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

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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.

1. 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, has 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 its cleavage specificity for proteins and polypeptides, and the ability to reduce the risk of bacterial contamination during the hydrolysis process due to 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 determines 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

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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 on their degree on hydrolysis.

2. Materials and methods

2.1. Materials

Piscidin-1 (Bio world), R_2A 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.

2.2. Bacteria

Aeromonas media (ATCC 33907 fish farm effluent), *Aeromonas salmonicida* (ATCC 33659, trout), *Flavobacterium psychrophilum* (ATCC 49511, kidney of diseased rainbow trout fry), *Flavobacterium araucananum* (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), *E. coli* (K12 E851), *M. luteus* (ATC 4698), *B. cereus* (ATC12826), and *Enterococcus faecalis* (ATCC 51299) were used in this study.

2.3. 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. 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 a substrate.

2.4. 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 by a mincer and stored in polyethylene bags, at −20 °C, until they were used for digestion by pepsin.

2.5. 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 (13,500 rpm) for 30 s (Barkia et al., 2010). Aliquots from the homogenate were centrifuged (6000*g*, 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 a 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 measured 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 10,000*g* 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 hydrolyzed 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 acids amounts to 125 g/mol, h_{tot} was set at 8.3 g per kg protein (Nielsen, Petersen, & Dambmann, 2001). B