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ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY OF HYDROLYSATES OBTAINED FROM MUSCLE FOOD INDUSTRY BY-PRODUCTS – A SHORT REPORT

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Enzymatic hydrolysate (*Alcalase* catalysed) was obtained from cracklings (CEH) and acid hydrolysates were prepared from cracklings (CAH) and chicken feathers (FAH). The degree of hydrolysis (DH) of CEH, CAH, and FAH were 14.0%, 53.8%, and 46.2%, respectively. The results of the SE-HPLC confirmed that small molecular weight peptides composed a considerable fraction of all hydrolysates. The crude hydrolysates exhibited angiotensin I-converting enzyme (ACE) inhibitory activity as determined using hippuryl-His-Leu as the substrate. The inhibition of ACE by CEH, CAH, and FAH was 72.3%, 50.0%, and 49.6% under identical assay conditions.

INRTODUCTION

Hypertension is one of the major risk factors of developing cardiovascular diseases (heart failure, stroke, coronary heart disease, myocardial infraction) [Kannel, 1996] that affect a considerable part of the world adult population. The search for diet-related preventive measures for hypertension is of interest within the scope of functional foods [Li *et al.*, 2004]. Certain peptides obtained from hydrolysed food proteins may have angiotensin I-converting enzyme (ACE) inhibiting activity which is a key mechanism that prevents an increase in blood pressure. Such peptides have a promising value to be utilized for therapeutic purposes to prevent hypertension.

Many ACE inhibitory peptides have been obtained from enzymatic hydrolysates of animal origin food proteins such as casein [Kim & Chung, 1999; Tauzin *et al.*, 2002], whey protein [Pihlanto-Leppälä *et al.*, 2000; Hernández-Ledesma *et al.*, 2002], muscle protein [Arihara *et al.*, 2001; Nakashima *et al.*, 2002], fish protein [Curis *et al.*, 2002], chicken egg [Yoshii *et al.*, 2001], gelatin [Kim *et al.*, 2001], and blood plasma proteins [Wanasundara *et al.*, 2002]. These examples show that animal proteins such as casein, collagen, lactalbumin, myosin, ovalbumin, and serum albumin are potent in developing ACE inhibitory peptides upon hydrolysis.

Antioxidative activity of the hydrolysates obtained from muscle food industry by-products has been reported in our previous study [Flaczyk *et al.*, 2003]. The present study reports an ACE inhibitory activity of protein hydrolysates obtained from cracklings and poultry feathers.

MATERIAL AND METHODS

Materials. Cracklings of pork and feathers of chicken were obtained from the local food industry in Poznań (Poland). Cracklings were ground and defatted with hexane in Soxhlet apparatus. Feathers were thoroughly washed with deionised water and cut into small pieces.

Chemical analysis. The content of total nitrogen (N_{tot}) in hydrolysates was determined by the Kjeldahl N analysis. α-Amino nitrogen (N_{NH2}) and the degree of hydrolysis (DH) of the samples were determined using the trinitrobenzenesulphonic acid (TNBS) method [Panasuik *et al.*, 1988].

Enzymatic hydrolysis. The enzymatic hydrolysate from cracklings (CEH) was prepared using *Alcalase* 2.4 L (*Novo Nordisk Company*, Bagsverd, Denmark; a declared activity of 0.6 Anson units AU per gram). The hydrolysis was carried out in a 250 mL thermostatic vessel equipped with a stirrer and pH-meter, set at a temperature of 55±1°C for 1 h at pH 8.5, at an enzyme/substrate ratio of 0.15 AU/g material as described by Shahidi *et al.* [1995]. After hydrolysis, the enzyme was inactivated by heating the mixture at 80°C for 5 min. Non-hydrolysed material was precipitated by adding chloroform at room temperature and then insoluble material was removed by centrifugation. The pH of the supernatant was adjusted to 7.0, and the hydrolysate was lyophilised.

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Acid hydrolysis. Acid hydrolysis was carried out using 4.75 mol/L HCl at 110°C for 4 h (cracklings) or 12 h (feathers), at a material to aqueous HCl ratio of 3:7 (w/v). After hydrolysis, pH of the hydrolysate was brought up to 5.7 with sodium carbonate fine powder. The mixtures were then shaken with activated charcoal (1.8%, w/v 15 min at 60–70°C), filtered through Whatman No. 3 filter paper, stored at 4°C for 4 weeks and then finally spray dried. Hydrolysates were marked as CAH (from cracklings) and FAH (from feathers).

Size exclusion chromatography of hydrolysates. Size exclusion chromatography of the hydrolysates were performed by HPLC using a Waters HPLC system. The HPLC system comprising a pump (Waters 600EP), an autosampler (Waters 715 ultra wisp), a system controller (Waters 600), and a photodiode array detector (Waters 996) was used. A mobile phase of 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) was utilised at a flow rate of 0.2 mL/min. The separation was done by TSK G 2000 SW_{xl} column (TosoHaas, Montgomerywille, PA, USA) protected by a SW_{xl} precolumn. The sample was dissolved in mobile phase (4 mg/mL) and passed through a 0.45 μm filter. The injection volume was 10 μL and the detection was at 220 nm. Bovine serum albumin (66 000 Da), cytochrom c (12 500 Da), bovine lung aprotinin (6 500 Da), bovine insulin chain B oxidised (3 495 Da), human angiotensin II (1 040 Da), leucine encephalin (556 Da) and Thr-Tyr-Ser (369 Da) were employed as molecular weight standards.

ACE inhibitory assay. The activity of ACE was determined using hippuryl-His-Leu (Sigma) as the substrate [Cushman & Cheung, 1971] with the modification of Mehanna and Dowling [1999]. The assay was conducted in a Tris buffer (50 mmol/L, pH 8.3) containing 300 mmol/L NaCl. The same buffer was used to dilute the hydrolysates, enzyme, and substrate. The initial assay volume consisted of 50 μL of the substrate (3 mmol/L), 50 μL of the enzyme (*i.e.* ACE from porcine kidney, Sigma) solution containing 1.25 mU of declared enzyme activity, and 50 μL of assay sample. All these solutions were incubated for 30 min at 37°C in a water bath first without mixing and then for an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop ACE activity. The reaction mixture was separated by HPLC to determine the hippuric acid content produced due to ACE activity on the substrate. The HPLC method employed to quantify free hippuric acid was as follows. A reversed phase C18 column (LUNA, 5 μm, 250 x 4.6 mm; Phenomenex), mobile phase was an isocratic system consisting of 12.5% (v/v) acetonitrile in deionised water, and its pH was adjusted to 3.0 by adding glacial acetic acid, injection volume was 10 μL and eluted hippuric acid was detected at 228 nm. A series of standard hippuric acid solutions were prepared to construct a calibration curve of peak area against hippuric acid concentration. The control reaction mixture contained 50 μL of buffer instead of the assay sample and the control was expected to liberate the maximum amount of hippuric acid from the substrate due to uninhibited ACE activity. Same Waters HPLC system described above was employed for this analysis.

The percent inhibition of enzyme activity was calculated as follows:

$$\%\ Inhibition = \frac{[hippuric\ acid]_{control} - [hippuric\ acid]_{sample}}{[hippuric\ acid]_{control}} \times 100\%$$

All analyses were carried out in triplicate samples.

RESULTS AND DISCUSSION

The content of α-amino nitrogen (N_{NH2}) and the degree of hydrolysis (DH%) of the hydrolysates are presented in Figure 1. The highest content of total nitrogen (N_{tot}) was observed for CEH. This hydrolysate was characterised by the lowest level of N_{NH2} (1.7%) and the lowest degree of hydrolysis (14.0%), indicating that only a small fraction of protein was hydrolysed due to *Alcalase* catalysis. The contents of N_{tot} in CAH and FAH were 7.9% and 8.2%, respectively. The N_{NH2} of 3.8% was observed for both acid hydrolysates. The degrees of hydrolysis for the acid

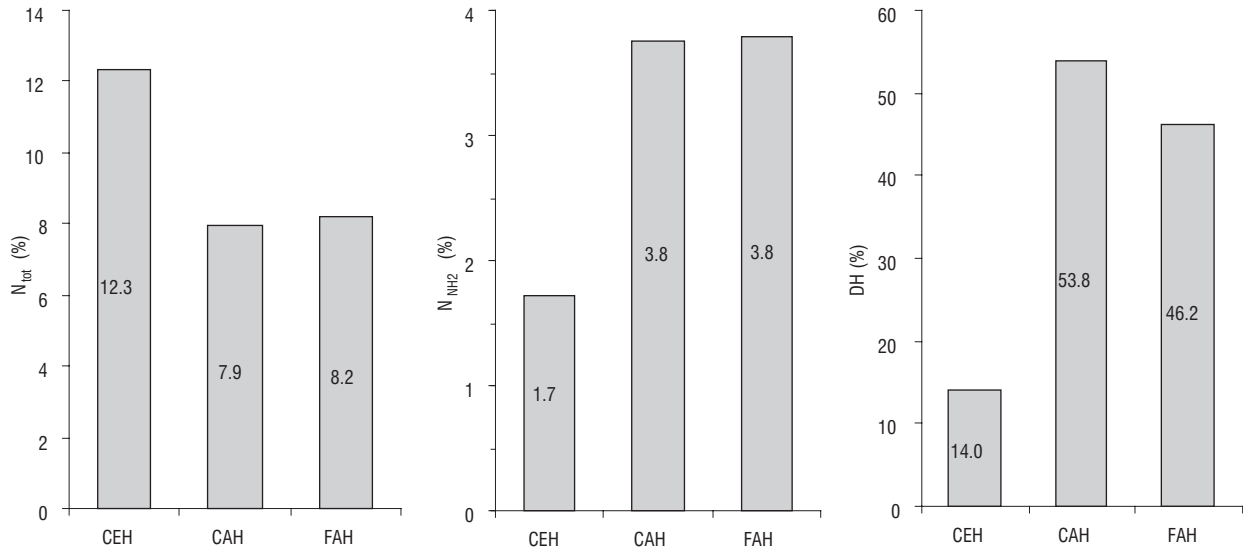

FIGURE 1. Chemical characteristics of the investigated hydrolysates.

hydrolysate (CAH) was 3.8 times higher than that for the enzymic hydrolysate (CEH), indicating that acid-catalysed hydrolysis was able to break more peptide bonds than the *Alcalase*-assisted hydrolysis. The DH% of CEH was in the range of values reported in the literature for various protein sources hydrolysed by *Alcalase* [Gwiazda *et al.*, 1994; Kim *et al.*, 1990; Ponnampalam *et al.*, 1987; Hoyle & Merritt, 1994; Shahidi *et al.*, 1994].

Figure 2 shows the separation of molecular mass standards and hydrolysates on the TSK G2000 SW_{XL} column; this packing material and applied conditions of size exclusion chromatography enabled good separation of the standards in a broad range of molecular masses: from 66 000 Da to 365 Da. The same column under similar separation conditions was used for the analysis of hydrolysates. The HPLC chromatogram of CEH and CAH is characterised by three main peaks. Five peaks are noted on the chromatogram of FAH. Molecular weight of peptides present in the hydrolysates was less than 6 500 Da.

All the hydrolysates showed ACE inhibiting activity. The control which has no external inhibitor added released 283.7 μmol/sample (Figure 3) hippuric acid from the substrate hippuryl-His-Leu. The addition of hydrolysates to the sample decreased the liberation of hippuric acid from the substrate. The lowest level of hippuric acid was released for CEH (78.5 μmol/sample), indicating a stronger inhibition of ACE activity than other hydrolysates CAH and FAH liberated almost the same amounts of hippuric acid (141.8 and 142.9 μmol/sample). Figure 4 shows the percentage inhibition of ACE activity by the three hydrolysates. The enzymic hydrolysate of cracklings, CEH exhibited 72.3% of inhibition ACE activity compared to the acid hydrolysate of the same substrate, CAH. The acid hydrolysed proteins of feathers (FAH) were able to inhibit (49.6%) ACE activity at as closer levels as CAH (50%). This indicated that even at low concentration (as shown by α-amino nitrogen and DH%), the *Alcalase*-assisted hydrolysis generated comparatively stronger ACE inhibitory peptides than the acid hydrolysis of cracklings. These results indicate that porcine skin proteins of cracklings and poultry feather proteins produce peptides with ACE inhibitory activity upon hydrolysis.

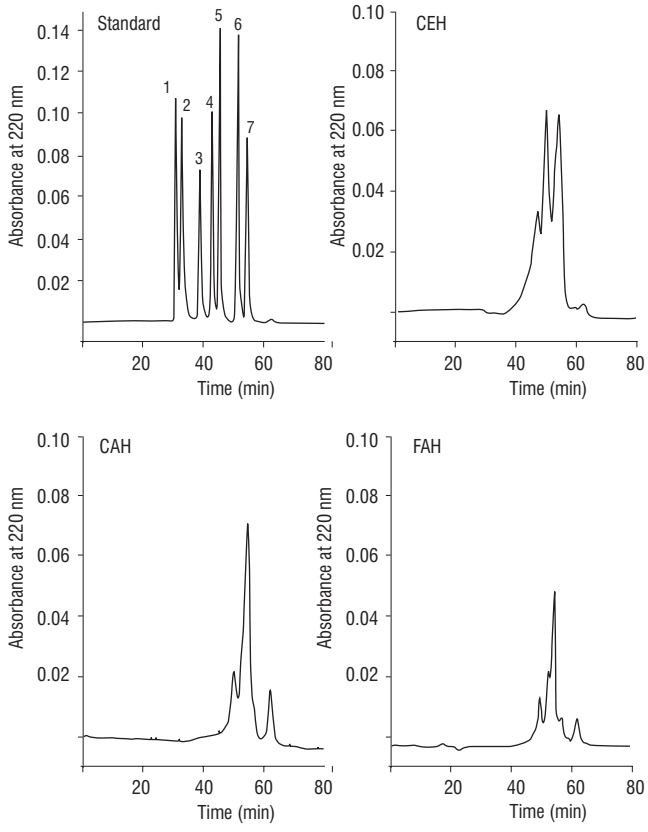

FIGURE 2. SE-HPLC chromatogram of molecular weight standards and hydrolysates; (1 – bovine serum albumin (66 000 Da), 2 – cytochrom c (12 500 Da), 3 – bovine lung aprotinin (6 500 Da), 4 – bovine insulin chain B oxidised (3 495 Da), 5 – human angiotensin II (1 040 Da), 6 – leucine encephalin (556 Da), 7 – Thr-Tyr-Ser (369 Da).

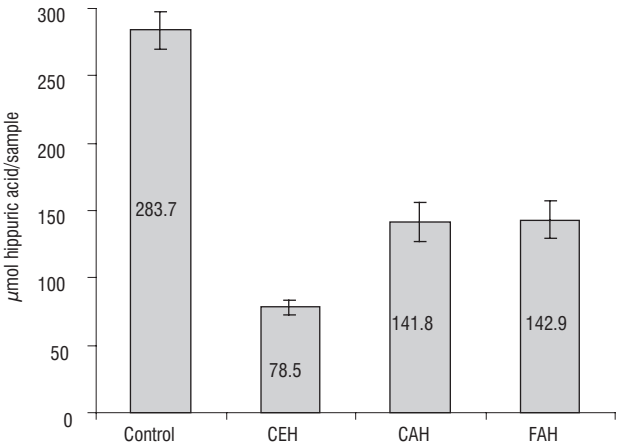

FIGURE 3. Content of released hippuric acid by control and assay samples determined using the HPLC method.

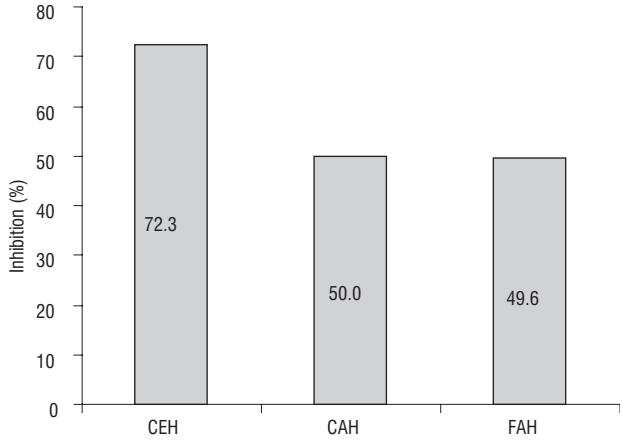

FIGURE 4. Percentage inhibition of angiotensin I-converting enzyme activity by the investigated hydrolysates.

CONCLUSIONS

The inhibitory effect of hydrolysates prepared from cracklings and chicken feathers on ACE activity make these products and raw materials potential candidates to be developed and applied within the concept of functional foods. The separation of the most active peptide/peptides from the hydrolysates and elucidation of their chemical structure is in progress.

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HYDROLIZATY BIAŁKOWE OTRZYMANE Z PRODUKTÓW ODPADOWYCH PRZEMYSŁU SPOŻYWCZEGO JAKO INHIBITORY ENZYMU KONWERTUJĄCEGO ANGIOTENSYNĘ I (ACE) – KRÓTKIE DONIESIENIE

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Enzymatyczny hydrolizat białkowy otrzymano z odtłuszczonych skwarek (CEH) stosując enzym alkalazę. Surowcem do uzyskania kwasowych hydrolizatów były skwarki (CAH) i kurze pierze (FAH). Stopień hydrolizy CEH, CAH i FAH wynosił odpowiednio: 14.0%, 53.8% i 46.2%. Analiza SE-HPLC potwierdziła obecność w hydrolizatach niskocząsteczkowych peptydów. Stosując jako substrat hipuryl-His-Leu wykazano za pomocą RP-HPLC, że badane hydrolizaty wykazują zdolność do inhibicji enzymu konwertującego angiotensynę I (ACE). W warunkach testu inhibicja ACE przez CEH, CAH i FAH wynosiła odpowiednio: 72.3%, 50.0% i 49.6%.