Fractionation and Characterization of the Macromolecular Meaty Flavor Enhancer from Beef Meat Extract

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ABSTRACT: Macromolecular meaty flavor enhancer was fractionated from a commercial beef extract. The macromolecular fraction was obtained by dialysis and separated by anion-exchange chromatography, Cu^{2+}-chelate chromatography, and gel filtration chromatography. Two fractions were isolated as active meaty flavor enhancers. To elucidate the partial structures, the active fractions were treated with endoprotease Glu-C followed by high-performance liquid chromatography (HPLC) separation of the peptide fragments. Determinations of the amino acid compositions and amino acid sequences of the isolated fragments showed that the 2 active fractions consisted of collagen and tropomyosin. The macromolecular material obtained from heated collagen and tropomyosin in the low-molecular-weight fraction of beef soup stock enhanced the meaty flavor. These results suggested that collagen and tropomyosin were precursors of the macromolecular meaty flavor enhancer.

Keyword: beef extract, meaty flavor, tropomyosin, collagen, heating process

Introduction

Beef extract is used in food manufacturing to generate a meaty aroma and meaty flavor designated as mouthfeel, continuity, and thickness.

In a previous study (Shima and others 1998), the comparison of the taste between beef bouillon and a mixed reference solution was investigated. The mixed reference solution of beef bouillon was prepared from 36 chemicals (amino acids, nucleotides, sugars, organic acids, and others) on the basis of analytical data. It was reported that the reference solution lacked the brothy flavor designated as "brothy sour taste" and thickness. Furthermore, the authors purified the taste-active compound, which enhanced "brothy sour taste," and identified the compound as N-(4-methyl-5-oxo-1-imidazolin-2-yl)-sarcosine. This result suggested the existence of an unknown flavor enhancer in beef bouillon.

Several studies have been carried out on the effects of macromolecular substances, such as gums and starches, on the taste of chicken broth (Rosett and others 1996). It was reported that the addition of these materials increased the intensity of thickness and decreased the intensity of saltiness of chicken broth. These results suggested that the addition of macromolecular materials could change the taste and flavor of foods.

Although many studies have been performed on the taste of di- or tri-peptides (Arai and others 1973; Hamilton and others 1974; Noguchi and others 1975), few have been conducted on the taste effects of longer peptides or proteinaceous materials. Recently, it was reported that a peptide fraction (MW 1000 to 10000) obtained from beef soup stock increased mildness and mouthfeel when it was added to beef soup (Ishii and others 1995). Furthermore, it was reported that several peptides from aged (2 °C for 1 wk) pork meat troponin-T decreased sourness when they were added to the soup prepared from non-aged pork meat (Nishimura 2001). However, there are few reports on the structure of the peptide or proteinaceous flavor enhancer.

The present study examined the fractionation and the characterization of macromolecular substances from beef extract, which increased the intensities of the continuity, mouthfullness, and thickness designated as meaty flavor.

Materials and Methods

Beef extract and beef soup stock

Commercial beef meat extract was purchased from Bordon Co. Ltd. (Sao Paulo, Brazil). The analytical data of the beef extract is as follows: moisture, 21.6% (w/w; dried at 105 °C for 4 h); total sugar, 6.6% (w/w); total nitrogen, 9.7% (w/w); NaCl, 2.8%; and creatinine, 7.5% (w/w). The beef extract was preserved for up to 1 y at 5 °C until used. Beef soup stock was prepared as previously reported (Kuroda and Harada 2000). Briefly, 6 kg of beef shank meat (obtained from market in Tokyo) was cut into pieces of 5 to 10 cm; 8 L distilled water was added and heated at 95 °C to 97 °C for 7 h. After removal of the beef meat, the soup was refrigerated for 16 h and the fat and the insolubles were removed by filtration. The soup stock obtained was freeze-dried and stored at –25 °C until use.

Preparation of macromolecular fraction and low-molecular-weight fraction

One kilogram of beef extract or 500 g of beef soup stock powder was diluted with 4 L distilled water and dialyzed using a dialyzing tube (Sanko Junyaku, Japan) against distilled water. Both fractions were concentrated with an evaporator, freeze-dried, and kept at –20 °C until use.

Chemical analyses

Samples were hydrolyzed with 6 *N* hydrochloric acid containing

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1% phenol at 120 °C for 24 h under a nitrogen gas phase using a Pico-Tag Work Station (Waters Corp., Milford, Mass., U.S.A.). Amino acid analyses were carried out with amino acid analyzer L-8500 (Hitachi Ltd., Tokyo, Japan). Peptide fragments were immobilized on a Sequelon-AA membrane (Millipore Ltd., Milford, Mass., U.S.A.). The N-terminal sequence of peptides was determined by automatic Edman degradation with Pro-Sequencer (Millipore Ltd.). Homology search of peptide sequences was carried out using the NBRF (Natl. Biomedical Research Foundation, Washington, D.C., U.S.A.) database.

The components in the beef extract samples were quantified as follows. The content of carbohydrates (total sugars) was analyzed by phenol-sulfuric acid method (Dubois and others 1956). The contents of crude fat were analyzed by the Soxhlet extraction method using diethyl-ether as the solvent. The contents of glycogen were analyzed by a slight modification of the method previously reported by Thivend and others (1965). Chemical analysis were performed 3 times.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were carried out with 5% to 20% polyacrylamide gradient gels according to Laemmli (1970). Isoelectric focusing electrophoresis was carried out using Phastsystem (Pharmacia Co., Inc., Uppsala, Sweden) according to Olsson and others (1988). The data analysis was performed using the Pharmacia Image Scanning system.

Enzymatic digestion of the macromolecular fraction

The macromolecular fraction was digested in the protease system or amylase system. For protease digestion, 500 mg of the macromolecular fraction (dry base) was dissolved in 0.1 *M* sodium phosphate buffer (pH 7.0), 10 mg of protease-Amano A (Amano Pharmaceutical Co., Tokyo, Japan) and 10 mg of protease-Amano M (Amano Pharmaceutical Co.) was added, and the enzymic reaction was allowed to proceed at 37 °C for 24 h. For amylase digestion, 500 mg of the macromolecular fraction was dissolved in 0.1 *M* sodium phosphate buffer (pH 7.0), 5 mg of α-amylase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 5 mg of glucoamylase (Sigma Chemical Co.) was added, and the enzymatic reaction was performed at 37 °C for 24 h. Enzymatic digests were heated at 100 °C for 10 min, dialyzed against distilled water, and freeze-dried.

Fractionation of macromolecular fraction by chromatographic method

Fractionation was performed according to the procedure shown in Figure 2.

Anion-exchange column chromatography. The macromolecular fraction from beef extract (100 g) was dialyzed against 0.02 *M* sodium phosphate buffer (pH 7.0) and applied into a diethylaminoethyl-(DEAE-) Toyopearl 650M (Tosoh Corp., Tokyo, Japan) column (252-mm inner dia × 150 mm) equilibrated with 0.02 *M* sodium phosphate buffer (pH 7.0). After washing, using the primary buffer, the adsorbed fraction was eluted by the linear gradient increase in the NaCl concentration (from 0 to 0.5 *M*, at a flow rate of 200 mL/min).

Cu^{2+}-chelate affinity chromatography. AF-chelate Toyopearl resin (Tosoh Corp.) was washed with 0.1 *M* $CuCl_2$ and equilibrated with 0.05 *M* sodium phosphate buffer (pH 7.8) containing 0.5 *M* NaCl. The active fraction obtained by DEAE-Toyopearl chomatography was dialyzed against 0.05 *M* sodium phosphate buffer (pH 7.8) containing 0.5 *M* NaCl and applied onto the Cu^{2+}-chelate column (118-mm inner dia × 150 mm). The adsorbed fraction was eluted with 0.05 *M* sodium acetate buffer (pH 5.0) containing 0.5 *M* NaCl and 0.05 *M* acetic acid containing 0.5 *M* NaCl. The elution was performed at a flow rate of 20 mL/min.

Gel filtration chromatography. Active fractions obtained by Cu^{2+}-chelate chomatography were dialyzed against distilled water and freeze-dried. These fractions were dissolved in elution buffer, 0.1 *M* sodium phosphate buffer (pH 7.0) containing 0.2 *M* NaCl, and applied into a Sephacryl S-300 (Pharmacia) column (50-mm inner dia × 900 mm). Chromatography was performed using the above elution buffer at a flow rate of 5.0 mL/min.

Isolation of peptide fragments from active fractions

The fractions obtained by chromatographies (50 mg) were hydrolyzed using 1.0 mg of endoprotease Glu-C (Boehringer Mannheim Ltd., Mannheim, Germany; sequencing grade, from *Staphylococcus aureus* V8; EC 3.4.21.19) in 0.1 *M* sodium acetate buffer (pH 5.0) at 3 °C for 24 h. The enzymatic digests were separated with 4 different high-performance liquid chromatography (HPLC) columns:

1. Asahipak GS-320P (Showa Denko Ltd., Tokyo, Japan; 20-mm inner dia × 700 mm); elution was performed with 10% acetonitrile containing 0.05% trifluoroacetic acid (TFA) at a flow rate of 5.0 mL/min.

2. Capcellpack C18 (Shiseido Co. Ltd., Tokyo, Japan; 20-mm inner dia × 250 mm); elution buffer A, 0.05% TFA; buffer B, 80% acetonitrile containing 0.05% TFA; gradient program, 2%→2%→50%B (0→5→45 min); flow rate, 5.0 mL/min.

3. Asahipak GS-320 (Showa Denko Ltd., 7.5-mm inner dia × 700 mm); elution was performed with 10% acetonitrile containing 0.05% TFA at a flow rate of 1.0 mL/min.

4. Vydac C18 (Grace Vydac Co. Ltd., Hesperia, Calif., U.S.A.; 4.6-mm inner dia × 150 mm); elution buffer A, B, and gradient program were same as (2); flow rate, 0.6 mL/min. Detection was carried out by monitoring at 214 nm UV. Each peptide fragment revealing a single peak on HPLC using a Vydac C18 column was applied to the determinations of amino acid composition and amino acid sequence.

Heating experiment of tropomyosin and/or gelatin

Bovine tropomyosin was purified from minced bovine neck meat by a method previously reported (Ojima and Nishita 1986). Tropomyosin and/or commercial bovine skin gelatin (Type B, approximately 225 bloom; Sigma Chemical Co.) were dissolved in distilled water or the solution of low-molecular-weight (LMW) fraction of beef soup stock (DM 25%) and heated at 95 °C for 6 h. After heating, the heated macromolecular material was obtained by dialysis against distilled water, freeze-dried, and subjected to sensory evaluation.

Sensory evaluation

The sensory evaluation was done by the method of Yamaguchi and Kimizuka (1979). The solution of the LMW-fraction (1.5% in distilled water, dry matter base) obtained from beef extract was used as a control solution for the sensory evaluation. Samples were dissolved in LMW solution and brought to 60 °C in a water bath. Approximately 50 mL of sample solution and 50 mL of control solution were served in plastic cups in the same time. Evaluation was done in a separated sensory booth. Panel was trained by evaluating various beef extract samples and made consensus for the intensities of continuity, mouthfeel, and thickness. For evaluation, the panel was asked to evaluate the samples using a 7-point rating scale (–3 to + 3; weaker than the control to stronger than the control) for 8 attributes (sweetness, sourness, saltiness, bitterness, umami, continuity, mouthfullness, and thickness). For evaluation of the macromolecular fraction, a 7-member panel (5 males and 2 females) was recruited.

Statistical analysis

Statistical analyses were performed using Excel 97 (Microsoft

Corp., Redmond, Wash., U.S.A.), and the *t* test was used to determine significance of differences between samples and control. Trends were considered significant when means of compared sets differed at $P < 0.05$.

Results and Discussion

Chemical composition of the macromolecular fraction

Table 1 shows the chemical composition of the macromolecular fraction from the beef extract. The results revealed that the macromolecular fraction contained fat, protein, and glycogen and also suggested that the major component was protein, especially soluble collagen (gelatin).

Flavor character of the macromolecular fraction

To ascertain the flavor characters of the macromolecular fraction of beef extract, an additional test on a solution of the LMW-fraction obtained from beef extract was carried out. The prepared macromolecular fraction, a brown powder, had little aroma or basic taste in distilled water at a concentration of 0.2%. Figure 1 shows the results of an additional test. The addition of the macromolecular fraction did not influence the intensities of basic tastes such as saltiness, sweetness, sourness, bitterness, and umami. However, the results indicated that the addition of the macromolecular fraction significantly increased the intensities of continuity, mouthfullness, and thickness. From these results, it was suggested that the macromolecular fraction from the beef extract might function as a flavor enhancer as a result of synergistic effects with compounds in the LMW fraction.

Evaluation of the macromolecular fraction treated with enzymes

The results of the sensory evaluation of the enzyme-treated macromolecular fraction are shown in Table 2. It was revealed that amylase treatment did not affect the intensity of the meaty flavor such as continuity, mouthfullness, and thickness. On the other hand, protease treatment markedly decreased the intensities of meaty flavor of the macromolecular fraction. These results suggest that the macromolecular meaty flavor mainly consisted of proteinaceous materials.

Table 1—Major components in the macromolecular fraction obtained from beef extract[a]

Component	Contents (%, w/w)
Moisture	3.6 ± 0.6
Protein (total amino acids)[b]	60.5 ± 2.3
Gelatin[c]	45.8 ± 1.8
Carbohydrate	4.2 ± 0.7
Glycogen	1.1 ± 0.2
Crude fat	0.5 ± 0.04

[a]Means ± standard deviation of triplicate determination.
[b]Protein contents calculated from the total contents of amino acids after 6 *N* HCl hydrolysis.
[c]Content of gelatin was calculated from the hydroxyproline content (Gelatin = Hyp × 7.52).

Table 2—Effects of enzymatic treatment on the flavor of the macromolecular fractions[a]

	Scores of flavor intensities		
Samples	Continuity	Mouthfullness	Thickness
Control (untreated)	2.5 ± 0.5	2.2 ± 0.4	2.2 ± 0.4
Protease-treated	0.8 ± 0.4*	0.9 ± 0.4*	0.6 ± 0.5*
Amylase-treated	2.3 ± 0.5	2.1 ± 0.4	2.3 ± 0.5
De-fatted	2.5 ± 0.5	2.1 ± 0.4	2.3 ± 0.5

[a]–3, Apparently weaker than the control; 3, apparently stronger than the control. Means ± standard deviation ($n = 7$). * = significantly different from control (untreated) at $P < 0.05$.

Chromatographic separation of the meaty flavor enhancer

The fractionation of the meaty flavor enhancer was carried out by anion-exchange chromatography with DEAE-Toyopearl, Cu^{2+}-che-

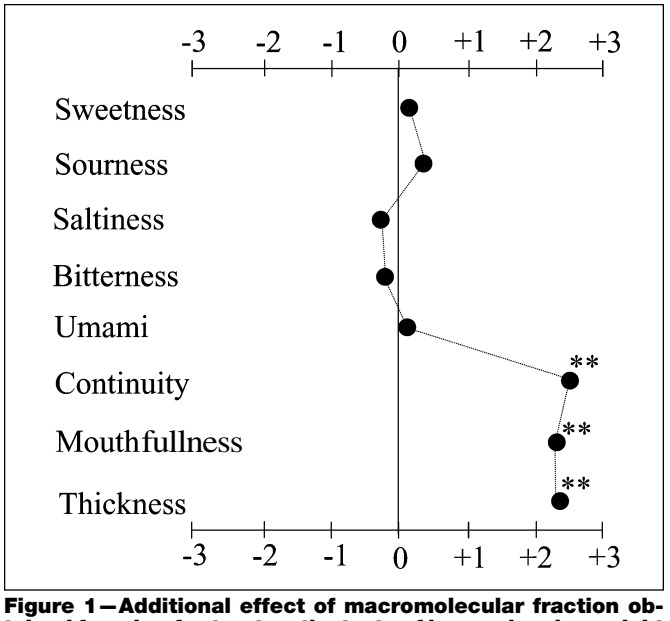

Figure 1—Additional effect of macromolecular fraction obtained from beef extract on the taste of low-molecular-weight (LMW) solution ($n = 7$). *, = significantly stronger than control, *$P < 0.05$, **$P < 0.01$ versus control (Student *t* test)**

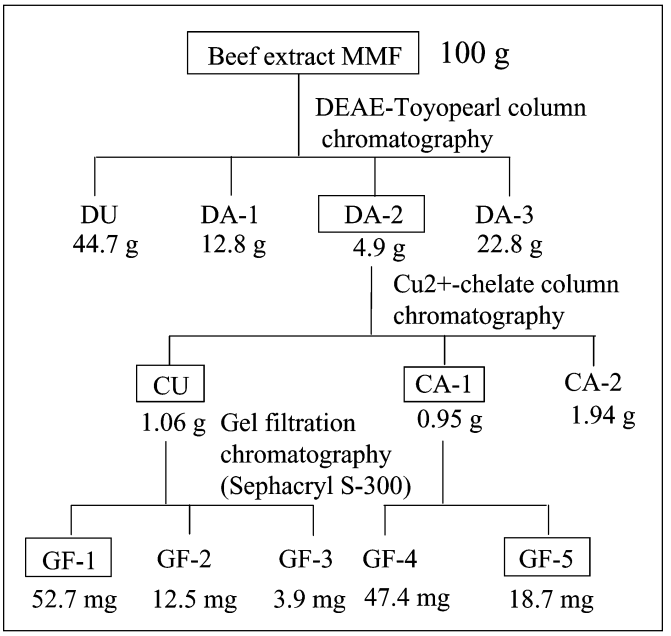

Figure 2—Fractionation procedure of macromolecular flavor enhancer from beef extract

late affinity chromatography, and gel filtration through Sephacryl S-300.The procedure of fractionation is summarized in Figure 2. As a result of DEAE-Toyopearl chromatography (Figure 3), the macromolecular fraction was separated into 4 fractions, unadsorbed (DU) fraction and 3 adsorbed fractions (DA-1, DA-2, and DA-3). The additional tests of each fraction on the LMW solution showed that the fraction designated as DA-2 influenced the intensities of continuity, mouthfeel, and thickness at a concentration of 0.02% (Table 3). Three other fractions showed few effects on the intensities of continuity, mouthullness, and thickness. From these results, the active fraction was shown to be an acidic substance. Fraction DA-2 was further fractionated by Cu^{2+}-chelate affinity chromatography.

Cu^{2+}-chelate affinity chromatography resulted in a separation of the DA-2 fraction into 3 fractions, an unadsorbed fraction (CU), a fraction eluted with pH 5 buffer (CA-1), and a fraction eluted with 0.05 *M* acetic acid (CA-2). As a result of the sensory evaluation shown in Table 4, each fraction had a weak effect; however, when fraction CU and fraction CA-1 were added to the same solution, a strong meaty flavor was generated. Fraction CA-2 did not have a major influence on the meaty flavor. This result suggests that the macromolecular meaty flavor enhancer consists of several types of components. Further fractionation of CU and CA-1 was carried out with gel filtration chromatography.

The chromatograms obtained by gel filtration chromatography are shown in Figure 4. As a result of gel filtration chromatography with Sephacryl S-300, the CU fraction was separated into 3 fractions (GF-1, GF-2, and GF-3), and the CA-2 fraction was separated into 2 fractions (GF-4 and GF-5). The sensory evaluation of fractions GF-1, GF-2, and GF-3 was carried out in the presence of fraction CA-1 (0.01%), and the evaluation of GF-4 and GF-5 was performed in the presence of fraction CU (0.01%). As shown in Table 5, in the presence of CA-1, the GF-1 fraction (added concentration, 0.01%) influenced the intensity of the meaty flavor, whereas in the presence of CU, GF-5 (added concentration, 0.01%) influenced the intensity of the meaty flavor. Furthermore, addition of fraction GF-1 and GF-5 in the same solution influenced the intensity of the meaty flavor. The remaining 3 fractions had weak effects on the meaty flavor. To elucidate the natures of these active fractions, the chemical natures of fractions GF-1 and GF-5 were investigated.

Characterization of the active fractions

The chemical natures of fractions GF-1 and GF-5 are summarized in Table 6. From the results of amino acid analyses, the contents of hydroxyproline in fractions GF-1 and GF-5 were 5.7% and 2.4%, respectively. These results suggest that both fractions consisted of collagen. The calculated collagen contents of GF-1 and GF-5 were 43.0% and 18.3%, respectively. Furthermore, the amino acid compositions showed that the contents of histidine and β-alanine in GF-5 were higher than those in GF-1. Recently, it was reported that carnosine in LMW was incorporated into the high-molecular-weight fraction and formed γ-glutamyl-β-alanyl-histidine isopeptide during heating of beef soup stock (Kuroda and Harada 2000). From these results, it was considered that GF-5 contained γ-glutamyl-β-alanyl-histidine isopeptide. Results obtained by SDS-PAGE analyses and reversed-phase HPLC analyses (Figure 5a) of active fractions showed that no clear bands and no clear peak were observed with these analyses. These results showed that these active fractions consisted of mixtures of numerous compounds.

Purification and analyses of peptide fragments from active fractions

Amino acid compositions of active fractions showed that both fractions consisted of collagen. To ascertain the precursor proteins

Table 3—Additional effects of fractions obtained by diethylaminoethyl-Toyopearl chromatography[a]

	Scores of flavor intensities		
Samples	Continuity	Mouthfullness	Thickness
DU 0.10%	0.4 ± 0.5	0.3 ± 0.5	0.4 ± 0.5
DA-1 0.10%	0.3 ± 0.5	0.3 ± 0.5	0.4 ± 0.5
DA-2 0.02%	2.3 ± 0.5*	2.1 ± 0.6 *	2.3 ± 0.5*
DA-3 0.05%	0.4 ± 0.5	0.4 ± 0.5	0.3 ± 0.5

[a]–3, Apparently weaker than the control; 3, apparently stronger than the control. Means ± standard deviation (n = 7); *significantly different from control at $P < 0.05$.

Table 4—Additional effects of fractions obtained by Cu^{2+}-chelate affinity chromatography[a]

	Scores of flavor intensities		
Samples	Continuity	Mouthfullness	Thickness
DA-2 0.02%	2.3 ± 0.5*	2.1 ± 0.6*	2.3 ± 0.5*
CU 0.02%	0.3 ± 0.5	0.4 ± 0.5	0.4 ± 0.5
CA-1 0.02%	0.4 ± 0.5	0.2 ± 0.4	0.3 ± 0.5
CA-2 0.02%	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4
CU 0.01% + CA-1 0.01%	1.9 ± 0.4*	2.2 ± 0.4*	1.6 ± 0.5*
CU 0.01% + CA-2 0.01%	0.4 ± 0.5	0.4 ± 0.5	0.3 ± 0.5
CA-1 0.01%+CA-2 0.01%	0.5 ± 0.6	0.4 ± 0.5	1.2 ± 0.4*

[a]–3, Apparently weaker than the control; 3, apparently stronger than the control. Means ± standard deviation (n = 7). *significantly different from control at $P < 0.05$.

Table 5—Additional effects of fractions obtained by gel filtration chromatography[a]

	Scores of flavor intensities		
Samples	Continuity	Mouthfullness	Thickness
CA-1 0.01%+GF-1 0.01%	1.7 ± 0.5*	1.7 ± 0.5*	1.5 ± 0.5
CA-1 0.01%+GF-2 0.01%	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.4
CA-1 0.01%+GF-3 0.01%	0.3 ± 0.5	0.2 ± 0.4	0.2 ± 0.4
CU 0.01% + GF-4 0.01%	0.3 ± 0.5	0.3 ± 0.5	0.5 ± 0.7
CU 0.01% + GF-5 0.01%	1.5 ± 0.8*	1.7 ± 0.5*	1.7 ± 0.5*
GF-1 0.01%+GF-5 0.01%	1.3 ± 0.5*	1.5 ± 0.5*	1.3 ± 0.5*

[a]–3, Apparently weaker than the control; 3, apparently stronger than the control. Means ± standard deviation (n = 7). *significantly different from control at $P < 0.05$.

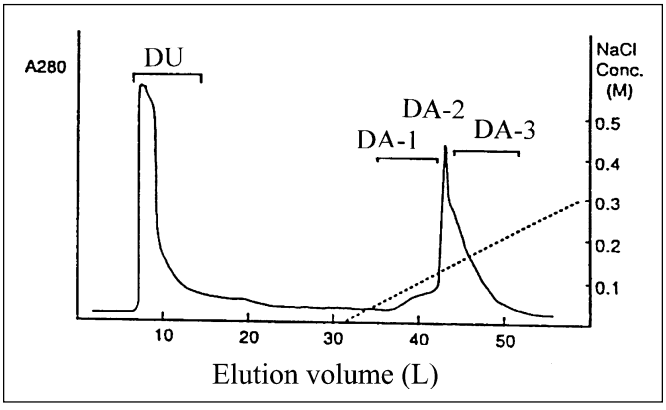

Figure 3—Fractionation of macromolecular fraction from beef extract using diethylaminoethyl-(DEAE-) Toyopearl 650M column; column, 252-mm inner dia × 150 mm; flow rate, 200 mL/min; elution buffer, 0.02 *M* sodium phosphate buffer (pH 7.0); gradient, from 0 to 0.5 *M* NaCl in 0.02 *M* sodium phosphate buffer (pH 7.0).

other than collagen, the fractions were digested by endoprotease Glu-C from *S. aureus* V8 and purified using HPLC. Although both fractions showed no clear peak on reversed-phase HPLC, chromatograms obtained with the digests of the fractions showed several peaks (Figure 5b). This result showed that there were common sequences in the active fractions. Peptide fragments were purified using 4 different HPLC columns. As a result of purification of peptide fragments, 20 peptides were purified from the enzymatic digest of GF-1, while 27 peptides were obtained from the digest of GF-5. Five of 20 fragments from GF-1 and 6 of 27 fragments from GF-5 were derived from collagen because they contained hydroxyproline. From the results shown in Table 7, 5 fragments from GF-1 and GF-4 fragments from GF-5 were homologous to the sequences in mammalian muscular tropomyosin. Other remaining fragments could not be assigned because of the length of fragments (di- or tripeptides). From these results, both of the active fractions GF-1 and

Table 6—Chemical natures of GF-1 and GF-5[a]

	GF-1	GF-5
Amino acid composition (%)		
Hydroxyproline	5.72 ± 0.21	2.43 ± 0.11
Aspartic acid	6.37 ± 0.17	6.07 ± 0.21
Threonine	2.54 ± 0.08	2.08 ± 0.07
Serine	3.50 ± 0.17	2.62 ± 0.08
Glutamic acid	15.34 ± 0.76	10.80 ± 0.68
Proline	8.79 ± 0.36	4.34 ± 0.20
Glycine	12.14 ± 0.65	6.35 ± 0.36
Alanine	7.64 ± 0.41	4.98 ± 0.29
Cystine	0.68 ± 0.03	1.40 ± 0.08
Valine	2.47 ± 0.12	2.06 ± 0.11
Methionine	1.30 ± 0.09	1.01 ± 0.06
Isoleucine	1.54 ± 0.09	1.56 ± 0.08
Leucine	4.04 ± 0.12	3.38 ± 0.08
Tyrosine	0.86 ± 0.05	1.37 ± 0.12
Phenylalanine	1.48 ± 0.11	1.44 ± 0.12
Lysine	5.49 ± 0.27	3.78 ± 0.18
Histidine	0.26 ± 0.02	4.96 ± 0.29
Arginine	4.89 ± 0.09	2.93 ± 0.07
Total of amino acids	85.0 ± 3.54	63.2 ± 2.56
Molecular weight		
Gel filtration HPLC	about 27 kD (broad)	6 to 7 kD (broad)
SDS-PAGE	10 to 30 kD (broad)	5 to 15 kD (broad)
Isoelectric points	4.6 to 4.8	4.4 to 4.7

[a]Means ± standard deviation of triplicate determination. HPLC = high-performance liquid chromatography; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

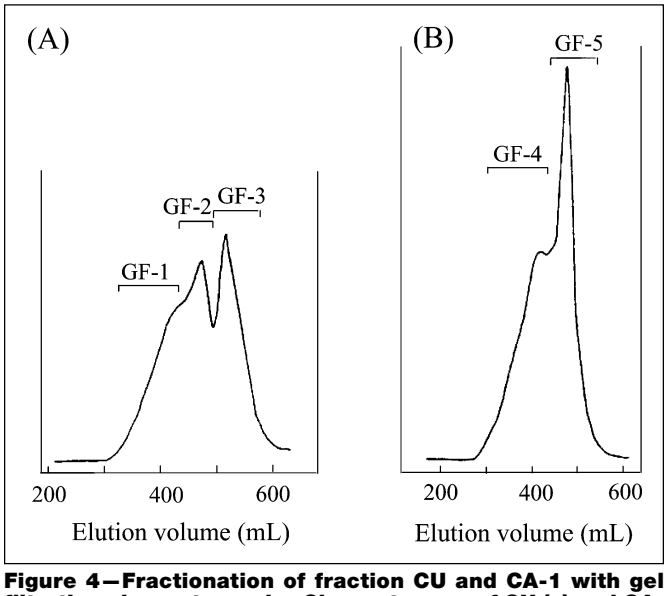

Figure 4—Fractionation of fraction CU and CA-1 with gel filtration chromatography. Chromatogram of CU (a) and CA-1 (b); column, Sephacryl S-300, 50-mm inner dia × 900 mm; flow rate, 5 mL/min; elution buffer, 0.2 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 7.0).

Table 7—Amino acid sequences of peptide fragments obtained from proteolytic digests of GF-1 and GF-5

Peptide fragment	Amino acid sequence	Assignment
From GF-1 digest:		
Nr 1	Ala-Glu-Thr-Arg-Ala-Glu	TM[a](α,β) 235-240
Nr 2	Glu-Ala-Glu-Lys-Ala-Ala-Asp-Glu	TM(α,β) 115-122
Nr 3	Lys-Lys-Ala-Thr-Val-Ala-Glu	TM(α,β) 76-82
Nr 4	Lys-Met-Glu-Ile-Gln-Glu	TM(β) 140-145
Nr 5	Leu-Asp-Lys-Tyr-Ser-Glu	TM(α) 57-62
From GF-5 digest:		
Nr 6	Val-Glu-Lys-Tyr-Ser-Glu	TM(β) 57-62
Nr 7	Asp-Lys-Tyr-Glu-Glu	TM(α) 218-223
Nr 8	Val-Tyr-Ala-Gln-Lys	TM(β) 260-264
Nr 9	Leu-Tyr-Ala-Gln-Lys	TM(α) 260-264

[a]TM = tropomyosin.

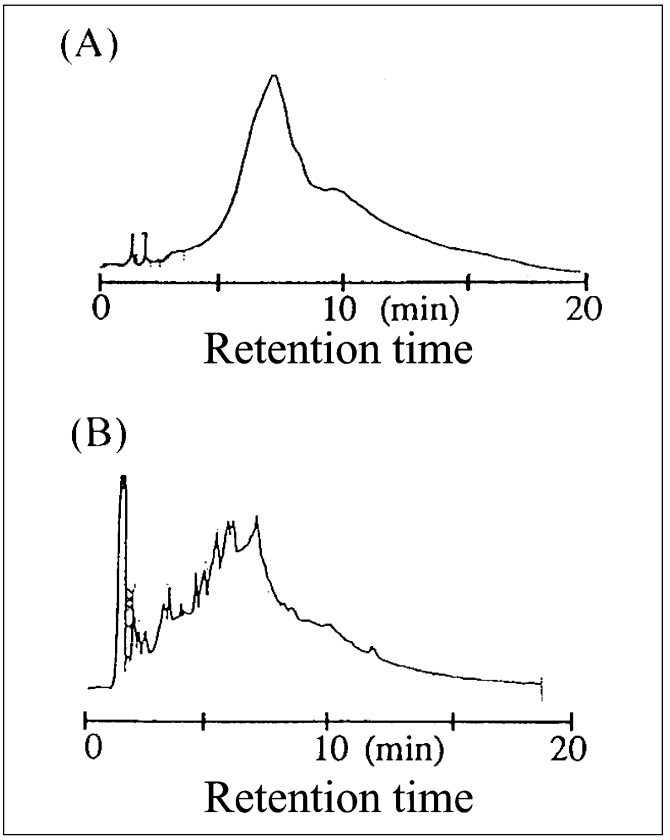

Figure 5—Reversed-phase high-performance liquid chromatography (HPLC) chromatogram of GF-1 fraction and enzymatic digest of GF-1. Chromatogram of GF-1 (a) and proteolytic digest of GF-1 with endoprotease Glu-C (b). Column, Capcellpack C-18, 4.6-mm inner dia × 250 mm; flow rate, 1.0 mL/min; detection, UV 214 nm; eluent A, 0.05% TFA; B, 80% acetonitrile in 0.05% TFA; Gradient program, 2%→2%→50%B (0→5→45 min).

GF-5 consisted partially of collagen and tropomyosin. Tropomyosin is a component of a thin filament of skeletal muscle (Kendrick-Jones and others 1970), and it is known to be contained in meat foods (Asghar and others 1985), raw fish foods (Sikorski and others 1976), and surimi (Sano and others 1989; Martinez and others 1992). However, there have been no studies reporting the existence of tropomyosin in meat soup or meat extract. In the present study, native tropomyosin was not detected in fractions obtained from beef extract. It is suggested that tropomyosin was denatured and modified by heating during the production of beef extract. Results of SDS-PAGE analyses (Table 6; Figure 6) showed that the molecular weight of GF-1 was 10 to 30 kD, and that of GF-5 was 5 to 15 kD. These results showed that the fragmentation of tropomyosin (MW of subunit = 33 kD) and collagen (MW of subunit = approximately 100 kD) occurred during the production of the beef extract. It is known that the isoelectric point of collagen is 7.0 to 8.0. From the fact that the active fractions were acidic (pI of GF-1 = 4.6 to 4.8; pI of GF-5 = 4.4 to 4.7), it is suggested that collagen fragments in the active fractions were the result of the deamidation or conjugation with other acidic proteins.

Sensory evaluation of nonheated and heated gelatin and tropomyosin

To confirm the contribution of gelatin and tropomyosin to the meaty flavor, the effect of these proteins (native form and heated form) on the generation of the meaty flavor was investigated. Tropomyosin, gelatin, or heated protein was dissolved in 2% (w/w) LMW solution at a concentration of 0.05% (dry base). As shown in Table 8, neither unheated gelatin nor tropomyosin had an effect on the meaty flavor. Furthermore, heated gelatin and heated tropomyosin in water had no effect on the meaty flavor. However, the macromolecular material obtained from gelatin and tropomyosin heated in LMW fraction of beef soup stock enhanced the intensity of meaty flavor when it was added to the LMW solution of beef soup stock. From these results, it was suggested that gelatin and tropomyosin were the precursors of the macromolecular meaty flavor enhancer. Furthermore, it was also considered that the macromolecular meaty flavor enhancer was formed during the heating process. It was reported that the meaty flavor (continuity, mouthfullness, and thickness) of beef extract or chicken extract increased during the heating process (Yonemitsu and others 1997). Therefore, the formation of the macromolecular meaty flavor enhancer from gelatin and tropomyosin occurs during the heating process. Recently, it was reported that carnosine in LMW was incorporated into the high-molecular-weight fraction and formed γ-glutamyl-β-alanyl-histidine isopeptide during heating of beef soup stock (Kuroda and Harada 2000). Furthermore, a positive correlation between the contents of the isopeptide and the meaty flavor of commercial beef extract (Kuroda 2003). From these results, it seems that the formation of the above isopeptide partially causes the increase of the continuity, thickness, and mouthfeel (designated as meaty flavor) during heating of the precursor proteins. Studies of the detailed conditions for the formation of the meaty flavor enhancer from soluble collagen (gelatin) and tropomyosin are currently in progress in our laboratory.

Table 8—Additional effects of gelatin, tropomyosin, and the macromolecular materials obtained from heated gelatin and tropomyosin[a]

	Scores of flavor intensities		
Samples	Continuity	Mouthfullness	Thickness
Unheated gelatin	0.2 ± 0.4	0.3 ± 0.5	0.2 ± 0.4
Un-heated tropomyosin	0.3 ± 0.5	0.2 ± 0.4	0.2 ± 0.4
Gelatin/tropomyosin heated in DW	0.3 ± 0.5	0.2 ± 0.4	0.2 ± 0.4
Gelatin heated in LMW	0.3 ± 0.5	0.3 ± 0.5	0.4 ± 0.5
Tropomyosin heated in LMW	0.2 ± 0.4	0.2 ± 0.4	0.3 ± 0.4
Gelatin/tropomyosin heated in LMW	1.7 ± 0.5*	1.4 ± 0.5*	1.5 ± 0.5*

[a]–3, Apparently weaker than the control; 3, apparently stronger than the control. Means ± standard deviation (n = 7). *significantly different from control at $P < 0.05$. DW = distilled water; LMW = low molecular weight.

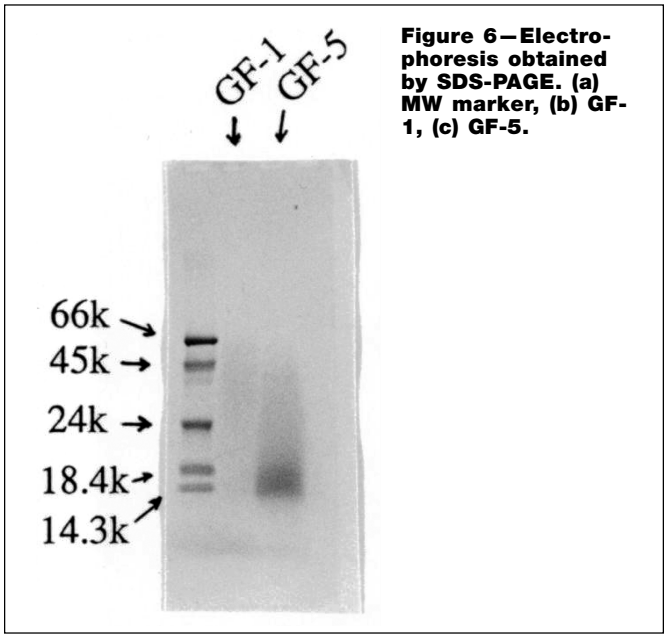

Figure 6—Electrophoresis obtained by SDS-PAGE. (a) MW marker, (b) GF-1, (c) GF-5.

Conclusions

Macromolecular meaty flavor enhancer was separated by chromatography and 2 fractions were obtained. The amino acid sequence analysis of fragments obtained by enzymatic treatment showed that both fractions consist of collagen and tropomyosin. From the fact that the macromolecular material obtained from heated soluble collagen and tropomyosin enhanced the meaty flavor, it is suggested that collagen and tropomyosin were precursors of the macromolecular meaty flavor enhancer.

References

Arai S, Yamashita M, Noguchi M, Fujimaki M. 1973. Taste of L-glutamyl oligopeptides in relation to their chromatographic properties. Agric Biol Chem 37:151–6.
Asghar A, Samejima K, Yasui T. 1985. Functionality of muscle proteins in gelation mechanisms of structured meat products. Crit Rev Food Sci Nutr 22:27–106.
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–6.
Hamilton JS, Hill RD, Leeuwen HV. 1974. A bitter peptide from Cheddar cheese. Agric Biol Chem 38:375–9.
Ishii K, Nishimura T, Okitani A, Tamura Y, Hatae K, Shimada A. 1995. Role of peptides in the taste of cooked beef. J Home Econ Jpn 46:307–12.
Kendrick-Jones J, Lehman W, Szent-Gyorgyi AG. 1970. Regulation in molluscan muscles. J Mol Biol 54:313–26.
Kuroda M. 2003. Studies on the novel isopeptide, γ-glutamyl-β-alanyl-histidine, in meat extract. [DPhil dissertation]. Tsukuba, Ibaraki, Japan: Tsukuba Univ. 107 p.
Kuroda M, Harada T. 2000. Incorporation of histidine and β-alanine into the macromolecular fraction of beef soup stock during heating. J Food Sci 65:596–603.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–5.
Martinez I, Solberg C, Lauritzen K, Ofstad R. 1992. Two-dimensional electrophoretic analyses of cod (Gadus morhua, L.) whole muscle proteins, water-

soluble fraction and surimi. Effect of the addition of $CaCl_2$ and $MgCl_2$ during the washing procedure. Appl Theor Electrophor 2:201–6.
Nishimura T. 2001. Role of peptides in food taste. Kagaku Seibutsu 39:177–83.
Noguchi M, Arai S, Yamashita M, Kato H, Fujimaki M. 1975. Isolation and identification of acidic oligopeptides occurring in a flavor-potentiating fraction from a fish protein hydrolysate. J Agric Food Chem 23:49–53.
Ojima T, Nishita K. 1986. Troponin from Akazara scallop striated adductor muscles. J Biol Chem 261:16749–54.
Olsson I, Axio-Fredriksson UB, Degerman M, Olsson B. 1988. Fast horizontal electrophoresis. I. Isoelectric focusing and polyacrylamide gel electrophoresis using PhastSystem. Electrophoresis 9:16–22
Rosett TR, Kendregan SL, Gao Y, Schmidt SJ, Klein BP. 1996. Thickening agents effects on sodium binding and other taste qualities of soup systems. J Food Sci 61:1099–104.
Sano T, Noguchi SF, Tsuchiya T, Matsumoto JJ. 1989. Contribution of tropomyosin to fish muscle gel characteristics. J Food Sci 54:258–64.
Shima K, Yamada N, Suzuki E, Harada T. 1998. Novel brothy taste modifier isolated from beef broth. J Agric Food Chem 46:1465–8.
Sikorski Z, Olley J, Kostuch S. 1976. Protein change in frozen fish. CRC Crit Rev Food Sci Nutr 8:97–129.
Thivend VP, Mercier C, Massy AG. 1965. Application of glucoamylase to starch quantification. Starke 17:278–83.
Yamaguchi S, Kimizuka A. 1979. Psychometric studies on the taste of monosodium glutamate. In: Filer LJ, Garattini S, Kare MR, Reynolds WA, Wurtman RJ, editors. Glutamic acid: Advances in biochemistry and physiology. New York: Raven Press. p 35–54.
Yonemitsu M, Okamura T, Nishikawa A, Ohmura H. 1997. Manufacture of concentrated extract. Jpn Kokai Tokkyo Koho Appl JP 97-135673.