Enzymatic Determination of Carnosine in Meat and Fish using β -Ala-Xaa Dipeptidase and Histidine Ammonia-Lyase derived from *Pseudomonas putida* NBRC100650

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Abstract

Carnosine is a naturally occurring dipeptide and functional component in foods and shows health-promoting effects. Generally, food-derived carnosine is quantified via high-performance liquid chromatography (HPLC). Herein, we developed a method for quantifying carnosine in foods using microbial enzymes, β -Ala-Xaa dipeptidase (BapA) and histidine ammonia-lyase (HAL). Carnosine concentration in extracts of chicken, pork, beef, bonito, and tuna was determined via both HPLC and enzymatic determination. The carnosine contents measured via enzymatic determination were in agreement with those determined via conventional HPLC analysis. Relative standard deviation values of the conventional HPLC method and the enzymatic determination of carnosine in foods were 0.728–5.76% and 0.504–4.58%, respectively. The recovery of carnosine in food extracts via enzymatic determination was 97–103%. Therefore, the developed enzymatic determination technique using BapA and HAL can be used for the determination of carnosine in meats and fishes with comparable accuracy to that of conventional HPLC analysis.

Keywords: carnosine, imidazole dipeptide, β -Ala-Xaa dipeptidase, histidine ammonia-lyase, enzymatic determination.

Introduction

Carnosine is a naturally occurring dipeptide comprising L-histidine and β -alanine and is present in mammalian tissues such as brain and skeletal muscle tissues.^{1,2} Carnosine and its naturally occurring derivatives, anserine, ophidine (balenine), homocarnosine, and acetyl carnosine are the most common imidazole dipeptides (Fig. 1). Livestock-derived meat such as beef and pork contains a high concentration of carnosine; chicken meat contains a high concentration of anserine.³ Anserine is also present in tissues of fishes such as tuna and rainbow trout.^{4,5} Ophidine is found in snake and whale muscle tissues.⁶ Whereas, homocarnosine is found in human urine and the brain tissues of pigs, dogs, and cats.⁷ Acetyl carnosine is found in mammalian cardiac muscles.⁸ Carnosine has various physiological functions such as the maintenance of the pH balance and anti-glycation, anti-oxidation, and anti-crosslinking effects.^{9–13} Moreover, it was reported that carnosine supplementation may represent an effective strategy for inducing anti-senescence and anti-fatigue and prevention of type 2 diabetes.^{14–16} Therefore, carnosine is used as a functional component in foods that show health-promoting effects; various dietary supplements containing carnosine are commercially available. In Japan, the system of “Foods with function claims” was launched in 2015. Food products can be labeled with a function claim, which is based on scientific evidence, under food business operators’ responsibility. Fresh produces are subject to labeling under this system, and a homebred chicken (Hakata Jidori) labeled with carnosine concentration is in the market. Simple and cost-effective methods to determine carnosine in food can enable food business operators to indicate the concentration of carnosine on labels of various foods, and customers can use the information to maintain their health.

Generally, the concentration of carnosine and its derivatives in food is determined using

high-performance liquid chromatography (HPLC). It is difficult to separate carnosine and anserine via HPLC using conventional octadecylsilyl columns. Therefore, an appropriate column such as a hydrophilic interaction chromatography column or carbon column is used for the analysis of imidazole dipeptides.^{17,18} HPLC analysis shows high accuracy; however, it requires expensive equipment and trained staff for operating the equipment. In contrast, enzyme assays can be used for quantification because they reduce the initial cost and are easy to perform. Enzymatic determination is also suitable for high-throughput analysis. For example, a flow-injection analytical (FIA) system with an immobilized cholesterol oxidase capillary and an electrochemical detector for cholesterol determination was developed recently.¹⁹ Cholesterol oxidase converts cholesterol to hydrogen peroxide and cholestenone in the capillary, and hydrogen peroxide is detected with a three electrode system. The FIA system with cholesterol oxidase can determine cholesterol without complex sample preparation method and at a low cost. A unique spectrophotometric method to determine polyhexamethylene biguanide (PHMB) with glucose oxidase has also been reported.²⁰ PHMB promotes the glucose oxidase reaction with ferricyanide ion. Utilizing this promotion effect, PHMB can be determined using a microplate reader. Thus, we attempted to develop a method to determine carnosine in food with enzymes. To the best of our knowledge, the determination of imidazole dipeptides via enzyme assays has not been reported.

Carnosinase from vertebrates^{21,22} and β -Ala-Xaa dipeptidase (BapA) from *Pseudomonas* sp.²³ catalyze the conversion of carnosine to L-histidine and β -alanine. The use of histidine ammonia-lyase (HAL) from *P. putida* to determine L-histidine in plasma and urine samples has been reported.²⁴ Therefore, we considered that L-histidine generated from carnosine could also be determined with HAL. In the analysis using two

kinds of enzymes, it is desirable that the optimum temperature and pH for each enzymatic reaction are similar. Because we expected that the optimum reaction conditions for enzymes from an identical organism might be similar, BapA and HAL from *P. putida* were selected as enzymes to determine carnosine concentration (Fig. 2). HAL catalyzes the conversion of L-histidine to urocanic acid and ammonia. Since urocanic acid shows an absorbance at 277 nm,²⁵ the concentration of carnosine can be determined by measuring the absorbance of urocanic acid using a spectrophotometer. To the best of our knowledge, the use of HAL to determine L-histidine in food samples has not been tested. Herein, we demonstrate the enzymatic determination of L-histidine in meats and fishes using HAL.

Experimental

Reagents and chemicals

Carnosine and L-alanine *p*-nitroanilide (L-Ala-*p*NA) were obtained from Peptide Institute (Osaka, Japan). Anserine was purchased from Bachem AG (Bubendorf, Switzerland). Acetyl carnosine was purchased from Cayman Chemical (Ann Arbor, MI, USA). D-Alanine *p*-nitroanilide (D-Ala-*p*NA) was obtained from Watanabe Chemical Industries (Hiroshima, Japan). 3-Methyl-L-histidine was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Polypepton was obtained from Nihon Pharmaceutical (Tokyo, Japan). Yeast extract was obtained from Oriental Yeast (Tokyo, Japan). Restriction enzymes and kits for performing genetic manipulation were obtained from Takara Bio (Kyoto, Japan), Nippon gene (Tokyo, Japan), New England Biolabs (Beverly, MA, USA), Bioneer (Alameda, CA, USA), and Roche (Indianapolis, IN, USA). The DNeasy UltraClean Microbial Kit was obtained from QIAGEN (Venlo,

Netherland). All other reagents were of analytical grade and were obtained from Nacalai Tesque (Kyoto, Japan) and FUJIFILM Wako Pure Chemical Corporation.

Enzyme assays

The dipeptidase activity of BapA was assayed by using D-Ala-*p*NA as a substrate.²³ Dipeptidase activity was assayed by monitoring the increase in absorbance at 405 nm (V-730BIO spectrophotometer; JASCO, Tokyo, Japan) due to the production of *p*-nitroaniline at 30 °C in a 1 mL standard reaction mixture containing 5 mM D-Ala-*p*NA and 0.1 M Tris-HCl buffer (pH 8.0). One unit (U) of enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *p*-nitroaniline/min in the reaction. Specific activity was expressed as U/mg of protein. The concentration of *p*-nitroaniline was determined from the molar extinction coefficient of 11,500 M⁻¹ cm⁻¹.²⁶

HAL activity was assayed by monitoring the increase in absorbance at 277 nm due to the production of urocanic acid at 30 °C in a 1 mL standard reaction mixture containing 3.3 mM L-histidine and 0.1 M Tris-HCl buffer (pH 8.0). One U of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of urocanic acid/min in the reaction. Specific activity was expressed as U/mg of protein. The concentration of urocanic acid was determined from the molar extinction coefficient of 18,800 M⁻¹ cm⁻¹.²⁵

Microorganisms and culture conditions

Pseudomonas putida NBRC100650 was cultivated in Luria-Bertani (LB) medium at 30 °C with reciprocal shaking for 20 h. *Escherichia coli* DH5α was cultivated in LB medium in a reciprocal shaker at 37 °C overnight. Recombinant Rosetta-gami B(DE3)

cells were cultured aerobically in LB medium containing 30 µg/mL chloramphenicol and 0.1 mg/mL ampicillin at 37 °C for 18 h. The culture was supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside and grown for a further 3 h. The cells were harvested via centrifugation (7,000 × g, 20 min, 4 °C), washed with 0.85% NaCl, and stored at -80 °C until use.

Cloning of P. putida-derived bapA and hutH genes

Genomic DNA was isolated using the DNeasy UltraClean Microbial Kit. The gene encoding BapA (*bapA*; PP_3844, UniProtKB database accession number Q88G81) was amplified via polymerase chain reaction (PCR) in a 25 µL reaction mixture containing 1× *pfuUltra* High-Fidelity DNA polymerase reaction buffer, 0.2 mM deoxynucleotide triphosphates, 13 pmol of each primer (5'-GGAATTCCATATGAAAGACACGATGAAGCCACG-3' and 5'-CCCAAGCTTACCATTCGCCGCTCCCGGCCAGC-3'), 83 ng of *P. putida* NBRC100650 genomic DNA, and 1.3 U of *pfuUltra* High-Fidelity DNA polymerase. PCR was performed as follows: pre-incubation at 95 °C for 2 min and then 30 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, and final elongation at 72 °C for 10 min. The PCR product was digested using NdeI and HindIII and ligated into pET21a, which had been previously digested using the same restriction enzymes. The resultant plasmid was transformed into Rosetta-gami B(DE3) to produce the recombinant histidine-tagged BapA.

The gene encoding HAL (*hutH*; PP_5032, UniProtKB database accession number Q88CZ7) was amplified via PCR in a 25 µL reaction mixture containing 1× Prime STAR GXL buffer, 2.5 mM deoxynucleotide triphosphates, 6.3 pmol of each primer (5'-GGAGATATACATATGACCGAACTCACCTCAAGCCTG-3' and

5'-GTGGTGGTGGTGGTGCAGGCTTGGCAGTACACCCGCCGGC-3'), 83 ng of *P. putida* NBRC100650 genomic DNA, and 0.63 U of Prime STAR GXL DNA polymerase. PCR was performed as follows: pre-incubation at 95 °C for 2 min and then 30 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, and a final elongation at 72 °C for 10 min. The DNA fragment derived from pET21a was amplified via PCR in a 25 µL reaction mixture containing 1× Prime STAR GXL buffer, 2.5 mM deoxynucleotide triphosphates, 6.3 pmol of each primer (5'-CATATGTATATCTCCTTCTTAAAGTTAAACAAA-3' and 5'-CACCACCACCACCACCACCTGAGATCCGGCTGCTAA-3'), 47 ng of pET21a plasmid, and 0.63 U of Prime STAR GXL DNA polymerase. PCR was performed as follows: pre-incubation at 95 °C for 2 min and then 30 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 5 min 30 s, and a final elongation at 72 °C for 10 min. PCR products containing *hutH* were ligated together with the DNA fragment derived from pET21a using the In-Fusion HD cloning Kit according to the manufacturer's instructions. The resultant plasmid was transformed into Rosetta-gami B(DE3) to produce the recombinant histidine-tagged HAL.

Purification of the recombinant enzyme

Histidine-tagged BapA-expressing Rosetta-gami B(DE3) cells were suspended in a binding buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), and 1 mM phenylmethylsulfonyl fluoride, and disrupted on ice via sonication. Subsequent processes were performed at 4 °C. The crude extract obtained via sonication was applied to a nickel-nitrilotriacetic acid His•Bind Resin (Novagen, Madison, WI, USA) column equilibrated using the binding buffer. Histidine-tagged BapA was eluted using a linear gradient of 0.06–1 M imidazole in the binding buffer. The enzyme

solutions were pooled and concentrated using VIVASPIN 20, 30,000 MWCO, PES (Sartorius, Göttingen, Germany), and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM 2-mercaptoethanol and 0.1 mM ethylenediaminetetraacetic acid (EDTA) twice. Histidine-tagged HAL was purified using the same method with a modified dialysis step. HAL was dialyzed against 20 mM potassium phosphate buffer (pH 7.2) twice. The final preparation of both histidine-tagged enzymes was stored at -80 °C until use for molecular characterization of the enzymes.

Since the HAL-catalyzed reaction was inhibited by EDTA, BapA was dialyzed using 20 mM Tris-HCl buffer (pH 8.0) before use for enzymatic determination of carnosine in food. HAL was incubated with 130 mM Tris-HCl buffer (pH 9.0) containing 1.3 mM 2-mercaptoethanol and 0.1 mM MnCl₂ at 25 °C for 15 min to activate the enzyme before use for enzymatic determination of carnosine in food.²⁷

Kinetic analysis

The initial rates of the BapA and HAL-catalyzed reactions were measured using various concentrations of substrate. Data were incorporated in the hyperbolic Michaelis-Menten equation, and kinetic parameters were calculated using nonlinear least-squares regression using Kaleida Graph version 4.0 (Adelbeck Software, Reading, PA, USA).

Preparation of food extracts

Extraction of carnosine from foods was performed according to the method described by Mori *et al.*,¹⁸ with minor modifications. Minced meats (chicken, pork, and beef) and sliced raw fishes (bonito and tuna) were obtained from local commercial sources in Kochi, Japan. Sliced raw fishes were minced on ice. Each minced food (10 g)

was suspended in cold water (50 mL). The food suspensions were heated at 95 °C for 30 min, and then centrifuged at $20,000 \times g$ for 60 min at 4 °C. The supernatant was collected and used as a food extract in subsequent experiments.

HPLC analysis of carnosine, anserine, and L-histidine in food extracts

HPLC analysis was performed according to the method described by Mora *et al.*¹⁷ Carnosine, anserine, and histidine present in food extracts were analyzed using an HPLC system (system controller, SCL-10A; column oven, CTO-10AC; pump, LC-10AD; detector, SPD-M10AC; degasser, DGU-14A; Shimadzu, Kyoto, Japan) equipped with an Atlantis HILIC Silica column ($\phi 4.6 \times 150$ mm, 3 μ m, Waters, Milford, MA, USA). The column was maintained at 40 °C. Mobile phases consisted of solvent A containing 0.65 mM ammonium acetate (pH 5.5) in water:acetonitrile (25:75), and solvent B containing 4.55 mM ammonium acetate (pH 5.5) in water:acetonitrile (70:30). The separation conditions involved a linear gradient from 0 to 100% of solvent B in 13 min at a flow rate of 1.4 mL/min. The food extract was filtered through a 0.22 μ m membrane filter and injected. The absorbance of the effluent at 214 nm was continuously monitored for carnosine, anserine, and histidine. The column was equilibrated for 15 min under the initial conditions before each injection.

Enzymatic determination of L-histidine in food extracts

The concentration of L-histidine in food extracts was determined using a 1 mL L-histidine assay mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 0.1 mg/mL HAL, and food extract. The L-histidine assay mixture was incubated at 30 °C for 60 min and then heated at 95 °C for 5 min to denature HAL. After stopping the HAL-catalyzed reaction, denatured HAL in the L-histidine assay mixture was removed via

centrifugation at 12,000 rpm for 5 min at room temperature (20–28 °C). The absorbance of the supernatant at 277 nm associated with the production of urocanic acid by the HAL-catalyzed reaction was measured. A blank solution (L-histidine assay mixture without food extract) was heated at 95 °C for 5 min, centrifuged at 12,000 rpm for 5 min at room temperature, and the absorbance of the blank solution at 277 nm was measured. The absorbance value of the blank solution at 277 nm was subtracted from the absorbance value of the L-histidine assay mixture at 277 nm, and the concentration of L-histidine was calculated by incorporating the subtracted absorbance value at 277 nm into the standard curve.

Enzymatic determination of carnosine in food extract

The concentration of carnosine in food extracts was determined using a 1 mL carnosine assay mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 2.0 mg/mL BapA, 0.1 mg/mL HAL, and food extract; the mixture was incubated at 30 °C for 60 min and then heated at 95 °C for 5 min to denature both enzymes. After stopping the BapA and HAL-catalyzed reactions, both the denatured enzymes in the carnosine assay mixture were removed via centrifugation at 12,000 rpm for 5 min at room temperature. The absorbance of the supernatant at 277 nm associated with the production of urocanic acid by the BapA and HAL-catalyzed reactions was measured. This absorbance was attributed to urocanic acid, which was converted from both carnosine and L-histidine in food extracts. To determine the concentration of carnosine present in food extracts, the absorbance value associated with L-histidine was subtracted. Hence, the blank solution A (carnosine assay mixture without BapA) was heated at 95 °C for 5 min, centrifuged at 12,000 rpm for 5 min at room temperature, and then the absorbance at 277 nm was measured. The absorbance value of the blank solution A at 277 nm which was attributed

to urocanic acid converted from L-histidine and the residual denatured HAL. The blank solution B (2.0 mg/mL BapA) was processed in the same manner as blank solution A and then the absorbance at 277 nm was measured. The absorbance value of blank solution B at 277 nm was attributed to the residual denatured BapA. The absorbance values of the blank solutions A and B at 277 nm were subtracted from the absorbance value of the carnosine assay mixture at 277 nm, and the subtracted absorbance value was incorporated into the standard curve of carnosine.

Recovery of carnosine in food extract via enzymatic determination of carnosine

The extracts from meats (chicken, pork, and beef) and fishes (bonito and tuna) were diluted with distilled water by 80-fold and 960-fold, respectively. Carnosine at final concentrations of 4.52 and 9.04 $\mu\text{g/mL}$ was added to the diluted food extracts and the enzymatic determination of carnosine was performed.

Results and Discussion

*Properties of BapA derived from *P. putida* NBRC100650*

The amino acid sequence identity between BapA (PP_3845) derived from *P. putida* NBRC100650 and previously reported BapA from *Pseudomonas* sp. MCI3434 is 64%.²³ BapA derived from *P. putida* NBRC100650 has not been characterized previously. Therefore, we analyzed the properties of histidine-tagged BapA derived from *P. putida* NBRC100650 to determine the reaction conditions for enzymatic determination of carnosine. The optimum temperature of the BapA-catalyzed reaction was determined by measuring the activity in a standard reaction mixture, and varying the temperature from 20 to 70 °C (Fig. S1 A, Supporting Information). The enzyme

showed maximum activity at 55 °C, and 70% of the maximum activity was observed at 30 °C. The optimum pH of the BapA-catalyzed reaction was analyzed using various 0.1 M buffer solutions at 30 °C (Fig. S1 B, Supporting Information). The enzyme showed maximum activity at pH 9.0 in glycine-NaOH buffer. After incubation of BapA at different temperatures for 1 h in a 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA, the enzyme activity was measured in the standard reaction mixture at 30 °C (Fig. S1 C, Supporting Information). BapA was stable at up to 35 °C, and 93% of the enzyme activity was lost at 50 °C. The pH stability of BapA was examined by incubating the enzyme for 1 h at 30 °C in various buffers prior to performing assays in the standard reaction mixture at 30 °C (Fig. S1 D, Supporting Information). More than 80% of the enzyme activity was retained between pH 7.0 and pH 8.5. The K_m and V_{max} values of BapA for D-Ala-*p*NA were 2.3 ± 0.2 mM and 15 ± 1 U/mg, respectively. The K_m and V_{max} values of BapA for L-Ala-*p*NA were 25 ± 3 mM and 1.2 ± 0.1 U/mg, respectively. BapA derived from *P. putida* NBRC100650 may potentially metabolize other N-terminal β -alanyl dipeptides, because BapA derived from *Pseudomonas* sp. MCI3434 metabolizes N-terminal β -alanyl dipeptides such as β -alanyl-L-alanine, β -alanylglycine, β -alanyl-L-leucine, and β -alanyl- β -alanine.²³ ZnCl₂ inhibited the BapA-catalyzed reaction whereas CoCl₂, NiCl₂, and CuCl₂ activated the reaction (Table S1, Supporting Information). EDTA (50 μ M) had no effect on the BapA-catalyzed reaction.

Properties of HAL derived from P. putida NBRC100650

Histidine-tagged HAL was characterized to determine the optimal reaction conditions for enzymatic determination of carnosine. The optimum temperature of the HAL-catalyzed reaction was determined by measuring the activity in a standard reaction

mixture, and varying the temperature from 20 to 60 °C (Fig. S2 A, Supporting Information). HAL showed maximum activity at 40 °C, and 94% of the maximum activity was observed at 30 °C. The optimum pH of the HAL-catalyzed reaction was analyzed using various 0.1 M buffer solutions at 30 °C (Fig. S2 B, Supporting Information). HAL showed maximum activity at pH 8.0 in Tris-HCl buffer. After incubation of HAL at different temperatures for 1 h in 20 mM potassium phosphate buffer (pH 7.2), the enzyme activity was measured in the standard reaction mixture at 30 °C (Fig. S2 C, Supporting Information). HAL was stable at up to 30 °C, and 96% of the enzyme activity was lost at 60 °C. The pH stability of HAL was examined by incubating the enzyme for 1 h at 30 °C in various buffers prior to performing assays in the standard reaction mixture at 30 °C (Fig. S2 D, Supporting Information). More than 85% of the enzyme activity was retained between pH 7.0 and pH 10.0. The K_m and V_{max} values of HAL for L-histidine were 3.9 ± 0.3 mM and 4.8 ± 0.1 U/mg, respectively. $CuCl_2$ activated the HAL-catalyzed reaction whereas $CoCl_2$, $NiCl_2$, and $BaCl_2$ inhibited the reaction (Table S1, Supporting Information). L-Cysteine (5.0 mM) inhibited the reaction by 81%. EDTA (50 μ M) inhibited the reaction by 94%. Therefore, purified BapA solution containing 0.1 mM EDTA was dialyzed using 20 mM Tris-HCl buffer (pH 8.0) before use for enzymatic determination of carnosine.

HPLC analysis of carnosine, anserine, and L-histidine in food extracts

The retention times for carnosine, anserine, and L-histidine were 9.9 min, 11.9 min, and 6.1 min, respectively. The linear range and correlation coefficient of the carnosine calibration curve were 0–0.5 mg/mL and 0.998, respectively. The linear range and correlation coefficient of the anserine calibration curve were 0–0.5 mg/mL and 0.997, respectively. The linear range and correlation coefficient of the L-histidine

calibration curve were 0–0.5 mg/mL and 0.997, respectively. Thus, the concentrations of carnosine, anserine, and L-histidine could be determined using the same HPLC conditions. The amounts of carnosine, anserine, and L-histidine present in foods (chicken, pork, beef, bonito, and tuna) determined using the conventional HPLC method are summarized in Table 1. The relative standard deviation (RSD) values of HPLC analysis of carnosine in chicken, pork, beef, bonito, and tuna were 1.97%, 0.728%, 1.47%, 5.76%, and 4.15%, respectively. RSD values of HPLC analysis of L-histidine in bonito and tuna were 1.13% and 2.38%, respectively.

Enzymatic determination of L-histidine and carnosine in food extracts

The linear range and correlation coefficient of the L-histidine calibration curve generated using HAL were 0–15.5 µg/mL and 0.999, respectively (Fig. 3). The limit of detection (LOD) and quantification (LOQ) of the enzymatic determination of L-histidine were 0.675 µg/mL and 2.14 µg/mL, respectively. The linear range and correlation coefficient of the carnosine calibration curve generated using BapA and HAL were 0–22.6 µg/mL and 0.999, respectively (Fig. 3). The LOD and LOQ of the enzymatic determination of carnosine were 0.844 µg/mL and 2.73 µg/mL, respectively. The amounts of L-histidine and carnosine in chicken, pork, beef, bonito, and tuna analyzed via enzymatic determination are summarized in Table 1. The results of the enzymatic determination of carnosine and L-histidine in food samples were in agreement with the results of conventional HPLC analysis. RSD values of the enzymatic determination of L-histidine using HAL in bonito and tuna were 1.79% and 1.50%, respectively. RSD values of the developed enzymatic determination of carnosine using BapA and HAL in chicken, pork, beef, bonito, and tuna were 1.52%, 1.17%, 0.504%, 4.58%, and 3.56%, respectively. The repeatability was considered satisfactory

since the RSD of the enzymatic determination process was below 5% and is comparable to that of HPLC analysis. The recovery of carnosine added to the food extracts was 97–103% (Table 2). Therefore, enzymatic determination using BapA and HAL can be used for determining L-histidine and carnosine in meats and fishes with comparable accuracy to that of conventional analysis using the HPLC method.

Anserine, ophidine, homocarnosine, and acetyl carnosine are carnosine derivatives. HPLC analysis indicated that BapA catalyzed the reaction of anserine to β -alanine and 1-methyl histidine; however, HAL did not metabolize 1-methyl histidine. Therefore, anserine was undetected by the developed enzymatic determination system. Previously, it was reported that L-histidine present in plasma and urine samples can be estimated using HAL, and the presence of histidine derivatives such as 1-methyl histidine and 3-methyl histidine do not affect the determination of L-histidine.²⁴ The determination of carnosine in food extracts using BapA and HAL was not affected by the carnosine derivative, anserine, present in food extracts. The reaction mixture containing 10 mM 3-methyl histidine, 0.1 M Tris-HCl buffer (pH 8.0), and 1.8 μ g/mL HAL was incubated at 30 °C, and an increase of absorbance at 277 nm of this reaction mixture was not observed. Thus, ophidine, which is composed of β -alanine and 3-methyl histidine, was not detected with the developed enzymatic method. Komeda *et al.* reported that homocarnosine was not hydrolyzed by BapA.²³ Therefore, homocarnosine cannot be detected using the developed enzymatic method. The reaction mixture containing 20 μ g/mL acetyl carnosine, 0.1 M Tris-HCl (pH 8.0), 2.0 mg/mL BapA, and 0.1 mg/mL HAL was incubated for 30 °C for 60 min. There was no increase in the absorbance of the mixture at 277 nm. Thus, acetyl carnosine cannot be detected using the developed enzymatic method. N-Terminal β -alanyl dipeptides are good substrates for BapA²³; thus, BapA probably distinguishes the β -alanine moiety of imidazole dipeptides. Moreover,

HAL is specific to L-histidine and its reaction is not interfered with histidine analog, such as 1-methyl histidine and 3-methyl histidine. Because two kinds of enzymes, which act on different substrates, were used in the developed enzymatic method, carnosine in food extracts was determined with a high accuracy. Zn^{2+} strongly inhibited the BapA-catalyzed reaction and Co^{2+} , Ni^{2+} , and L-cysteine strongly inhibited the HAL-catalyzed reaction. Moreover, it was reported that 4-fluorohistidine and 4-nitrohistidine are poor substrates and competitive inhibitors of HAL.²⁸ Therefore, the developed method may not be applicable for food samples containing a large amount of these compounds. HPLC analysis requires filtration of the sample before injection, whereas pretreatment of the sample is not required for the developed enzymatic method. The developed method is easy to operate and does not involve disposal of fluid waste such as acetonitrile which is used as a mobile phase in HPLC analysis. The developed method may not only be used for carnosine analysis in foods, but also in quality management for dietary supplements containing carnosine. Further, the developed enzymatic method may be applied in a microplate-based assay and is suitable for high-throughput analysis.

Conflicts of interest

The authors declare no conflicts of interest.

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Supporting Information: Enzymological properties of BapA and HAL. This material

is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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Table 1 HPLC determination and enzymatic determination of the amount of carnosine, anserine, and L-histidine in food

Foods	HPLC determination			Enzymatic determination	
	Carnosine/ mg 100 g ⁻¹	Anserine/ mg 100 g ⁻¹	L-Histidine/ mg 100 g ⁻¹	Carnosine/ mg 100 g ⁻¹	L-Histidine/ mg 100 g ⁻¹
Chicken	251 ± 5	630 ± 20	N. D. ^a	258 ± 4	N. D. ^a
Pork	320 ± 2	N. D. ^a	N. D. ^a	317 ± 4	N. D. ^a
Beef	390 ± 6	96.0 ± 2.6	N. D. ^a	385 ± 2	N. D. ^a
Bonito	46.5 ± 2.7	253 ± 1	1,660 ± 20	47.8 ± 2.2	1,650 ± 30
Tuna	175 ± 7	741 ± 12	945 ± 23	178 ± 6	956 ± 14

All experiments were performed in triplicate and all data are expressed as the mean ± standard deviation.

HPLC, high-performance liquid chromatography.

a. Not detected.

Table 2 Recovery of carnosine added to the food extracts

Foods	Concentration added/ $\mu\text{g mL}^{-1}$	Concentration present/ $\mu\text{g mL}^{-1}$	Concentration found/ $\mu\text{g mL}^{-1}$	Recovery, %
Chicken	0	6.18	—	—
	4.52	10.7	10.6 ± 0.3	99.4 ± 2.4
	9.04	15.1	14.6 ± 0.4	97.0 ± 2.4
Pork	0	8.48	—	—
	4.52	13.0	13.2 ± 0.4	102 ± 3
	9.04	17.5	17.3 ± 0.1	98.9 ± 0.5
Beef	0	9.28	—	—
	4.52	13.8	13.6 ± 0.2	98.4 ± 1.3
	9.04	18.3	18.2 ± 0.4	99.2 ± 1.9
Bonito	0	0.0900	—	—
	4.52	4.61	4.74 ± 0.27	103 ± 5
	9.04	9.13	9.20 ± 0.02	101 ± 1
Tuna	0	0.380	—	—
	4.52	4.90	4.94 ± 0.05	101 ± 2
	9.04	9.43	9.30 ± 0.13	98.6 ± 2.4

Concentration of carnosine in the food extracts was determined via high-performance liquid chromatography analysis. Authentic carnosine was added to each diluted food extract and analysis of carnosine was performed via enzymatic determination. All experiments were performed in triplicate and all data are expressed as the mean $\pm$ standard deviation ($n=3$).

Figure Captions

Fig. 1 Structures of imidazole dipeptides.

Fig. 2 Enzymatic reactions catalyzed by BapA and HAL. BapA, β -Ala-Xaa dipeptidase; HAL, histidine ammonia-lyase.

Fig. 3 Standard curves for carnosine and L-histidine obtained via enzymatic determination. The standard curve of carnosine was generated for a 1 mL mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 2.0 mg/mL BapA, 0.1 mg/mL HAL, and 0–22.6 μ g/mL carnosine. The standard curve of L-histidine was generated for a 1 mL mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 0.1 mg/mL HAL, and 0–15.5 μ g/mL L-histidine. All experiments were performed in triplicate and all data are expressed as the mean $\pm$ standard deviation. BapA, β -Ala-Xaa dipeptidase; HAL, histidine ammonia-lyase.

Fig. 1

Fig. 2

Fig. 3

Graphical Index