacterial peptides consist of approximately 50 % hydrophobic residues (Cheng et al., 2013; Yeaman et al., 2003). As already described, the hydrophobicity of the antibacterial peptides had a considerable influence on the antibacterial activity because the membrane interactions (especially partitioning into the lipid bilayer and the specificity of the antimicrobial peptides), as well as the membrane permeability by peptides, were affected by peptide hydrophobicity (Wieprecht et al., 1997; Yeaman et al., 2003).

6.2.1. Concentration of antibacterial peptides by ion-exchange chromatography and gel filtration

Based on the influencing factors (peptide charge and size), trout hydrolysate was concentrated by anion-exchange chromatography and gel filtration (chapter 5). Similar purification processes have been described by Beaulieu et al. (2010), Cheng et al. (2013) and Doyen et al. (2012).

First, trout protein hydrolysate with a DH of 30 % was separated into cationic and anionic peptides (chapter 5). Trout protein hydrolysate contained a higher proportion of cationic peptides (63 % vs. 29 % anionic peptides), which are known to be important for the initial electrostatic attraction of antibacterial peptides to the negatively charged phospholipid membranes of bacteria (chapter 1, section 2.4). The purification process produced an overall yield of 92 %, consistent with the results of Beaulieu et al. (2010), who obtained an 88 % yield of peptides using cation-exchange chromatography.

Thereafter, the cationic and anionic peptides were separated via gel filtration into peptide fractions of molecular weight > 3 kDa, 1 kDa - 3 kDa and < 1 kDa. This was based on the finding that the active fractions of fish and/or crustacean protein hydrolysate (*Chionoectes opilio*, *Nile tilapia*, *Barbus callensis*, and *Cancer irroratus*) were between 200 and 3,624 Da in molecular weight (Beaulieu et al., 2013; Doyen et al., 2012; Robert et al., 2015; Sila et al., 2014). Due to the high degree of hydrolysis (30 %), most peptides of the trout protein hydrolysate were di- and tripeptides with molecular weights of less than 1 kDa (53.4 % cations / 41.3 % anions) and oligopeptides with molecular weights between 1 and 3 kDa (37.1 % cations / 57.4 % anions); only a small fraction of the polypeptides were > 3 kDa in molecular weight (9.6 % cations / 1.3 % anions) (Figure 5.2).

Cationic peptides with molecular weights of less than 1 kDa to >3 kDa and anionic peptides with molecular weights of 1 kDa-3 kDa exhibited antibacterial effects against certain bacteria at different concentrations (chapter 5, Table 3). Cationic peptides exhibited the broadest spectrum of activity. They also inhibited the growth of gram-positive and gram-negative bacteria that cause food spoilage and fish diseases. The broadest spectrum of activity was possessed by peptides with a molecular weight of < 1 kDa; they were able to inhibit the growth of 11 species of bacteria. A strong correlation between peptide cationicity and antibacterial activity was demonstrated by Bessalle et al. (1992). The high specificity of AMPs towards bacteria is often

attributed to the interaction of cationic peptides with the negatively charged bacterial membrane, which contains lipopolysaccharides and teichoic acids (chapter 1, section 2.4) (Epanand et al., 1999; Yeaman et al., 2003).

Anionic peptides with a molecular mass of 1 kDa-3 kDa exhibited predominantly an inhibitory effect against food contaminants; only the fish farming bacteria *Weisella minor* was inhibited by this fraction. Anionic antibacterial peptides isolated from fish protein hydrolysates derived from *Chionoectes opilio* (Doyen et al., 2012) and *Cancer irroratus* (Beaulieu et al., 2013) were previously shown to possess antimicrobial activity against *E. coli*, *L. innocua*, *Aeromonas* sp., *Listeria monocytogenes*, *Morganella morganii*, *Shewanella putrefaciens*, *Staphylococcus* sp. and *Vibrio vulnificus*.

After purification, the peptide concentration necessary to achieve complete inhibition (MIC) of bacterial growth was reduced up to 9-fold. Using an antibacterial peptide derived from *Chionoectes opilio* hydrolysate via electroseparation, Doyen et al. (2012) achieved a comparable degree of purification (10-fold) with a MIC value of 50 mg/ml after purification. Thus, the efficacy of purified *Chionoectes opilio* hydrolysate fraction KC11 (14 V/cm, 50 kDa) against *E. coli* (10^7 CFU/ml) should be regarded as significantly lower than that of the purified trout peptides (approximately 5 mg/ml). The antibacterial peptides from trout by-products showed the highest activity against the fish farming bacteria *F. psychrophilum*, *F. araucanum* and *R. salmoninarum*. Similar MIC values were achieved with the antibacterial peptide piscidin-1.

In some cases, the purification process led to only a slight reduction in the MIC value. This could be attributed to synergistic effects between the peptides and the non-peptides present in the mixture. Synergistic effects between various antibiotic agents were described by Rushing et al. (1963) with respect to the combination of sorbic acid and benzoic acid and by Lin et al. (2013) in analyses of the combination of peptide and non-peptide antibiotics. In addition, cationic and non-cationic peptides appear to have a synergistic effect in improving the immune response (Beaulieu et al., 2013; Marshall et al., 2003).

The peptides especially influenced the lag phase of bacterial growth; bacterial proliferation was thereby significantly delayed. Concentrations below the MIC value led to significant prolongation of the lag phase. At the end of the incubation period, the bacterial content was

approximately 50 % that of the positive control. In contrast, piscidin-1 prolonged the lag phase of bacterial growth only slightly (Chapter 5; Figure 5.3).

6.3. Bibliography

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7. Summary

In the present thesis, trout by-products were evaluated as a natural source of antibacterial peptides obtained by enzymatic digestion with trout pepsin. The impact of different degrees of hydrolysis on the antibacterial activity of the hydrolysates was investigated. Active peptides were isolated by a combination of ion-exchange chromatography and gel filtration, and the effectiveness of the peptides in relation to its charge, size and amino acid composition was illustrated.

Chapter 2 reports the isolation and characterization of pepsinogen from the stomach of *Oncorhynchus mykiss*. For enzyme isolation, a series of chromatographic separations (anion-exchange chromatography and gel filtration) preceded by ammonium sulphate precipitation was used. This procedure achieves high enzyme purity and allows detailed enzyme characterization. Three pepsinogen isolates that exhibited differences in their optimal conditions for activity (pH, temperature), their catalytic constants K_M and k_{cat} for proteolysis of acid-denatured haemoglobin, their mechanisms of pepsinogen activation and their N-terminal amino acid sequences were isolated. As indicated by electrophoretic tests, pepsinogen isolate I consists of two isoforms, and pepsinogen isolate III contains four isoforms. All trout pepsins show high enzyme activity at low temperatures (relative activity 35-70 % at 20°C) and over a wide range of pH (pH of 1-6). In addition, the denaturing temperatures (approximately 50 °C) of all the trout pepsins are lower than that of porcine pepsin.

Chapter 3 reports the isolation of trout pepsin by aqueous two-phase (ATPs) extraction and polyelectrolyte precipitation with pectin. This purification method is rapid and cost-effective and produces high enzyme yields of 25-30 %. The highest specific enzyme activity was achieved by the phase combination of 20 % PEG 1500 and 20 % $MgSO_4$. The separation of pepsinogen from PEG was achieved by polyelectrolyte precipitation with pectin (2 % w/w). Under acid conditions (pH 2.5), pepsinogen was separated from the soluble ATPs extract and partially converted to pepsin. In conclusion, it can be shown that pectin is an appropriate alternative to chitosan, which is predominantly used for enzyme precipitation. Both polyelectrolytes facilitated enzyme yields of approximately 25 % with a 2 % polyelectrolyte solution. The formation of insoluble macroaggregates between pectin molecules and the enzyme was demonstrated by turbidimetric measurement. Subsequent separation of the components was performed by resuspending the

aggregates in distilled water, placing the suspension into dialysis tubing, embedding it in PEG 20000 and separating the peptides from the polyelectrolyte-containing sediment by centrifugation. The isolated enzyme exhibited cleavage activity against animal and plant proteins; the activity against animal proteins was significantly higher.

Chapter 4 reports the enzymatic digestion of trout by-products to release antibacterial peptides and evaluation of the impact of different degrees of hydrolysis on the antibacterial effectiveness. The degree of hydrolysis was found to exert a considerable influence on antibacterial activity; a significant increase in the observed inhibitory effect against food contaminants and fish farming bacteria occurred at the beginning of hydrolysis. After a period of hydrolysis of 150 minutes (DH 25 %), the hydrolysate demonstrated no further change in its antibacterial activity ($P > 0.005$) or molecular weight distribution. The undigested proteins, as well as hydrolysates with low degrees of hydrolysis, exert a growth-promoting effect on the bacteria. Depending on the bacterial species, the bacterial concentration increased up to 10-fold in comparison with the positive control. Trout hydrolysate with a high degree of hydrolysis (< 20 %) demonstrated a broad spectrum of activity and inhibited the growth of both gram-positive and gram-negative bacteria. Depending on the bacteria, peptide concentrations of 47 mg/ml to 2 mg/ml resulted in complete growth inhibition (incubation time 24 h, bacterial concentration $1-2 \times 10^6$ cfu/ml). The hydrolysates exhibited the greatest antibacterial effect and therefore the lowest MIC values for *Flavobacterium* sp. and *R. salmoninarum* (2 mg/ml and 5 mg/ml, respectively). The effect of the hydrolysate on *Flavobacterium* sp. was significantly higher than the effect of nisin and matched that of oxytetracycline at a concentration that was 75 times greater. Compared to piscidin-1, the antibacterial effect of trout protein hydrolysate (DH 30 %) was up to 75-fold lower. In general, the antibacterial effect on food-borne microorganisms was lower than the antibacterial effect on fish farming pathogens.

By characterization of the hydrolysates, it was shown that a marked change in chemical composition occurred as a result of hydrolysis. By separating the undissolved, uncleaved substances after hydrolysis, we were able to minimize the fat content (55 % to 0.74 %) and increase the protein content (32 % to 68 %) of the hydrolysate compared to the raw substrate. The trout hydrolysates displayed high solubility over a wide pH range (3-9). Amino acid analysis of the trout hydrolysate (DH 30 %) indicated a high content of amino acid residues with polar side chains (12.9 %) and hydrophobic side chains (22.7 %). The highest concentrations were found for

leucine (4.63 %), lysine (5.11 %), alanine (4.35 %), arginine (4.80 %), aspartic acid (5.84 %), glutamic acid (8.71 %) and glycine (5.34 %).

Chapter 5 reports the isolation of active peptides from trout hydrolysate by ion-exchange chromatography and gel filtration. The purification process led to a significant (up to 9-fold) reduction in the minimum concentration required to inhibit bacterial growth. However, the antimicrobial effect could not be attributed to individual peptides because cationic peptides > 3 kDa, > 1 kDa and < 1 kDa and anionic peptides with molecular weights between 1 and 3 kDa exhibited antibacterial activity against several species of bacteria. Anionic peptides especially inhibited the growth of bacteria that cause food spoilage, whereas cationic peptides also inhibited bacteria that cause disease in fish. Cationic peptides with molecular weights below 1 kDa showed the broadest spectrum of activity and inhibited the growth of 11 of the studied species of bacteria. Only 3 species of bacteria were not inhibited by these peptides. In addition to low molecular weight, these peptides possessed a high proportion (66 %) of amino acids with hydrophobic side chains.

The peptides especially influenced the lag phase of bacterial growth; bacterial proliferation was thereby significantly delayed. The antibacterial peptides showed a high content (between 7.9 and 20.1 %) of amino acids with positive net charges. The broad spectrum of activity and the prolongation of the lag phase of bacterial growth demonstrate the high potential of these peptides (especially cationic peptides with molecular weights of 1-3 kDa) as preservatives in food processing and as agents that can be used to reduce bacterial growth in fish farming.

8. Zusammenfassung:

In der vorliegenden Arbeit wurde die antibakterielle Wirksamkeit von hydrolysierten Forellennebenprodukten (Innere Organe) untersucht. Die Freisetzung der antibakteriellen Peptide erfolgte über eine enzymatische Hydrolyse mittels Forellenpepsin. Hierbei wurde der Einfluss unterschiedlicher Hydrolysezeiten auf die antibakterielle Wirksamkeit der Hydrolysate untersucht. Die Isolation der aktiven Peptide erfolgte über eine Kombination aus Ionenaustauschchromatography und Gelfiltration. Im Anschluss wurde die Effektivität der Peptide in Anhängigkeit mit ihrer Ladung, Größe und Aminosäuren Zusammensetzung dargestellt.

Kapitel 2 beschreibt die Isolation und Charakterisierung von Pepsinogen und Pepsin aus dem Magen der Regenbogenforelle (*Oncorhynchus mykiss*). Die Enzymisolation erfolgte über eine Ammoniumsulfat Fällung, Anionen-Austauschchromatography und Gelfiltration. Dieser Prozess führte zu hohen Enzymreinheiten und ermöglichte eine detaillierte Enzymcharakterisierung. Drei Pepsinogenisolate, welche sich in ihren Enzymoptima (pH und Temperatur), katalytischen Konstanten für die Hydrolyse von Hämoglobin, dem Enzymaktivierungsmechanismus und der N-terminalen Aminosäure Sequenz unterschieden, konnten isoliert werden. Elektrophoretische Untersuchungen zeigten, dass Pepsinogenisolat I aus zwei Isoformen und Pepsinogenisolat III aus vier Isoformen aufgebaut sind. Alle Forellenpepsine zeigten eine hohe Enzymaktivität bei geringen Temperaturen (relative Enzymaktivität von 35 -70 % bei 20 °C) und über einen breiten pH Bereich (pH 1-6). Darüber hinaus lag eine deutlich geringere Denaturierungstemperatur (<50°C) verglichen mit Schweinepepsin (> 60°C) bei allen Forellenpepsinen vor.

Kapitel 3 beschreibt die Isolation von Forellenpepsin mittels der wässrigen 2 Phasen Extraktion und anschließender Polyelektrolyt Präzipitation mit Pektin. Diese schnelle und kosten günstige Aufreinigungsmethode führte zu Enzymausbeuten von 25-30%. Die höchste spezifische Enzymaktivität konnte bei der Phasenkombination 20 % PEG 1500 und 20 % MgSO₄ erreicht werden. Die anschließende Separation des Pepsinogens vom PEG erfolgte im sauren Milieu mittels einer 2 %igen Pektinlösung. Unter diesen Milieubedingungen (pH 2.5) wurde ein Teil des Pepsinogens zu Pepsin umgewandelt.

Es konnte aufgezeigt werden, dass Pektin eine alternative zu Chitosan bei der Isolation von Forellenpepsin darstellt. Beide Polyelektrolyte führten zu vergleichbaren Enzymausbeuten von

ca. 25 % bei einer 2 % igen Polyelektrolytlösung. Die Bildung der unlöslichen Makroaggregate zwischen Pektin und dem Enzym wurden mittels Turbidimetrie veranschaulicht. Eine anschließende Separation erfolgte über eine Resuspendierung in destilliertem Wasser. Nach einer Aufkonzentrierung wurde der enzymhaltige Überstand vom polyelektrolythaltigen Sediment mittels Zentrifugation abgetrennt. Das isolierte Enzym wies eine enzymatische Aktivität gegenüber pflanzlichen sowohl als auch tierischen Proteinen auf, wobei eine höhere Spaltungsaktivität gegenüber tierischen Proteinen aufgezeigt werden konnte.

Kapitel 4 beschreibt die Isolation von antibakteriellen Peptiden aus Forellennebenprodukten über eine enzymatische Hydrolyse mittels Forellenpepsin. Es konnte nachgewiesen werden, dass der Hydrolysegrad einen wesentlichen Einfluss auf die antibakterielle Wirksamkeit der Hydrolysate hat. Dabei konnte zu Beginn der Hydrolyse eine steigende antibakterielle Wirkung mit steigendem Hydrolysegrad beobachtet werden. Nach einer Hydrolysezeit von 150 min. konnte hingegen kein signifikanter Unterschied mehr in der antibakteriellen Wirksamkeit der Hydrolysate gezeigt werden. Im Gegensatz dazu zeigte die Verwendung der Rohproteine, wie auch Hydrolysate mit einem geringen Hydrolysegrad eine wachstumsfördernde Wirkung. Je nach Bakterienart führte dies zu einer 10-Fach höheren Bakterienkonzentration verglichen zur positiv Kontrolle am Ende der Inkubationszeit von 24 h und einer Bakterienanfängskonzentration von 1-2 KBE/ml (koloniebildende Einheiten). Die Forellenhidrolysate (DH > 20%) zeigten ein breites Aktivitätsspektrum, sie wirkten wachstumshemmend sowohl gegenüber gram-positiven als auch gram-negativen Bakterien. Je nach Bakterium führte eine Peptidkonzentration von 47 mg/ml bis 2 mg/ml zu einer vollständigen Inhibierung des bakteriellen Wachstums über eine Inkubationszeit von 24 h bei einer Bakterienkonzentration von $1-2 \times 10^6$ KBE/ml. Die höchste antibakterielle Wirksamkeit wiesen die Hydrolysate gegenüber *Flavobacterium* sp. und *Renibacterium salmoninarum* (Häufig auftretende Krankheitserreger in der Fischzucht) auf (MIC Wert: 2 mg/ml und 5 mg/ml). Das Forellenhidrolysat zeigte im Vergleich zu Nisin eine deutlich höhere Wirksamkeit gegenüber *Flavobacterium* sp. Im Vergleich zu Oxytetracyclin und Piscidin-1 lag hingegen eine deutlich geringere Wirksamkeit (75-fach) vor. Generell wiesen die Peptide eine höhere Wirksamkeit gegenüber Krankheitserregern in der Fischzucht als gegenüber Lebensmittelverderbskeimen auf.

Nach der Hydrolyse und Zentrifugation zeigten die Hydrolysate einen geringeren Fettanteil und einen höheren Proteinanteil im Vergleich zum Rohsubstrat. Die Aminosäuren Analyse zeigte auf,

dass das Hydrolysat einen hohen Anteil an Aminosäuren mit polaren Seitenketten und hydrophoben Seitenketten aufwies.

Kapitel 5 beschreibt die Isolation der aktiven Peptide aus dem Proteinhydrolysat über eine Ionenaustauschchromatography und Gelfiltration. Der Aufreinigungsprozess führte zu einer signifikanten Reduktion des MIC Wertes (bis zu 9-fach geringer), obwohl die antibakterielle Wirkung nicht auf einzelne Peptide zurückzuführen war. Sowohl kationische Peptide >3 kDa, >1 kDa und < 1 kDa als auch anionische Peptide mit einem Molekulargewicht zwischen 1 und 3 kDa zeigten eine antibakterielle Wirksamkeit gegenüber den Untersuchungskeimen. Anionische Peptide zeigten vor allem eine Inhibitorwirkung gegenüber Lebensmittelverderbskeimen wohingegen kationische Peptide ebenfalls das Wachstum von Krankheitserregern der Fischzucht hemmten. Kationische Peptide mit einem Molekulargewicht von <1 kDa zeigten das breiteste Aktivitätsspektrum und inhibierten das Wachstum von 11 Untersuchungskeimen. Lediglich 3 Bakterienarten wurden nicht durch diese Peptide gehemmt. Neben dem geringen Molekulargewicht wiesen diese Peptide (kationisch und < 1 kDa) verglichen zu den anderen Peptidclustern (>3 kDa, >1 kDa) den höchsten Anteil an hydrophoben Aminosäuren auf.

Die isolierten Peptide wirkten auf die Lag-Phase des Bakterienwachstums ein; wodurch deren Vermehrung deutlich verlangsamt werden konnte. Die aktiven Peptide zeigten einen hohen Anteil an Aminosäuren mit positiven Seitenketten. Das breite Wirkspektrum wie auch die Wirkweise (Verlängerung der Lag Phase) veranschaulicht das hohe Potential dieser Peptide zum einen für einen Einsatz in der Lebensmittelverarbeitung zur Lebensmittelkonservierung und zum anderen zur Reduktion der Bakterienkonzentration in der Fischzucht.

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