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Short communication

Angiotensin I-converting enzyme inhibitory activity in a hydrolysate of proteins from Northern shrimp (*Pandalus borealis*) and identification of two novel inhibitory tri-peptides

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ABSTRACT

The ACE inhibitory activity of a desalted protein hydrolysate from Northern shrimp (*Pandalus borealis*) was studied. Measurements by two independent methods both revealed higher *in vitro* ACE inhibitory activity, IC_{50} = 0.075 and 0.035 mg/ml, respectively, than earlier reported in comparable hydrolysates. Two novel ACE inhibitory tri-peptides, Phe-Thr-Tyr (IC_{50} = 275 and 59 μM) and Phe-Ser-Tyr (IC_{50} = 7.7 and 2.2 μM), were detected in the hydrolysate. An introductory feeding trial with spontaneously hypertensive rats indicated positive *in vivo* results when the rats were given 60 mg hydrolysate/kg body weight per day. Although further *in vivo* studies are necessary to verify the antihypertensive potential, the very high *in vitro* ACE inhibitory activity reveals that the shrimp protein hydrolysate is a promising candidate for nutraceutical application.

1. Introduction

In Norwegian fisheries the annual catch of Northern shrimp is 20 000–25 000 tons. The solid waste fraction from manufacturing, mainly heads and scales, is estimated to yield 40%, and about 8% of this fraction is protein [1,2]. Accordingly, annually 600–800 tons of shrimp byproduct protein may be obtained from Norwegian fisheries. If the shell and head fraction is treated with commercial enzymes, about 70% of the protein can be recovered as a water soluble protein hydrolysate, whereas the deproteinized scale fraction is suitable for chitin or chitosan production [1].

Angiotensin I-converting enzyme (ACE) plays an important role in the regulation of blood pressure, and peptides inhibiting the activity of this enzyme may be useful as therapeutic agents to lower high blood pressure. Most protein hydrolysates contain such peptides, and some of them have an ACE-inhibitory activity high enough to be useful as functional food to control mild hypertension [3]. Most inhibitory peptides are small with a hydrophobic amino acid at the carboxyl end [4], and the content of inhibitory peptides depends on the substrate protein, the proteolytic enzyme used and the hydrolytic conditions. It has been shown that ACE-inhibitory protein hydrolysates can be obtained by enzymic digestion of many different animal tissues [3], but several studies indicate that shrimp tissue hydrolysates often express higher ACE-inhibitory activity than other animal tissue hydrolysates investigated [5–7].

Earlier results showed that hydrolysates with high ACE inhibitory activity can be made from Northern shrimp byproducts by digestion with the commercial enzyme Alcalase (Novo Nordisk) [5]. This observation initiated an investigation were several enzyme preparations, and hydrolytic conditions were tested out to obtain a hydrolysate with high ACE-inhibitory activity and acceptable taste. The present paper characterizes a desalted shrimp waste protein hydrolysate selected according to these criteria and reveals the structure and activity of two new ACE-inhibitory peptides purified from this hydrolysate. ACE inhibitory activities were determined by two independent methods to secure reliable results. Results from an introductory feeding trial with spontaneously hypertensive rats are also presented.

2. Materials and methods

2.1. Materials

A dry, desalted Northern shrimp (*Pandalus borealis*) byproduct protein hydrolysate (DSPH) was provided by Marealis AS. Synthetic ACE-inhibitory peptides; Phe-Ser-Tyr (FSY), Phe-Thr-Tyr (FTY) and Pro-Ser-Tyr (PSY), were provided by GenScript USA Inc.

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2.2. Amino acid analyses

Free amines and amino acids were determined in an aqueous extract of DSPH, and total amino acids were determined in a hydrolysate (6 N HCl, 110 °C, 24 h) essentially as described by Pedersen et al. [8].

2.3. ACE inhibitory assays

ACE inhibitory activity was measured by two methods: (1) Essentially as given by Vermeirssen et al. [9] by incubation of ACE from rabbit lung tissue (Sigma, L 0796) with N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (Sigma, F 7131) as a substrate for 60 min at pH 8.2 and 37 °C. Activities were calculated from reduction in optical density at 340 nm from the start to the end of incubations. (2) Essentially as given by Dragnes et al. [10], a method first developed by Cushman and Cheung [11] applying Hippuryl-His-Leu as a substrate.

2.4. Purification of ACE-inhibitory peptides

DSPH (30.0 g) was solubilized in distilled water (300 ml) by stirring at room temperature and centrifuged (10 000 × *g*, 30 min at 4 °C). The clear, brown supernatant was ultra filtered at 10 °C on a 5 kDa cut-off filter (OMEGA, 76 mm) in a stirred filtration cell (Amicon B.V., Holland). The permeate from the 5 kDa filtration was then filtered on a 1 kDa cut-off filter yielding a low molecular weight permeate containing a mixture of short peptides and free amino acids. This permeate was lyophilized, and subjected to fractionation by cation exchange chromatography (Fractogel TSK SP-650, Merck). Lyophilized permeate powder (3.00 g) was solubilized in 0.02 M Na-citrate/HCl buffer pH 4.0 (200 ml) and applicated on a 250 ml (6 cm × 9 cm) column at 10 °C. After application and washing by 0.02 M buffer pH 4.0, the elution was initiated by changing to 0.02 M buffer pH 6.0. The highest ACE-inhibitory activity was measured in fractions with pH 5.0–5.5 (128 ml). After lyophilization samples of the active fraction (40 μg peptide/μl) was solubilized in 0.1% TFA and further fractionated by reverse phase chromatography on a Shimazu instrument (LCMS-2010 EV) equipped with a semi preparative C 18 column (Inertsil ODS-3, 10 mm × 250 mm). Peptides were eluted with a gradient of acetonitrile (2–98%). The 15 fractions (5 ml/fraction) eluted by 10–30% acetonitrile were collected.

2.5. Identification of ACE-inhibitory peptides

Fractions from the C 18 column were collected and lyophilized before ACE inhibitory activity was measured [10]. The fractions with highest activity was analysed by LCMS. Based on the MS-analyses some peptides were chosen for sequencing by LC–MS/MS (Waters, BioLynx) (Tromsø University proteomic platform (TUPP)).

2.6. In vivo experiments with hypertensive rats

An *in vivo* study with spontaneously hypertensive rats (SHR) was conducted by Systems Biology Worldwide Toxis Oy (Turku, Finland). Fifty male SHR, 9–11 weeks of age, were obtained from Charles River Laboratories (Germany). The SHR got RM1 (E) chow (Special Diets Services, UK) and tap water, both *ad libitum*. Groups I–IV ($n = 10$ per group) got dosages of 15, 30, 45 and 60 mg DSPH/kg body weight per day. The negative control group (Group V) ($n = 5$) got sterile water, and the positive control group (Group VI) ($n = 5$) got Captopril; 10 mg/kg body weight per day. The dosing solutions were made daily and used within 1 h after preparation. Dose volume was 10 ml/kg of body weight. DSPH was dissolved in sterile water and diluted to concentrations yielding correct daily doses. The test item was administered by oral route using a 5 ml syringe and feeding tubes. Blood pressure was measured by using a rat non-invasive tail measurement method (MLT125/R Rat Pulse Transducer and Tail Cuff, AD Instruments, Castle Hill, Australia) 6 h after administration of the test item. Prior to the measurements, the animals were warmed in a warming cabinet at 38 °C for ca. 10 min. Blood pressure was measured at day −4, 1, 5, 7, 15, 22 and 29.

2.7. Statistical analysis

Blood pressure levels were compared between treatment groups using a fixed effect repeated measures two-way ANOVA with time as repeated variable [12]. Post hoc tests were performed using one-way ANOVA comparing between treatments at different times and Bonferroni adjusted probabilities [12]. The data were tested for deviation from normal distribution (Kolmogorov–Smirnov test) adapted by Lilliefors, and tested for homogeneity of variance (Levene). At one sampling, the data proved to diverge from normal distribution, and showed heteroscedastic variance. ANOVA is generally robust against minor deviations from these assumptions [12], and comparison of results using a nonparametric method (Kruskal–Wallis) did not reveal any different result. Significance were assumed when $p < 0.05$, and statistical power of performed ANOVA was estimated according to Zar [12].

Table 1
Free extractable amino acids and dipeptides and total amino acids in desalted shrimp protein hydrolysate (DSPH) as compared to total amino acids in Atlantic cod muscle protein (g/100 g).

Amino acid/dipeptide	Desalted shrimp protein hydrolysate		
	Free	Total	Cod muscle protein[a]
Essential			
Histidine	0.06	2.60	2.94
Isoleucine	0.12	4.67	4.61
Leucine	0.61	6.85	8.13
Lysine	0.11	7.27	9.18
Methionine	0.14	2.59	2.96
Phenylalanine	0.34	4.64	3.90
Threonine	0.05	4.24	4.39
Tryptophan	0.04	1.06	1.12
Valine	0.12	5.27	5.15
Non-essential			
Tyrosine	0.13	3.57	3.38
Aspartic acid	0.02	8.42	10.24
Glutamic acid	0.10	11.56	14.92
Glycine	0.27	5.33	6.05
Serine	0.12	4.57	4.08
Arginine	0.20	7.13	5.99
Alanine	0.19	5.49	4.80
Proline	0.06	4.71	3.54
Hydroxyproline		0.22	
Cysteine/cystine		0.75	1.07
Other amino acids/dipeptides			
Asparagine	0.10		
Glutamine	0.02		
3-amino-propanoic acid	0.02		
4-amino-butanoic acid	0.01		
Carnosine	0.08		
Anserine	0.04		
Ornithine	0.04		
Taurine	0.02		

[a] Obtained from Shahidi et al. [13]

3. Results and discussion

3.1. Amino acid composition and ACE inhibitory activity of DSPH

Table 1 shows the total amino acid composition of DSPH, the content of free amino compounds and, as a reference, the amino acid composition of Atlantic cod muscle protein [13]. DSPH has an amino acid composition very similar to cod protein, and the well balanced composition of essential amino acids vouch for good nutritional value. The content of free amino acids is generally quite low except from free leucine and phenylalanine yielding 9 and 7% of total amounts of these amino acids. These results, combined with results from ultra filtration, show that about 50% of DSPH are small peptides (<1 kDa), mainly containing 2–10 amino acid residues, whereas free amino acids and dipeptides yields about 3%. In addition to the common amino acids asparagine and the dipeptide carnosine are present in fairly high concentrations (0.10 and 0.08 g/100 g DSPH, respectively). The presence of taurine may add value to DSPH as a nutraceutical, since it has been reported to inherit both antihypertensive and anti-carcinogenic properties [14,15].

About 2–4 times higher ACE inhibitory activities were measured by the Dragnes et al. [10] method, modified from Cushman and Cheung [11] than by the Vermeirssen et al. [9] method (Table 2). The reason for this is unknown, but significant differences in IC_{50}-values for defined peptides are frequently reported by different authors [3,16,17], and this underlines the importance of considering the method applied when ACE inhibitory activities of different peptides or preparations are compared. In the present work both methods applied reveal that DSPH has very high ACE inhibitory activity, yielding IC_{50}-values of 0.035 and 0.075 mg/ml by the Dragnes and Vermeirssen methods, respectively. This is significantly better than