# Investigation on the Formation and the Determination of γ-Glutamyl-β-alanylhistidine and Related Isopeptide in the Macromolecular Fraction of Beef Soup Stock

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To confirm the formation of γ-glutamyl-β-alanylhistidine and related peptide, a model solution (amide-containing amino acids and carnosine) has been heated, and the products are investigated. Spectroscopical analysis indicates that the major product from asparagine and carnosine is β-aspartyl-β-alanylhistidine, and that from glutamine and carnosine is γ-glutamyl-β-alanylhistidine. Furthermore, to confirm the increase of the above peptides during the heating process of food, an HPLC method for the determination of these isopeptides in food protein is constructed. The isopeptides are liberated by proteolytic digestion and fractionated by solid-phase extraction using Toyopack IC-SP cartridges. The fraction containing the isopeptides is derivatized with phenylisothiocyanate (PITC) and separated and quantified by HPLC using an octadecyl−silica column. As a result of quantification, an increase of the γ-glutamyl-β-alanylhistidine isopeptide in the macromolecular fraction of heated beef soup stock solution has been observed. These results suggest that the formation of the isopeptide occurs in the heating of various foods containing carnosine.

**Keywords:** *γ-glutamyl-β-alanylhistidine, isopeptide, high-performance liquid chromatography (HPLC), phenylisothiocyanate (PITC), phenylthiohydantoin (PTH), solid-phase extraction (SPE), beef, soup, heating process*

## INTRODUCTION

There have been many studies on the changes in amino acid residues during the heating process of food protein. The contents of lysine, arginine, and methionine residues were reported to be decreased when several kinds of protein were heated with reducing sugars (Hurrel and Carpenter, 1981). It has also been reported that polymerization of protein occurs during the process of heating in the presence or absence of reducing sugars (Weder and Scharf, 1981a,b; Okitani et al., 1984). Formation of ε-(γ-glutamyl) lysine cross-linkages has been observed during heating without reducing sugars (Otterburn, 1983). Furthermore, it has been reported that the lysinoalanine and related isopetides form when food protein is heated under alkaline condition (Friedman et al., 1984). These previous observations suggest that various changes in the amino acid residues occur in food protein during the heating process.

Previously, it was reported that levels of histidine and β-alanine in the macromolecular fraction increased during heating of beef soup stock solution (Kuroda and Harada, 2000). This suggested that the incorporation of carnosine into the macromolecular fraction occurs during heating of beef soup stock solution. Furthermore, results of isolation and characterization of incorporated carnosine suggested the existence of a tripeptide that was considered to be γ-glutamyl-β-alanylhistidine in the proteolytic digest of the macromolecular fraction from heated beef soup stock. These results suggested that a portion of carnosine was incorporated covalently into the macromolecular fraction and formed γ-glutamyl-β-alanylhistidine during heating.

In the present study, to confirm the formation of γ-glutamyl-β-alanylhistidine and related peptide, a model solution is heated and the products are investigated. Furthermore, to confirm the increase of the above peptides during the heating process of food, an HPLC method for the determination of these isopeptides in food protein is constructed and applied to the quantification of γ-glutamyl-β-alanylhistidine isopeptide in the macromolecular fraction of heated beef soup stock solution.

## MATERIALS AND METHODS

**Preparation of Beef Soup Stock Samples and the Macromolecular Fraction.** Preparation and heating of the beef soup stock solution were performed according to the method reported previously (Kuroda and Harada, 2000). The macromolecular fraction was prepared from heated beef soup stock solution as reported previously (Kuroda and Harada, 2000).

**Chemicals.** Carnosine was purchased from Sigma Chem. Co. (St. Louis, MO). L-Asparagine, l-glutamine, L-aspartic acid, and L-glutamic acid were from Ajinomoto Co., Inc. (Tokyo, Japan). Amino acid standard mixture (type H, A/N, and B), trifluoroacetic acid (TFA: protein sequencing grade), acetonitrile (HPLC grade), phenylisothiocyanate and triethylamine were purchased from Wako Pure Chem., Co., Ltd., (Osaka, Japan). All other reagents were of analytical grade.

**Enzymes.** *Streptomyces griseus* protease (Pronase E, EC3.4.24.31, type XXV) and bovine pancreas carboxypeptidase A (EC 3.4.17.1) were purchased from Boehringer Mannheim GmbH (Rhein, Germany). Porcine kidney leucine aminopep-

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tidase (EC 2.3.2.2, type III) and prolidase (EC 3.4.21.26) were from Sigma. Wheat carboxypeptidase W (EC 3.4.16.1) was from Seikagaku Kogyo (Tokyo, Japan).

**Heating of Amide-Containing Amino Acids and Carnosine.** L-Asparagine (1.0 M) or L-glutamine (1.0 M), and carnosine (1.0 M) were dissolved in distilled water, and pH was adjusted to 6.0 with 1.0 M NaOH. The reaction mixture was then heated at 95 °C for 24 h. After this incubation, the mixture was stored at −25 °C until chromatographic purification.

**Chromatography for Purification of the Products in the Model Systems.** The products from the reaction mixture were purified by HPLC by monitoring at UV 214 nm with an L-6000 UV detector (Hitachi Co., Ltd., Tokyo, Japan) under the following conditions:

*(1) Reversed-Phase HPLC with TSK-Gel Carbon C-500 Column (Tosoh Co., Ltd., Tokyo, Japan).* The reaction mixture was filtered through a 0.45-$\mu$m filter (Chromatodisk 25A, GL Science Co., Ltd., Tokyo, Japan). The filtrate was applied to a TSK-gel Carbon C-500 column (150 mm × 20 mm i.d., particle size 5 $\mu$m). The products were separated on a binary linear solvent gradient. Solution A was 0.05% TFA in distilled water, and solution B consisted of 80% (v/v) acetonitrile containing 0.05% TFA. The gradient profiles were as follows: 0−5 min, 2% B; 5−25 min, 2−20% B; 25−35 min, 20−50% B. The flow rate was 5 mL/min, and the column temperature was maintained at 40 °C. This purification process for each peak obtained was performed twice before amino acid analysis.

*(2) Reversed-Phase HPLC with a Vidac C18 Column (Vydac Co., Ltd.,).* The sample containing the major product was further purified by HPLC with a Vydac C18 column (150 mm × 4.6 mm i.d., particle size 5 $\mu$m). Elution was performed using 0.05% TFA, and the flow rate was 1.0 mL/min.

**HPLC Analysis of $\gamma$-Glutamyl-$\beta$-alanylhistidine.** *(1) Proteolytic Digestion.* Proteolytic digestion of the macromolecular sample was carried out as reported previously (Kuroda and Harada, 2000). Briefly, the macromolecular sample (5 mg/0.5 mL in 0.1 M borate buffer, pH 8.0) was digested by Pronase E (1.0 units/mg of protein for 24 h, 37 °C); after inactivation heating at 100 °C for 10 min, digestion was continued for 24 h by adding leucine aminopeptidase (1.0 unit/mg of protein), prolidase (0.5 units/mg of protein), and carboxypeptidase A (3.0 units/mg of protein) followed by the digestion with carboxypeptidase W (4.0 units/mg of protein for 24 h, 37 °C, pH 4.0).

*(2) Solid-Phase Extraction (SPE) of $\gamma$-Glutamyl-$\beta$-alanylhistidine.* The proteolytic digests obtained as described above were fractionated by solid-phase extraction using Toyopack IC-SP cartridges (500 mg of resin; Toso Co. Ltd., Tokyo, Japan). The proteolytic digest (from 5 mg of protein) was diluted in 0.1 M HCl and applied to a Toyopack IC-SP cartridge equilibrated with 0.1 M HCl. After washing with 0.1 M HCl (6 mL × 3), the cartridge was air-dried, and the fraction containing $\gamma$-glutamyl-$\beta$-alanylhistidine was eluted with 5 mL of 80% methanol containing 1% ammonia. The fraction thus obtained was dried by a vacuum concentrator.

*(3) Derivatization with PITC.* Derivatization with PITC was performed according to a slight modification of the method of Bidlingmeyer et al. (1984). The fraction obtained by SPE was dissolved in distilled water and pipetted into a glass tube (50 mm × 3 mm i.d.). The tube was placed in a reaction vial with a resealable enclosure (Waters, Milford, MA). Derivatization was performed using a Pico-Tag Work Station (Waters). The tube contents were dried under vacuum, and 10 mL of redrying solution consisting of a 2:2:1 mixture of methanol, water, and triethylamine (TEA) was added followed by drying again under vacuum. Then, 20 mL of derivatizing solution which consisted of a 7:1:1:1 mixture of methanol, water, TEA, and PITC was added, and derivatization was performed at 25 °C for 20 min. The excess reagent was removed under vacuum.

*(4) HPLC Separation of PTC−$\gamma$-Glutamyl-$\beta$-alanylhistidine.* Separation and detection were performed by the slight modification of the method reported by Sato et al. (1992). PTC derivatized samples were dissolved in 5 mM sodium phosphate buffer (pH 7.4) containing 10% (v/v) acetonitrile (Pico-Tag sample diluent, Waters), and the solution was filtered through a 0.45 mm filter (Chromatodisk 4A, GL Science). The filtrate (20 mL) was applied using an automatic sample injector into an Inertsil ODS-3 column (250 mm × 4.6 mm i.d., particle size 5 $\mu$m, GL Science). The separation of PTC derivative of $\gamma$-glutamyl-$\beta$-alanylhistidine was performed using a binary linear solvent gradient. Solution A consisted of 0.15 M ammonium acetate containing 7% (v/v) acetonitrile, and solution B consisted of acetonitrile and water (6:4). The gradient profiles were as follows: 0−15 min, 0% B; 15−25 min, 0−50% B; 25−35 min, 50−100% B. The flow rate was 1 mL/min, detection was performed at UV 269 nm, and column temperature was maintained at 40 °C.

**Conversion of the Phenylthiocarbamyl (PTC) Derivative of $\gamma$-Glutamyl-$\beta$-alanylhistidine to the Phenylthiohydantoin (PTH) Form.** The HPLC peaks corresponding to PTC−$\gamma$-glutamyl-$\beta$-alanylhistidine were collected and concentrated under vacuum, put into a glass tube (50 mm × 3 mm i.d.), and converted to the PTH form according to the method of Tarr (1986). Briefly, 10 mL of 25% (v/v) TFA was added, and tubes were set at 50 °C for 20 min. After drying under vacuum, addition of 25% TFA and holding at 50 ×C were repeated.

**Spectrometry.** Mass spectra were recorded with a JMS−HX110/HX110 tandem mass spectrometer (JEOL Co. Ltd., Tokyo, Japan). The ionization mode was set to fast-atom bombardment (FAB)−(+) with glycerol as the matrix. The mass of the PTH form of $\gamma$-glutamyl-$\beta$-alanylhistidine was measured by electron spray ionization (ESI) mass spectrometry using Model TSQ700 (Finnigan-Mat Inc.). $^1$H NMR, $^{13}$C NMR, and $^1$H−$^1$H COSY spectra were recorded with a Bruker AMX-600 apparatus. FG-HMBC analysis was performed using a JEOL α400 (JEOL). All NMR analyses were conducted using $D_2O$ as solvent and TMS as the internal chemical shift standard.

**Amino Acid Analysis.** The accuracy of the recovery following proteolytic digestion and SPE was confirmed by analyzing $\gamma$-glutamyl-$\beta$-alanylhistidine using a Model L-8500 amino acid analyzer (Hitachi) with lithium citrate buffer (PF-series for non-hydrolyzed amino acids and peptides analyses; Mitsubishi Chemical Inc., Tokyo, Japan) using ninhydrin as coloring reagent.

**Statistical Analysis.** Statistical analyses were performed using Excel version 5 (Microsoft Inc.), and the *t*-test was used to determine the significance of differences between means. Trends were considered significant when means of compared sets differed at $p < 0.05$.

## RESULTS AND DISCUSSION

**Chromatographic Separation of the Products from the Aspargine/Carnosine and Glutamine/Carnosine Systems.** As shown in Figure 1A, 14 peaks formed during the heating of asparagine and carnosine were detected by HPLC using a Carbon C-500 column. These peaks were designated as A1−A14. As shown in Figure 1B, 10 peaks formed during the heating of glutamine and carnosine. These peaks were designated as G1−G10. After repurification with the Carbon C-500 column, the amino acid composition of each peak was analyzed. Table 1 shows the amino acid composition of each peak formed from the reaction of asparagine and carnosine. The major peak designated as A7 consisted of aspartic acid, ***β***-alanine, and histidine in almost equal molar amounts. This result suggested that the major product A7 was the peptide which formed from aspargine and carnosine during heating in aqueous solution. Table 2 shows the amino acid composition of each peak formed from the reaction of glutamine and carnosine. The major peak designated as G6 consisted of glutamic acid, $\beta$-alanine, and histidine in almost equal molar amounts. This result suggested that the major

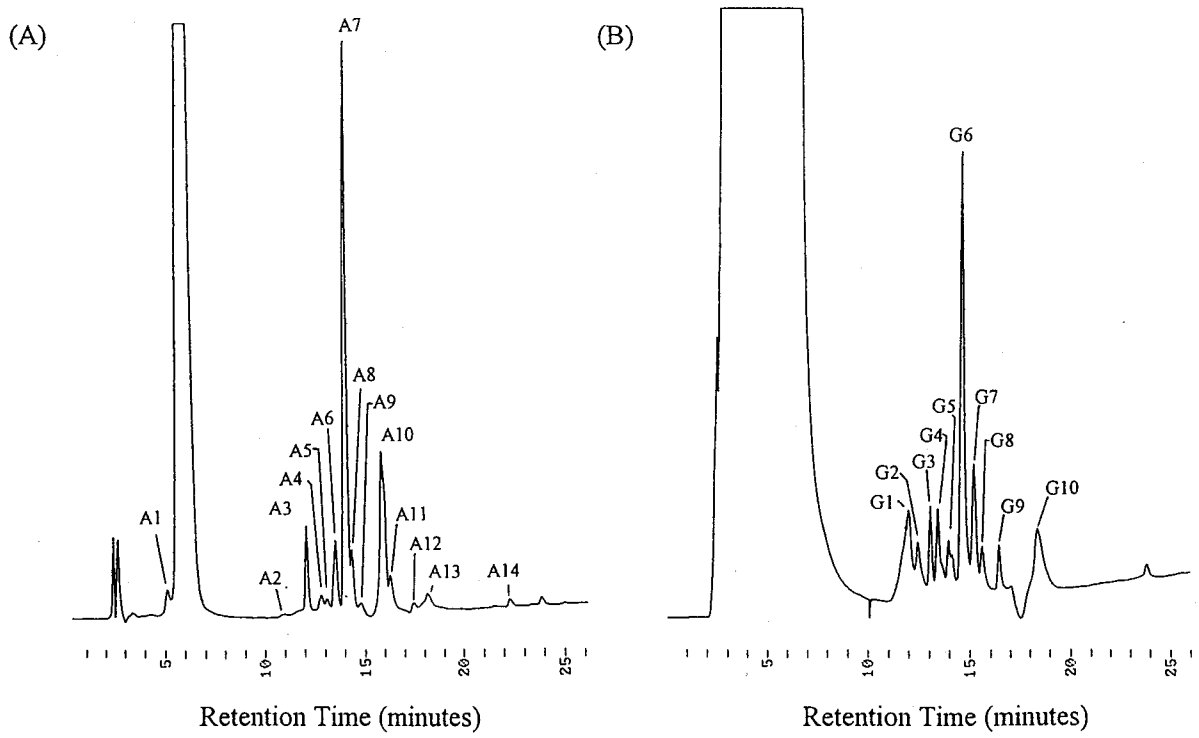


**Figure 1.** HPLC chromatogram obtained with carbon column for the reaction mixture: (A) Asparagine (1.0 M) and carnosine (1.0 M) heated at 95 °C for 24 h; (B) glutamine (1.0 M) and carnosine (1.0 M) heated at 95 °C for 24 h.

**Table 1. Amino Acid Components of Each Peak (A1−A14, μmol) Obtained from Aspargine and Carnosine during Heating at 95 °C for 24 H[a]**

| amino acid | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | 2.7 | 0.56 | 0.07 | 0.42 | 2.0 | 3.2 | 27.9 | 1.2 | 0.6 | 3.8 | 1.6 | 0.31 | 0.7 | 0.09 |
| b-Ala | 60.4 | 6.3 | 0.43 | 2.0 | 1.4 | 2.9 | 27.0 | 2.2 | 2.4 | 3.7 | 0.47 | 1.0 | 0.9 | 0.83 |
| His | 57.9 | 0.13 | 0.09 | 0.68 | 1.7 | 3.3 | 32.1 | 6.1 | 2.6 | 3.6 | 6.4 | 1.6 | 0.97 | 0.79 |

[a] The numbers show the molar amounts recovered in each peak obtained from 1 mL of the reaction mixture.

**Table 2. Amino Acid Componets of Each Peak (G1−G10, μmol) Obtained from Glutamine and Carnosine during Heating[a]**

| amino acid | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Glx | 133.1 | 1.3 | 0.28 | 0.79 | 1.0 | 2.6 | 0 | 0.74 | 0 | 0.08 |
| b-Ala | 28.8 | 0.7 | 1.1 | 0.37 | 5.6 | 2.8 | 0.25 | 0.44 | 0.09 | 0.34 |
| His | 87.6 | 1.3 | 1.5 | 2.6 | 6.5 | 2.7 | 4.7 | 0.89 | 0.23 | 0.62 |

[a] The numbers show the molar amounts in each peak obtained from 1 mL of the reaction mixture.

product G6 was the peptide formed from glutamine and carnosine during heating in aqueous solution. In the present study, we tried to characterize the other peaks, however, it was unsccessful to purify and identify the other peaks. Since the heated solution showed a light brown color (data not shown), it seemed possible that browning compounds formed via degradation of amino acids or carnosine and combined to amino acids or carnosine. Further investigtion on the other products is now in progress.

**Spectroscopic Analyses of A7 and G6.** The obtained A7 and G6 peaks were rechromatographed on a Vydac C18 column and subjected to spectroscopic analysis. Approximately 2.5 mg (on a dry basis) of A7 and 0.5 mg of G6 were obtained from 1 mL of reaction mixture. FAB-MS data for A7 revealed an $[M + 1]^+$ ion at $m/z = 342$. This result showed that the compound A7 was a tripeptide consisting of aspartic acid, β-alanine, and histidine (molar ratio = 1:1:1). FAB-MS data for peak G6 revealed an $[M + 1]^+$ ion at $m/z = 356$. This result indicated that compound G6 was a tripeptide consisting of glutamic acid, β-alanine and histidine (molar ratio = 1:1:1).

**Table 3. $^{13}C$ and $^{1}H$ NMR Spectral Data for A7**

| position | δ C (ppm) | δ H (ppm) | spin−spin (HMBC) | spin−spin ($^1H-^1H$ COSY) |
|---|---|---|---|---|
| 4 | 27.2 | 3.30−3.55 (2H, m) | 2,3,5,6 | 5 |
| 8 | 35.5 | 2.60−2.75 (2H, m) | 7,9 | 9 |
| 11 | 35.5 | 3.05−3.15 (2H, d) | 10,12,13 | 12 |
| 9 | 36.6 | 3.58−3.65 (2H, m) | 7,8,10 | 8 |
| 12 | 51.2 | 4.38−4.45 (1H, t) | 10,11,13 | 11 |
| 5 | 53.0 | 4.90−4.95 (1H, m) | 3,4,6,7 | 4 |
| 2 | 118.0 | 7.48 (1H, s) | 1,3 | |
| 3 | 129.7 | | | |
| 1 | 134.3 | 8.80 (1H, s) | 2 | |
| 10 | 171.7 | | | |
| 13 | 172.6 | | | |
| 6 | 174.6 | | | |
| 7 | 174.6 | | | |

Results obtained from $^1H$ NMR and $^{13}C$ NMR measurement for A7 are shown in Figure 2. Moreover, on the basis of the results of $^1H-^1H$ COSY spectroscopy analysis, each signal was assigned as shown in Table 3. Furthermore, the long-range coupled quaternary carbons were detected in the HMBC spectrum of A7. These results indicated an interaction of C10−H9. On the basis of these observations, the β-carboxyl group was considered to link to an amino group of β-alanine. These NMR data indicated that compound A7 was β-aspartyl-β-alanylhistidine (Figure 3).

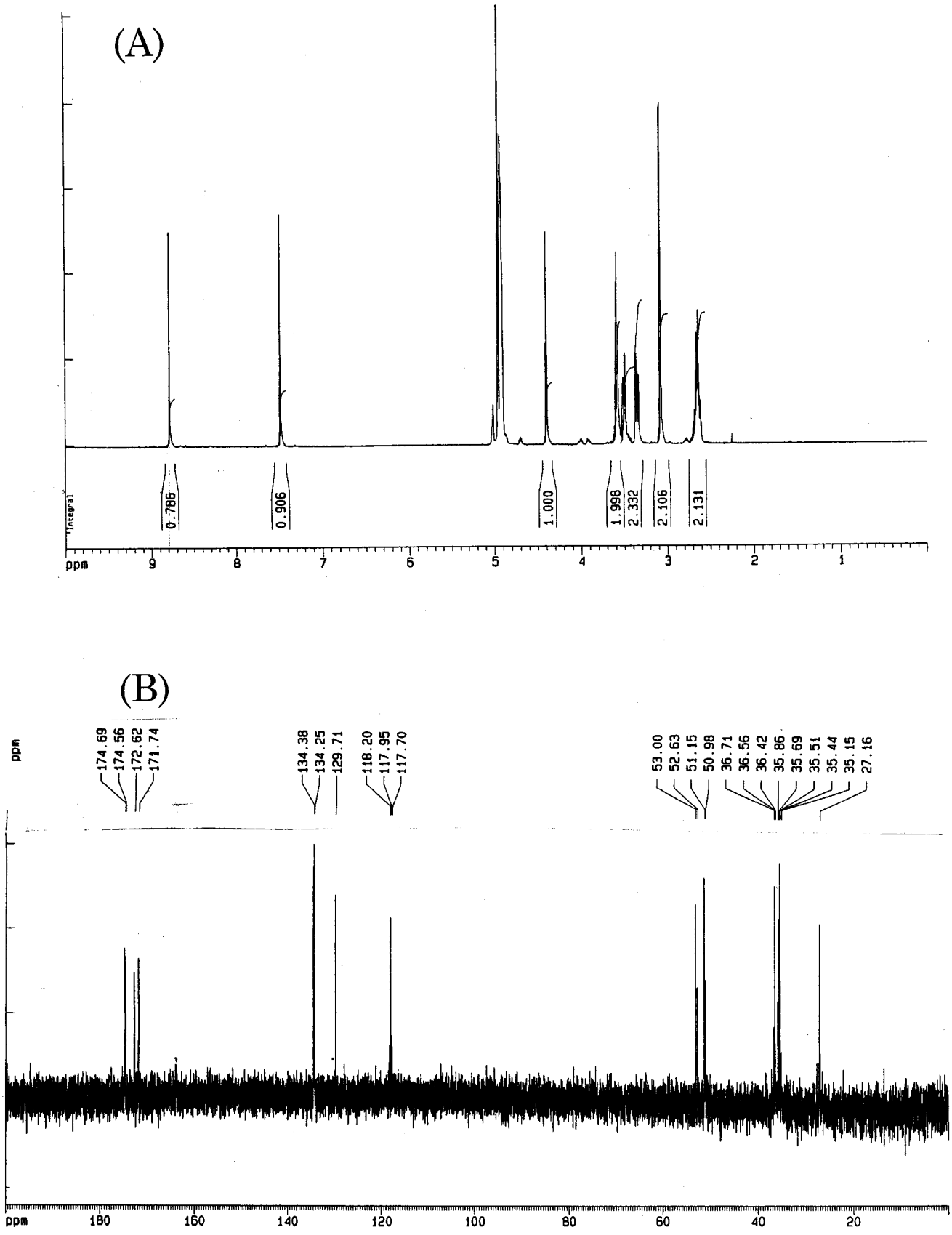


**Figure 2.** NMR spectrum of A7: (A) $^{1}$H NMR; (B) $^{13}$C NMR.

Results obtained from $^{1}$H NMR and $^{13}$C NMR measurements for G6 are shown in Figure 4. These spectra resembled those of ***β***-aspartyl-*β*-alanylhistidine. Moreover, from the results of $^{1}$H−$^{1}$H COSY spectroscopy, each signal was assigned as shown in Table 4. From the results of NMR analyses of G6 and the data obtained for A7 (*β*-aspartyl-*β*-alanylhistidine), compound G6 was considered to be *γ*-glutamyl-*β*-alanylhistidine (Figure 5).

**Heating of Aspartic Acid/Carnosine and Glutamic Acid/Carnosine Systems.** To confirm the mecha-

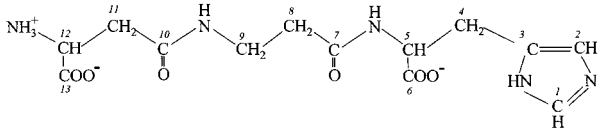

**Figure 3.** Interpreted structure of A7.

nism of formation of $\beta$-aspartyl-$\beta$-alanylhistidine and $\gamma$-glutamyl-$\beta$-alanylhistidine, the aspartic acid/carnosine (1.0 M/1.0 M) and M glutamic acid/carnosine (1.0 M/1.0 M) systems were heated and the products were investigated. For comparison, also asparagine- and glutamine-containing mixtures were heated. Quantification of

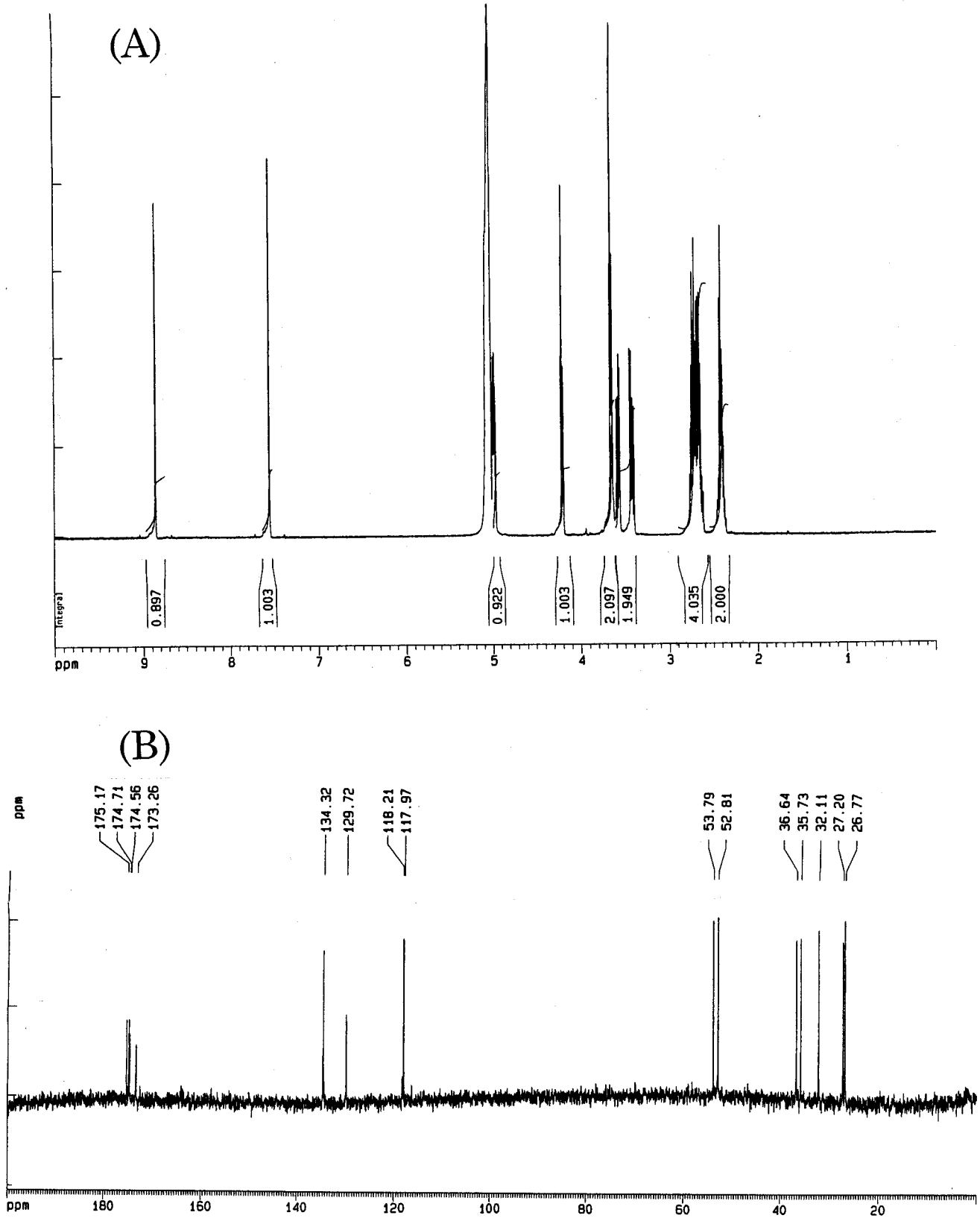


**Figure 4.** NMR spectrum of G6: (A) $^{1}$H NMR; (B) $^{13}$C NMR.

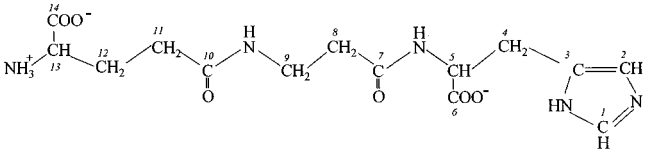

**Figure 5.** Interpreted structure of G6.

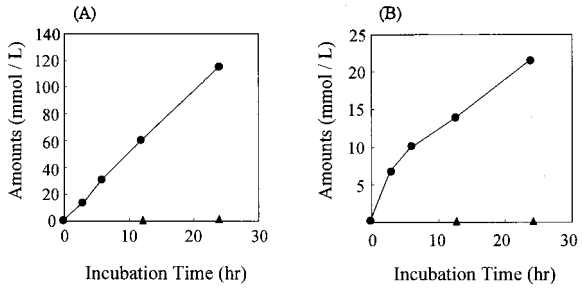


**Figure 6.** Time course of the formation of β-aspartyl-β-alanylhistidine and γ-glutamyl-β-alanylhistidine: (A) β-aspartyl-β-alanylhistidine from the asparagine/carnosine system (●) and aspartic acid/carnosine system (▲); (B) γ-glutamyl-β-alanylhistidine from the glutamine/carnosine system (●) and glutamic acid/carnosine system (▲).

**Table 4. $^{13}C$ and $^{1}H$ NMR Spectral Data for G6**

| position | δ C (ppm) | δ H (ppm) | spin−spin ($^{1}H-^{1}H$ COSY) |
|---|---|---|---|
| 12 | 26.8 | 2.35−2.50 (2H, m) | 11,13 |
| 4 | 27.2 | 3.40−3.60 (2H, m) | 5 |
| 11 | 32.1 | 2.60−2.84 (2H, m) | 12 |
| 8 | 35.7 | 2.60−2.84 (2H, m) | 9 |
| 9 | 36.6 | 3.62−3.70 (2H, t) | 8 |
| 13 | 52.8 | 4.19−4.28 (1H, t) | 12 |
| 5 | 53.8 | 4.95−5.02 (1H, m) | 4 |
| 2 | 118.0 | 7.67 (1H, s) | |
| 3 | 129.7 | | |
| 1 | 134.3 | 8.80 (1H,s) | |
| 6 + 7 + 10 + 13 | 173.3 | | |
| | 174.6 | | |
| | 174.7 | | |
| | 175.1 | | |

β-aspartyl-β-alanylhistidine and γ-glutamyl-β-alanylhistidine was performed by amino acid analyzer using the above peptides isolated as standard. As shown in Figure 6, only a trace amount of β-aspartyl-β-alanylhistidine (0.19 mmol/L at 24 h) and γ-glutamyl-β-alanylhistidine (0.15 mmol/L at 24 h) formed by heating of the aspartic acid/carnosine or glutamic acid/carnosine system. These results suggest that β-aspartyl-β-alanylhistidine and γ-glutamyl-β-alanylhistidine isopeptides mainly form from aspargine/carnosine and glutamine/carnosine.

**Pretreatment for the HPLC Analysis of γ-Glutamyl-β-alanylhistidine.** *(1) Recovery of γ-Glutamyl-β-alanylhistidine after Proteolytic Digestion.* A 20 nmol amount of authentic γ-glutamyl-β-alanylhistidine was applied to the sequential proteolytic digestion. The recovery was assayed using an amino acid analyzer ($n = 3$). The average recovery was 97.2%, indicating that γ-glutamyl-β-alanylhistidine was resistant to the digestion of the proteolytic enzymes used in this study.

*(2) Recovery of Free Amino Acids from the Macromolecular Fraction of Heated Beef Soup Stock after Proteolytic Digestion.* Amino acid analyses indicated that the recovery of total free amino acids after proteolytic digestion (ratio against the amounts of amino acids recovered by 6 M HCl hydrolysis) was approximately 70% and almost the same in the heated (from 0 to 6 h) soup stock. These results suggested that most of γ-glutamyl-β-alanylhistidine could be liberated by the proteolytic digestion.

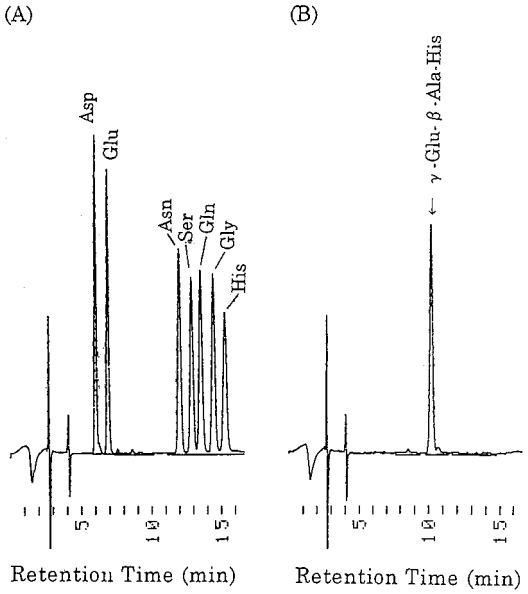


**Figure 7.** HPLC chromatogram of PTC derivatives of authentic amino acids (A) and authentic γ-glutamyl-β-alanylhistidine (B): (A) Standard amino acid mixture containing 1 nmol of each amino acid derivatized with PITC and PTC derivatives containing 100 pmol of each amino acid derivativeapplied to HPLC; (B) 1 nmol of γ-glutamyl-β-alanylhistidine derivatized with PITC and a PTC derivative containing 100 pmol of γ-glutamyl-β-alanylhistidine derivative applied to HPLC.

*(3) Recovery of Solid-Phase Extraction (SPE).* To confirm the recovery and reproducibility of the SPE process, authentic γ-glutamyl-β-alanylhistidine was subjected to SPE. γ-Glutamyl-β-alanylhistidine in the rinse solution (0.1 N HCl) and the eluent used for analysis (80% methanol containing 1% ammonia) was analyzed using an amino acid analyzer. Average recovery ($n = 10$) of γ-glutamyl-β-alanylhistidine in the eluent was 98.5%, and it was not detected in the rinse solution. The coefficient of variation of the recovery was 1.6%. These results suggested that SPE using Toyopack IC−SP could be used as a pretreatment for precise and reproducible analysis.

**Chromatographic Separation of PTC−γ-Glutamyl-β-alanylhistidine.** Figure 7 shows an HPLC chromatogram of PTC derivatives of standard amino acids (A) and the PTC derivative of authentic γ-glutamyl-β-alanylhistidine (B). The results show that sufficient resolution of γ-glutamyl-β-alanylhistidine (retention time, 10.2 min) from protein-constituting amino acids could be achieved using this HPLC method. To determine the accuracy of this method, 100 pmol of authentic PTC−γ-glutamyl-β-alanylhistidine was repeatedly analyzed ($n = 10$). The coefficient of variation was 1.0%, indicating that reproducible analysis could be achieved using this HPLC method. As shown in Figure 8, the relationship between peak area and the injected amount of PTC−γ-glutamyl-β-alanylhistidine was linear over the range of 5−500 pmol ($r = 0.999$). Figure 9 shows the elution pattern of the proteolytic digest of the macromolecular fraction obtained from heated (95 °C, 3 h) beef soup stock solution (DM = 30%). This result suggested that adequate separation between γ-glutamyl-β-alanylhistidine and other peaks was achieved using this HPLC method. To confirm the accuracy of separa-

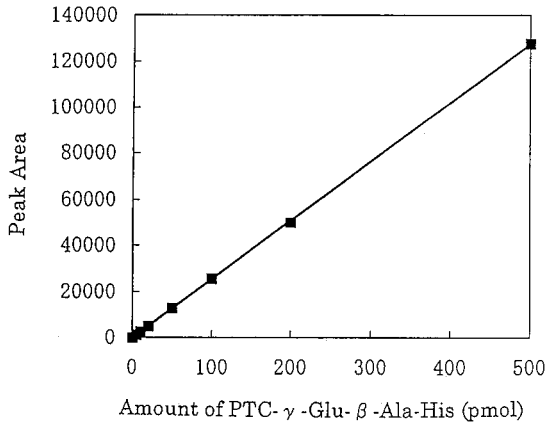


**Figure 8.** Relationship between peak areas and the concentration of PTC−γ-glutamyl-β-alanylhistidine.

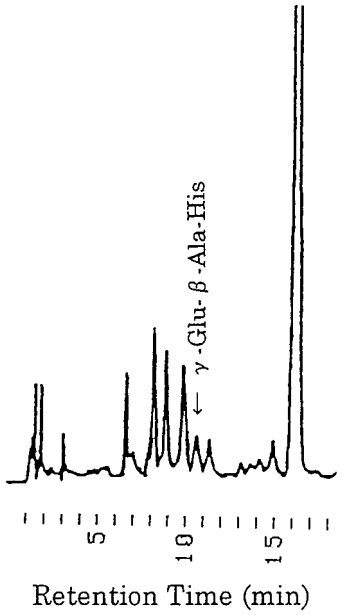


**Figure 9.** HPLC chromatogram of PTC derivatives of the γ-glutamyl-β-alanylhistidine-containing fraction from the macromolecular fraction of heated beef soup stock.

tion, the PTC−γ-glutamyl-β-alanylhistidine peak from beef soup stock was collected and analyzed by ESI-MS.

**ESI-MS Analysis of the PTH Form of γ-Glutamyl-β-alanylhistidine.** To confirm the identification of the PTC derivative, the HPLC peak of the authentic PTC−γ-glutamyl-β-alanylhistidine and that from beef soup stock were collected. The derivatives were converted to the stable PTH form, and their masses were determined by ESI-mass spectrometry. The results obtained by ESI-MS analysis are shown in Figure 10. The results demonstrated the existence of a major peak in each specimen with $m/z$ = 473.6 (authentic) and 473.5 (sample). This mass number was consistent with the parent ion ($MH^+$) of the PTH form of γ-glutamyl-β-alanylhistidine (MW = 472.4). Furthermore, it was suggested that the other peak with $m/z$ = 495.6 (authentic) and 495.2 (sample) was $MNa^+$. Although the ESI-MS patterns of several minor peaks were slightly different between the authentic specimen and that from the sample, the patterns of major peaks were very similar. These results suggested that the HPLC peak of the PTC derivative from heated beef soup stock consisted mainly of PTC−γ-glutamyl-β-alanylhistidine. Thus, the HPLC method described here could be used for determination of γ-glutamyl-β-alanylhistidine in the macromolecular fractions of heated beef soup stock solution.

**Table 5. Contents of γ-Glutamyl-β-alanylhistidine Isopeptide in the Macromolecular Fraction of Heated Beef Soup Stock Solution[a−c]**

| heating time (h) | contents of γ-Glu-β-Ala-His (nmol/g of DM) | contents of β-Ala (nmol/g of DM) | contents of His (nmol/g of DM) |
|---|---|---|---|
| 0 | 49.0 ± 9.2[A] | 270 ± 140[A] | 10 130 ± 2980[A] |
| 1 | 88.5 ± 15.6[B] | 1650 ± 400[B] | 13 580 ± 3950[AB] |
| 3 | 123.6 ± 24.2[C] | 2980 ± 910[C] | 15 370 ± 5860[B] |
| 6 | 133.5 ± 22.8[C] | 3880 ± 960[D] | 18 730 ± 7920[B] |

[a] Means ± standard deviation of 3 replicates (γ-Glu-β-Ala-His) or 11 replicates (β-Ala, His). [b] The numbers show the molar amounts recovered in the macromolecular fractions obtained from 1 g (dry matter) of beef soup stock. [c] Values in a column with different superscript letters are significantly different at $p < 0.05$.

**Measurement of γ-Glutamyl-β-alanylhistidine Isopeptide in Macromolecular Fractions of Heated Beef Soup Stock Solution after Enzymatic Digestion.** The present HPLC method was applied to the measurement of γ-glutamyl-β-alanylhistidine in the macromolecular fractions from heated beef soup stock solution. The results shown in Table 5 indicated that the contents of γ-glutamyl-β-alanylhistidine in the macromolecular fraction increased during the heating pro-

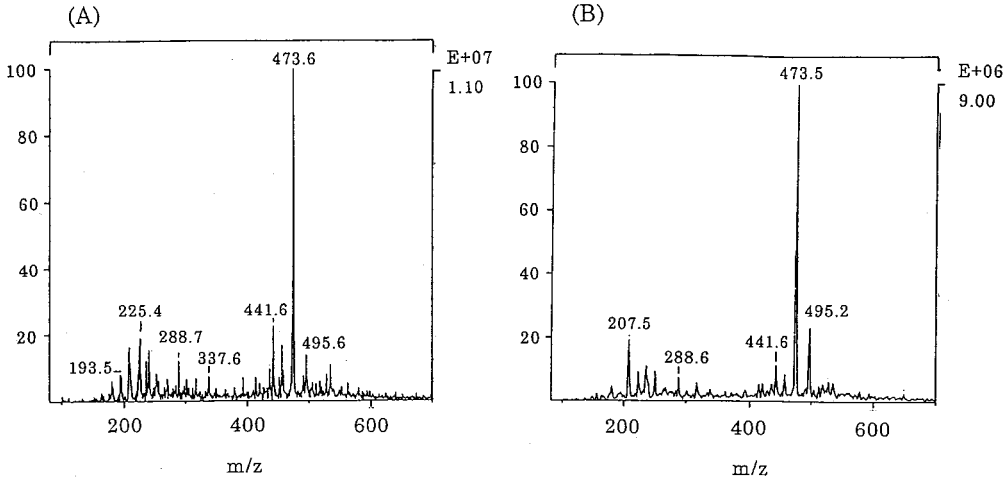


**Figure 10.** ESI-MS spectra of the PTH form of authentic γ-glutamyl-β-alanylhistidine (A) and γ-glutamyl-β-alanylhistidine from heated beef soup stock (B).

cess. This suggested that γ-glutamyl-β-alanylhistidine isopeptide was formed during the heating of beef soup stock solution. Furthermore, the content of γ-glutamyl-β-alanylhistidine was compared to the increases in levels of histidine and β-alanine in the macromolecular fractions determined by amino acid analyses followed by 6 N HCl hydrolysis (Table 5). The results indicate that the increased amount of β-alanine (HCl hydrolysis) is approximately half of the increased amount of histidine. This result suggests that a major part of the carnosine was incorporated and the β-alanine portion changed to a structure that cannot be recovered after HCl hydrolysis. The results in Table 5 also indicated that the amount of γ-glutamyl-β-alanylhistidine was increased by about 5% of the increase in β-alanine. These results suggested that other compounds containing β-alanine formed during the process of heating beef soup stock solution. Since the decrease in the levels of reducing sugar has been observed during the heating of beef soup stock (Kuroda and Harada, 2000), it is supposed that some of carnosine was incorporated via a Maillard reaction with reducing sugar. Recently, it has been reported that incubation (at 37 °C for 2 h) of several proteins with 4-hydroxynonenal (lipid-oxidation product) results in the modification of histidine residue and forms the cross-links (Uchida and Stadtman, 1992, 1993). From these previous observations, it seemed possible that some of carnosine has been incorporated into the macromolecular fraction by a reaction between the histidine moeity and lipid-oxidation products. In the present study, it has been shown that the heating of asparagine and carnosine in aqueous solution resulted in the formation of β-aspartyl-β-alanylhistidine. Identification of β-aspartyl-β-alanylhistidine was also attempted by the same strategy as described above. However, it was unsuccessful in detecting the HPLC peak corresponding to β-aspartyl-β-alanylhistidine. Further studies to identify other carnosine-containing compounds and the analysis of γ-glutamyl-β-alanylhistidine in various foods are currently in progress in our laboratories.

## ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment-mass spectrometry; NMR, nuclear magnetic resonance spectrometry; COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; TMS, tetramethylsilane. ESI-MS, erectron spray ionization-mass spectrometry; SPE, solid-phase extraction; PITC, phenylisothiocyanate; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; γ-Glu-β-Ala-His, γ-glutamyl-β-alanylhistidine.

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