Development of an effective process for utilization of collagen from livestock and fish waste

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Abstract

A procedure for the extraction of protein and production of peptides by enzymic hydrolysis from bone and skin wastes containing collagen was developed. Fat and inorganic components were first removed in a pretreatment step and a high molecular weight protein extracted under acidic conditions (pH 3) using a 1 h reaction time at 60 °C. The molecular weight of extract from pig skin was greater than 100 kDa. The extract had a high water retention capacity, was beneficial for repair of rough skin, had no odor problem and was demonstrated to be safe in skin patch tests. It was thus considered acceptable for use in cosmetic materials. Pretreated fish bone and pig skin were hydrolyzed with a commercial enzyme. The hydrolysates had a high anti-radical activity (IPOX 50, 0.18 and 0.45 mg ml⁻¹) and a high potential for decreasing blood pressure (IC 50, 0.16 and 0.41 mg ml⁻¹), suggesting the hydrolysates could be a useful additive in food materials. © 2002 Published by Elsevier Science Ltd.

Keywords: Collagen; Pig skin; Fish bone; Extraction; Enzymic hydrolysis

1. Introduction

Approximately 14.5 million head of cattle and swine and 333 million chickens are bred in Japan annually. Meat, milk and eggs are produced from these animals for domestic consumption. Residual parts, however, such as horn, hoof, skin and bone are wasted. The fishing industry also creates large amounts of scale and bone as waste byproducts. Assuming 10% of the animal weight is wasted, the total amount of waste generated from these sources can be as high as 900 000 tons per year.

Horn, hoof, skin, bone and scale contain large amounts of the structural proteins keratin and collagen. Keratin and collagen and associated hydrolysates have desirable characteristics for various industrial applications. For example, they are not hazardous to human health, are capable of water retention on the molecular scale, are essential biological nutrients and can serve as chromatographic carrier particles. Accordingly, they can be used in materials associated with or included in cosmetics, artificial organs, etc. and as such animal wastes in which these proteins are potentially valuable resources.

Effective degradation or hydrolysis of materials composed of keratin or collagen is difficult due to their stable hard protein structure, thus industrial utilization in materials for food, cosmetics, artificial organs etc. of these proteins have been little developed. Recently, though, an enzyme from commercially available sources was selected that is capable of degrading horn and hoof and established a process for the production of horn and hoof hydrolysates [1].

In this work, we report on the selection of an enzyme that can degrade bone and skin composed of collagen and the development of processes by which collagen is extracted from livestock and fishery wastes and peptides are produced by enzymic hydrolysis. The extract and hydrolysates were then characterized for their potential in commercial applications.
2. Materials and methods

2.1. Raw materials

The spine of a yellowtail fish were received and stored at \(-20 ^\circ C\) until use. Immediately prior to use, the materials were crushed into 3–5 mm chunks with a hammer and dried for more than 24 h under the conditions of 60 °C and reduced pressure at \(-76 \text{ mmHg}\). Fat and inorganic compounds were then removed (Section 2.2, below). Pig skin was received from a pork processing plant, cut into 3–5 cm squares with scissors and then chopped to smaller dimensions using a bone chopper (MKBC-32 #, Masuko Sangyo, Tokyo). It was then dried for more than 24 h under the conditions of 60 °C and reduced pressure. Sixteen commercially available proteases were used for investigation of enzymic hydrolysis of materials containing collagen.

2.2. Pretreatment

For removal of fat from bone and skin, 60 g of dried sample was placed in a glass roller bottle (3 l capacity) 400 ml of hexane was added to the bottle, which was then agitated on a roller-culture apparatus (Wheaton Instruments, NJ, USA) at a speed of scale 4 at room temperature for 12 h. The material in the roller-bottle was filtered through no. 5A filter paper (ADVANTEC, Tokyo) and dried for 24 h under the conditions of 60 °C and reduced pressure at \(-76 \text{ mmHg}\).

Removal of inorganic compounds from bone was carried out by soaking in a 0.6 N HCl solution at room temperature for 24 h. The concentration of bone in the solution was 10% (w/v). The de-ashed bone was filtered through no. 5A filter paper and washed three times with tap water (same volume as used for the 0.6 N HCl solution). The procedure for removal of inorganic compounds was performed twice, in which case the soaking and washing series described above was repeated. The sample was then dried for 24 h under the conditions of 60 °C and reduced pressure at \(-76 \text{ mmHg}\). The dried bone was ground to powder using a mill (Personal mill SCM-40A, Shibata Scientific Technology Ltd., Tokyo) and sieved with a no. 32 (0.50 mm) mesh sieve. The pig skin was not treated for removal of inorganic compounds due to the very low amount of this constituent. Furthermore, the pig skin was not ground with a mill.

2.3. Extraction of collagen

Pig skin from which fat was already removed was used for the extraction of high molecular weight collagen protein. The skin was placed in a 10 l flask of a rotary evaporator (Rotary Vacuum Evaporator N-11, EYELA, Tokyo) to a final concentration of 125 g l\(^{-1}\) with the addition of an acidic (pH 3) or an alkaline (pH 12) solution. The acidic solution was prepared by adjusting the pH of 1/15 M acetate buffer to 3 with 1 N HCl. The alkaline solution was prepared by diluting a NaOH solution with water to pH 12. Extraction was done at 60 °C at a rotary speed of scale 6 for 1 h. After extraction, the sample was filtered with a double layer of gauze and the filtrate was centrifuged at 8000 × g for 10 min. The supernatant was then lyophilized and the resulting extract was used for further investigations. The amount of extract (\%) was calculated as the ratio of extracted weight to initial weight of de-fatted pig skin.

2.4. Evaluation of enzymes

The first evaluation of enzymes was conducted with 20 mg of dried fish bone (of which fat and inorganic components were removed and then crushed as described above, hereafter called pretreated fish bone) and 2 ml of buffer solution (with suitable pH as required for each enzyme) combined in an 18 mmφ L-shaped tube and shaken at the designated temperature for each enzyme for about 10 min in a Monod-type shaker (Incubator MONOD-MINI, TAITEC, Tokyo). About 2 ml of enzyme solution, containing 2 mg-protein ml\(^{-1}\) of enzyme solution at final concentration, was added and the mixture was shaken at the required temperature for each enzyme for 60 min. After boiling the reaction mixture, the residual insoluble matter was removed by retention on a 0.45 μm membrane filter (cellulose nitrate, ADVANTEC) and its weight was measured after drying at 105 °C for 24 h. The degradation efficiency (\%) was calculated as the ratio of degraded weight of substrate to initial weight.

A second evaluation was conducted for six kinds of enzyme that had degradation efficiencies greater than 60%. Forty milligrams of dried fish bone and 4 ml of buffer solution (with pH as required for each enzyme) were placed in an 18 mmφ L-shaped tube and shaken at the designated temperature for each enzyme for about 10 min on a Monod-type shaker. Four milliliter of enzyme solution, containing 2 mg-protein ml\(^{-1}\) of enzyme solution at final concentration, was added and the mixture was shaken at the required temperature for each enzyme for 60 min. After boiling the reaction mixture, the residual insoluble matter was filtered (as the first evaluation) and its weight was measured after drying at 105 °C for 24 h.

2.5. Enzymic hydrolysis in a reactor

One hundred twenty five gram of substrate (pretreated fish bone and de-fated pig skin) and 6.25 g of the selected enzyme L were placed in a reactor with a working volume of 1 l (MBF-250M, EYELA) and agitated at 200 rpm for 60 min at 60 °C and a pH of
8.0. The reaction mixture was then centrifuged at 8000×g for 10 min and the weight of precipitate was measured after drying at 105 °C for 24 h.

2.6. Analytical

2.6.1. Organic and inorganic materials

Organic matter in this research was defined as the amount of sample material that vaporized during incineration at 600 °C for 30 min (i.e. amount of sample weight loss on ignition) and, conversely, inorganic matter was defined as the amount of residual solids following ignition (i.e. fixed solids or mineral ash). Organic matter (%) and inorganic matter (%) were calculated as the k percentage of reduced weight and retained weight in incineration to dried weight.

2.6.2. Fat content

To determine fat content, approximately 1 g of dried sample was taken and weighed accurately. The sample was placed in a thimble filter (35 mmφ × 120 mm, ADVANTEC) and the filter with sample was placed in a Soxhlet extractor (SOXTEC SYSTEM HT2, AK-TAK Ltd., Tokyo). The fat content was then determined according to Methods of Analysis in Health Science [2].

2.6.3. Protein content

For determination of the protein content, 60 mg of dried sample and 5 ml of 1 N NaOH solution were added to a test tube and mixed well. The tube with sample was then heated in boiling water for 10 min and cooled to room temperature. The extracted solution under alkaline conditions was filtered and made up to 100 ml. The protein content was then determined by the Lowry–Folin method [3]. Collagenpeptide reagent (Wako Pure Chemical Industries Ltd., Osaka) was used as the standard.

2.6.4. Molecular weight

The molecular weight of the extract was determined by polyacrylamide gel electrophoresis with sodium dodecylsulphate (SDS-PAGE) on a 12% polyacrylamide gel at a voltage of 200 V according to the method of Laemmli [4].

2.6.5. Water retention capacity

The water retention capacity of the extract was determined by direct application to human skin. The person being tested entered a test room maintained at 20 °C and remained seated on a chair and inactive for about 20 min. A sample solution of 0.1 ml volume was then applied to the forearm and excess solution was wiped off with paper after 60 s. A commercially used collagen solution (collagen B) and distilled water were applied for the positive and negative control test instead of a sample solution. The water content on the skin of the forearm was then determined with a skin surface hygrometer (SKIKON-200, I.B.S Co., Hamamatsu, Japan). The water content on the skin was measured at 60 s intervals for 10 min.

2.6.6. Water vaporization potential

The water vaporization potential was determined by direct application to human skin. About 0.2 ml of sticky plaster (Libatape Pharmaceutical Co., Tokyo) containing 10% sodium dodecylsulphate solution (w/v) was applied to the forearm skin of a person for 6 h to make the skin rough. Subsequently, 0.1 ml of sample solution was applied to the forearm skin once a day for 3 days. After 14 applying the sample solution, the amount of vapourized water on the forearm skin was measured with a Tewameter (TM210, Nippon Eurotec Co., Tokyo). The measurements were performed in a test room at 20 °C on a person in a seated, inactive posture.

2.6.7. Extract odour

The extract odor was determined on 0.05 g-nitrogen 1−1 sample dilutions with pH adjustments at 2 U intervals from 2 to 10. Odour intensity was measured on 1.0 ml dilution aliquots in a fragrance sensor (SF-105, Sogo Pharmaceutical Co., Tokyo) following a 5 min holding period.

2.6.8. Skin patch test

To evaluate the degree of safety of the extract to human skin, a patch test was conducted according to the Association of Industrial Information’s method [5]. About 0.2 ml sticky plaster (Libatape Pharmaceutical Co.) containing 0.03% (w/v) of the extract was applied to the forearm skin of a person for 24 h. The plaster was then removed and the skin of the person was observed after 30 min and 24 h visually by unaided eyesight and at 50-fold magnification using a microscope.

2.6.9. Peptide molecular weight

One milliliter of peptide solution and 4 ml of 6 N HCl were added to a screw cap test tube and mixed well. The cap was closed tightly under a nitrogen atmosphere and was then heated at 110 ± 1 °C for 24 h. Subsequently, 4 ml of 6 N NaOH solution was added to the tube and the reaction mixture was made up to suitable volume with distilled water. Next, 0.1 ml of hydrolyzed solution or 0.1 ml of peptide solution without hydrolysis was added to 0.9 ml of 4% (w/v) NaHCO₃ solution, and was added to 1 ml of 1% (w/v) 2,4,6-trinitrobenzenesulphonic acid (TNBS), respectively, followed by incubation at 40 °C for 2 h. The solutions were then diluted 10 times with a 4% (w/v) NaHCO₃ solution and absorbance was measured at 420
nm. The standard curve was prepared using glycine. The concentration of amino residue in each sample, with and without hydrolysis, was then calculated by Eq. (1) and the mean degree of polymerization was calculated by Eq. (2):

\[
x - \text{amino residue (g l}^{-1}) = \frac{\text{Concentration as glycine standard (g l}^{-1}) \times 14.01}{75.09}
\]

Degree of polymerisation(%) = \frac{\text{Concentration of } x-\text{amino residue of hydrolyzed sample}}{\text{Concentration of } x-\text{amino residue of non-hydrolyzed sample}} \times 100

The mean molecular weight was calculated according to both the mean degree of polymerization and the amino acid composition, as described below.

2.6.10. Amino acid composition

For the determination of amino acid composition, a sample was hydrolyzed under both acidic and alkaline conditions. For acidic hydrolysis, 1 mg of sample and 1 ml of 6 N HCl solution were added to an ample tube. The tube was then sealed under a vacuum and held at 110 ± 1 °C for 24 h. For alkaline hydrolysis, 1 mg of sample, 3 mg of starch and 2 ml of 2.5 N NaOH solution were added to the ample tube. The tube was then sealed under a vacuum and held at 110 ± 1 °C for 24 h. Hydrolyzed samples were analyzed by high-performance liquid chromatography (HPLC, JASCO, Tokyo) using fluorescence detection (JASCO). For chromatographic separation, an AA-pak Na⁺ column was used at a constant oven temperature of 60 °C with four kinds of eluent to form a pH gradient. The separated amino acids were combined with o-phthalaldehyde (OPA) for fluorescence detection. The intensity of fluorescence was detected with excitation at 340 nm and emission at 450 nm.

2.6.11. Physiological activities

Anti-radical (antioxidant) activity was determined using luminol-enhanced chemiluminescence according to the method reported by Kanazawa et al. [6]. IPOX₅₀ (inhibitory potential of 50% for peroxylradicals) indicates the concentration of sample required to quench 50% of the chemiluminescence in the reaction mixture. Potential for reducing blood pressure was evaluated as the inhibitory activity of the angiotensin I converting enzyme (ACE). ACE inhibitory activity was determined using a modified method of Cushman and Cheung [7]. IC₅₀ (inhibitory concentration of 50% for ACE activity) indicates the concentration of sample required to reduce 50% of the ACE activity in the reaction mixture.

3. Results and discussion

3.1. Pretreatment of bone and skin

Fig. 1 (left side) shows the compositions (as raw materials) of pig skin and fish bone used on a dry-weight basis. ‘Other organic matter’ in Fig. 1 indicates the amount remaining after subtracting the weight of protein and fat from total organic matter. Fish bone, due to its high fat and inorganic matter contents, was selected to conduct a preliminary optimization study on pretreatment procedures. Initially, with an extraction time of 12 h, the influence of bone concentration in the extraction solution was investigated. At bone concentrations of 100 and 150 mg l⁻¹ the residual fat content following extraction was about 6%; however, at a bone concentration of 175 mg l⁻¹ the residual fat increased to 10%. A bone concentration of 150 g l⁻¹ was thus used to conduct further study on the influence of mixing time. With a 6 h mixing time, the residual fat content was reduced to 8%, and with 12 and 24 h mixing times, residual fat was reduced to 6%. An
3.1. Extraction of collagen

The extraction time of 12 h was thus selected for further use.

Removal of inorganic matter was initially investigated by soaking the sample in 0.5 M EDTA solution, dilute HCl (pH 2) or 0.6 N HCl. The amount of inorganic residue after soaking for 8 h was 39% with the EDTA solution, 50% with dilute HCl and 19% with 0.6 N HCl. The 0.6 N HCl solution (with the lowest amount of residue) was thus used for further study on the influence of soaking time. By soaking a 10% de-fatted fish bone solution (w/v) in 0.6 N HCl solution for 8, 16, and 24 h, the amount of inorganic residue measured decreased with an increase in treatment time, however, even with a 24 h treatment time, 5% inorganic matter still remained. With a second consecutive 24 h treatment period, the inorganic residual decreased to zero. Thus, it was evident that a duplicate extraction procedure was essential for effective removal of inorganic matter.

Following the optimized procedure above, the composition of fish bone and pig skin after pretreated were as shown in Fig. 1 (right side). The reported values (g dry-weight) indicate the amount remaining following pretreatment of 100 g dry-weight raw material. Removal of inorganic matter from pig skin was not attempted because the content in the raw material was only 1%. In pretreatment of bone, more than 80% of fat and 90% of inorganic matter were removed. Recovery of protein from fish bone, 53%. Recovery of protein from pig skin, however, was as high as 91%, evidently because the procedure for removal of inorganic material was not conducted. The amount (g dry-weight) of protein recovered from 100 g of raw material was 19 g in fish bone and 42 g in pig skin.

3.2. Extraction of protein

A preliminary study was conducted to determine the optimal conditions for extraction of protein following pretreatment. Pig skin, which had a high protein recovery during pretreatment, was used with both acidic (pH 3) and alkaline (pH 12) extraction solutions at 60 °C and otherwise as per the method for production of gelatin [8]. The amount of extracted protein from defatted pig skin was about 72% using the acidic solution and 80% with the alkaline solution. Extracted protein at pH 12 showed a large amount of small proteins of less than 100 kDa indicating that the collagen molecules were being fractured. Conversely, at pH 3, a higher molecular weight protein of over 100 kDa was dominant.

Collagen is widely used in cosmetic materials due to its excellent water retention characteristics, low incidence of causing allergic reactions [9] and effectiveness in repairing damaged skin [10]. The water retention capacity of collagen obtained in this study as evaluated in skin tests is shown in Fig. 2, where a higher electric conductivity indicates a higher water retention capacity. As shown in Fig. 2 (left side), the acidic extract had a high water retention capacity, essentially the same as that of collagen B, a commercially available form of collagen. The alkaline extract, however, had a low water retention capacity—not improved over that of water alone (control). Also shown in Fig. 2 (right side) are the results obtained using the cosmetic solutions prepared using extracts obtained at a concentration of 0.05 g-nitrogen l⁻¹. The cosmetic solution containing the acidic extract had a higher water retention capacity than that with the alkaline extract. The acidic extract
which had a higher molecular weight also had higher water retention capacity than that of the alkaline extract, indicating the possible significance of molecular weight as an indicator for water retention capacity.

The effect of collagen obtained in this research on recovery of rough skin was also investigated. In this assessment, the amount of water that can be vapourized from skin serves an indication of its roughness, i.e. rough skin offers little protection against vapourization. As shown in Fig. 3, the amount of water vapourized from rough skin was higher than that from normal skin. By applying the acidic extract or collagen B to rough skin, the amount of vaporization decreased and approached the level of normal skin. The acidic extract obtained in this research had the most beneficial effect for the recovery or repair of rough skin.

Since the collagen extracted in this research, especially under acidic conditions, demonstrated excellent properties for cosmetic materials, the safety of these materials for contact with human skin and odour intensity were also investigated. For the acidic extract, 28 out of 30 persons had no red colour spots and only two persons developed a slightly red discoloration. To judge the safety of the extracts using the results of the patch tests, a stimulation index has been proposed by Sugai et al. [11,12]. The index is calculated by Eq. (3) with score values as follows: is 0, ± is 0.5, + is 1.0, ++ is 2.0, +++ is 3.0, and ++++ is 4.0 [13].

Stimulation index for skin safety

\[
\text{Stimulation index} = \left( \frac{\text{Total score}}{\text{number of tests}} \right) \times 100 \tag{3}
\]

A sample is considered safe when the index is below five [13]. From the results of patch test using the acidic extract, the index was 3.33 (\((0.5 \times 2)/30) \times 100\) indicating a wide margin of safety. For patch test using the alkaline extract, all 30 tests had negative responses, thus the extracts from pig skin were shown to be safe for use in cosmetic materials.

Some materials produced from livestock or fishery waste tend to maintain their characteristic odours. If these materials are used, it would be necessary to mask the odour by adjusting the amount of material added or by the addition of a perfume. Difficulty was encountered in evaluating the odours of some materials because responses depended to some degree on personal preference. Thus, an odour sensor was used for the evaluation of the extracts from pig skin. The pH levels of the acidic and alkaline extracts were adjusted to 2, 4, 6, 8 and 10, and the intensity of the odour was measured at each level. As shown in Fig. 4, the intensity of

![Fig. 3. Effects of various extracts on recovery of rough skin.](image)

![Fig. 4. Influence of pH on odor response of acidic and alkaline extracts.](image)
Table 1
Evaluation of 16 commercially available enzymes on degradation of pretreated fish bone

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Origin</th>
<th>Reaction conditions</th>
<th>Degradation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Aspergillus niger</td>
<td>pH 3.0, Temperature 60°C</td>
<td>63.2</td>
</tr>
<tr>
<td>B</td>
<td>Aspergillus oryzae</td>
<td>pH 6.0, Temperature 50°C</td>
<td>37.4</td>
</tr>
<tr>
<td>C</td>
<td>Aspergillus melleu</td>
<td>pH 8.0, Temperature 50°C</td>
<td>64.8</td>
</tr>
<tr>
<td>D</td>
<td>A. oryzae</td>
<td>pH 7.0, Temperature 50°C</td>
<td>50.8</td>
</tr>
<tr>
<td>E</td>
<td>A. niger</td>
<td>pH 3.0, Temperature 60°C</td>
<td>60.3</td>
</tr>
<tr>
<td>F</td>
<td>A. niger</td>
<td>pH 3.0, Temperature 50°C</td>
<td>59.0</td>
</tr>
<tr>
<td>G</td>
<td>A. oryzae</td>
<td>pH 7.0, Temperature 50°C</td>
<td>36.5</td>
</tr>
<tr>
<td>H</td>
<td>Rhizopus delmar</td>
<td>pH 3.5, Temperature 55°C</td>
<td>50.8</td>
</tr>
<tr>
<td>I</td>
<td>Carica papaya L</td>
<td>pH 6.5, Temperature 60°C</td>
<td>55.2</td>
</tr>
<tr>
<td>J</td>
<td>A. niger</td>
<td>pH 3.0, Temperature 50°C</td>
<td>60.2</td>
</tr>
<tr>
<td>K</td>
<td>Bacillus subtilis</td>
<td>pH 7.0, Temperature 55°C</td>
<td>69.2</td>
</tr>
<tr>
<td>L</td>
<td>Bacillus sp.</td>
<td>pH 10.0, Temperature 60°C</td>
<td>85.8</td>
</tr>
<tr>
<td>M</td>
<td>A. oryzae</td>
<td>pH 3.0, Temperature 50°C</td>
<td>42.9</td>
</tr>
<tr>
<td>N</td>
<td>Rhizopus niveus</td>
<td>pH 3.0, Temperature 45°C</td>
<td>47.5</td>
</tr>
<tr>
<td>O</td>
<td>A. niger</td>
<td>pH 2.5, Temperature 55°C</td>
<td>40.0</td>
</tr>
<tr>
<td>P</td>
<td>C. papaya L</td>
<td>pH 6.5, Temperature 50°C</td>
<td>47.0</td>
</tr>
</tbody>
</table>

Fig. 5. Effects of enzyme concentration on degradation efficiency with 5 g l⁻¹ substrate at 60 °C.

odour for both the acidic and alkaline extracts increased at the lower and higher pH values. The minimum intensity occurred near a neutral pH for both extracts. These results were corroborative of the 124 Hz reported for commercial collagen B using the same test at pH 7. Thus, both extracts in this research were demonstrated to be acceptable with respect to odour.

As shown above, the acidic extract from pig skin had almost the same characteristics of commercial collagen. A cosmetic solution prepared using this acidic extract for the water retention capacity test was stored at 20 °C for 6 months. Following storage, no changes in previously determined characteristics including color and aggregation properties were observed. From these results, the acidic extract from pig skin appeared to be best suited for use in cosmetics.

3.3. Enzymic hydrolysis

Collagenase is a well characterized collagen degrading enzyme; however, for practical considerations, more readily available commercially produced proteases were evaluated for use in this research. Sixteen commercial enzymes were selected to evaluate their effectiveness for degradation of pretreated fish bone. As shown in Table 1, the degradation efficiencies of six of the enzymes were over 60%. Enzyme L had the highest degradation efficiency of 85.8% and enzyme K had the second highest efficiency of 69.2%. Enzymes L and K, which originated from Bacillus species as did the alkaline protease, appeared to be superior to those originating from fungi for the degradation of collagen among the 16 enzymes evaluated.
The influence of enzyme concentration among the six enzymes shown to have degradation efficiencies over 60% (Table 1) was investigated using pretreated fish bone at a constant substrate concentration of 5 g l⁻¹. Fig. 5 shows the results for enzymes K and L. Enzyme L demonstrated a degradation efficiency of 83% with an enzyme concentration of 0.25 g l⁻¹. The five other enzymes tested, including enzyme K (Fig. 5, left side), had degradation efficiencies of less than 70%, also with 2.5 g l⁻¹ enzyme. From these results, enzyme L was clearly the best for degradation of collagen among the 16 enzymes.

For enzyme L with the optimal 83% degradation efficiency using 5 g l⁻¹ of substrate and 0.25 g l⁻¹ of enzyme, the ratio of substrate to enzyme was 20:1. The influence of substrate concentration was then investigated at a constant substrate to enzyme ratio of 20:1. Under these conditions, degradation efficiencies greater than 80% were obtained with less than 125 g l⁻¹ of substrate. However, the efficiency decreased to about 70% with 250 g l⁻¹ of substrate. Thus, a substrate concentration of 125 g l⁻¹ was selected for further investigation of enzymic hydrolysis of collagen.

With a 1 h reaction time in a reactor with 1 l working volume at 60 °C at pH 8.0, 125 g l⁻¹ of fish bone was degraded using 6.25 g l⁻¹ of enzyme L. With pretreated fish bone as substrate, degradation efficiencies from 75 to 80% were obtained. However, with de-fatted pig skin, efficiencies from 90 to 95% were obtained under the same conditions. Using the residual pig skin after extraction as substrate, the degradation efficiency was also the same as that in the de-fatted pig skin.

3.4. Properties of hydrolysate

The mean degree of hydrolysate polymerization Eq. (2) was investigated using the TNBS method as described in Section 2. All hydrolysates from fish bone and pig skin showed a mean degree of polymerization of about three, and the hydrolysates appeared to be a composite of oligopeptides. The amino acid compositions of extract and hydrolysate were also determined as shown in Table 2. Though the amount of glycine in the hydrolysate and extract was about 33% for pig skin, the glycine content of hydrolysate for fish bone was lower (ca. 27%). It might be caused by contamination of proteins other than collagen in bone. Furthermore, the amounts of hydroxyproline and proline were from 7.6 to 7.9% and 11.4 to 12.6%, respectively. These results are typical of collagen amino acid composition, thus it was evident that both the hydrolysate and the extract were products of collagen protein.

Many studies addressing useful applications of peptides have been conducted, including such applications as anti-radical (antioxidant) activity and blood pressure reduction activity. In this study as well, these two activities were investigated for extract and hydrolysate. In Table 3, the relatively high values of parameters for extract indicate that it has low anti-radical activity and 11 no ability to reduce blood pressure. Conversely, the

Table 2
Amino acid composition of extract and hydrolysate obtained in this research (Unit, mol%)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Extract</th>
<th>Hydrolysate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid extract obtained from pig skin</td>
<td>Pig skin</td>
<td>Fish bone</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>7.9</td>
<td>7.6</td>
<td>6.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>4.6</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.6</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Serine</td>
<td>3.0</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.8</td>
<td>8.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Proline</td>
<td>12.6</td>
<td>11.9</td>
<td>11.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>33.8</td>
<td>32.7</td>
<td>27.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.3</td>
<td>11.0</td>
<td>16.7</td>
</tr>
<tr>
<td>Cystine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>2.3</td>
<td>2.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.4</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.0</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.4</td>
<td>2.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.3</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.6</td>
<td>1.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.8</td>
<td>0.8</td>
<td>N.T.*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.7</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.9</td>
<td>4.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* N.T.: not measured.
Table 3
Evaluation of antioxidant activity (IPOX 50) and potential for reduction of blood pressure (IC 50) of extract and hydrolysate obtained in this research

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean molecular weight</th>
<th>IPOX 50 (mg/ml)a</th>
<th>IC 50 (mg/ml)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>Acid extract obtained from pig skin</td>
<td>&gt;100 000</td>
<td>1.73</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>Pig skin</td>
<td>350–350</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Fish bone</td>
<td>350–350</td>
<td>0.18</td>
</tr>
</tbody>
</table>

a For both parameters, a lower value indicates a higher activity.
b N.D., not detected.

Fig. 6. Flow diagram of total procedure for extraction of protein and production of peptides by enzymic hydrolysis from pig skin and fish bone.

IPOX 50 and IC 50 of hydrolysate were less than 1 mg ml⁻¹, indicating both significant anti-radical activity and potential for reducing blood pressure. These results are corroborative of those of Kim et al. [14] who reported a maximum IC 50 of 0.593 mg ml⁻¹ for hydrolysate obtained from cow skin using various enzymes. The hydrolysate obtained using enzyme L had a high potential for reducing blood pressure, which coupled with its ease of assimilation suggest that it could have useful applications in food materials.

3.5. Development of an effective process

From the results described above, a complete procedure for effective utilization of livestock and fishery wastes containing collagen could be developed as shown in Fig. 6. With this process, wastes could be changed to valuable materials. In the pretreatment step, fat and inorganic materials were removed. Fat removed in this process can be used for production of cooking oil and the inorganic component can be used for production of calcium apatite, which are avenues of further research being pursued in our lab. Following pretreatment, a high molecular weight protein could be extracted with a 1 h reaction time at 60 °C under acidic condition (pH 3). The extract obtained could have useful applications in cosmetics due to its high water retention capacity, ability to repair rough skin, lack of any odour problem and absence of harmful effects on skin. The residues of the protein extraction process were easily hydrolyzed with commercially available enzyme L. The hydrolysate was considered suitable for use as a food additive due to its high anti-radical activity and high potential for lowering high blood pressure.

We found no reports of an effective process for general utilization of livestock and fishery wastes. When pig skin was utilized in this process, 25 g of extract and 9 g of hydrolysate could be produced from 100 g of dried pig skin, and the recovery ratio of collagen protein was as high as 90% (Fig. 6).

4. Conclusions

A process for effective utilization of collagen contained in livestock and fish waste has been developed. The process consists of pretreatment, extraction of high...
molecular weight protein, and enzymic hydrolysis of the residue of the extraction process. The extract had useful properties for use as a cosmetic material due to its high water retention capacity, ability to repair rough skin, lack of any odour problem and absence of harmful effects on skin. The hydrolysate had suitable properties for use as a food additive due to its high anti-radical activity and high 12 potential for lowering high blood pressure.

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References