Novel Brothy Taste Modifier Isolated from Beef Broth

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The essential components which impart the favorable "brothy taste" as the characteristic flavor of beef bouillon were investigated. Broth prepared from beef was performed successively by dialysis, electrodialysis, gel filtration chromatography, chelate affinity chromatography, and carbon partition chromatography, and finally three fractions which gave the "brothy taste" were obtained. Among these, one fraction, *A8,* contained the component responsible for this taste in highest purity. Structural analysis was carried out using FAB-MS and various NMR methods, and the main compound of *A8* was thus elucidated to be the novel compound *N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine.

Keywords: *Beef; extract; sour; brothy; taste; amino acid derivative*

INTRODUCTION

Beef bouillon is widely used as a base for various dishes and processed foods due to its flavor. The flavor is thought to be the result of complex sensations arising from taste and aroma. Many investigations have been carried out on the aroma of beef bouillon, and many components responsible for the aroma have been identified along with their mechanisms of formation (Gasser and Grosch, 1990; Farmer and Patterson, 1991; Cerny and Grosch, 1992, 1993; Madruga and Mottram, 1995). On the other hand, the components which impart the brothy taste have not been determined much, with the exception of "the delicious peptide" that has the primary structure Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, isolated from beef extract digested with papain (Yamasaki and Maekawa, 1978, 1980), and several other peptides (Spanier and Edwards, 1987; Nishimura et al., 1988; Spainer et al., 1990; Okitani et al., 1992; Ishii et al., 1995a,b; Tamura et al., 1997). Moreover, these peptides were found to be formed by enzyme processing or aging during storage, so almost no investigations have been performed on the components formed during cooking. Furthermore, many studies have been carried out on "the delicious peptide" (Tamura et al., 1989; Jorba et al., 1995; Wang et al., 1995a,b), but van Wassenaar (Wassenaar et al., 1995) and Hau (Hau et al., 1997) showed that this neither has flavoring properties nor occurs naturally.

Moreover, the taste qualities of the nonvolatile components of bouillon by chemical analysis were estimated (Warendorf et al., 1992). We prepared beef broth and estimated its characteristic taste. Our results indicated that the taste of broth could not be reproduced through the compounds already identified only. Therefore, we isolated and performed structural analysis of the component which imparts this brothy taste.

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MATERIALS AND METHODS

Preparation of Beef Broth. Six kilograms of beef shank on the market was cut into pieces of 5−10 cm, charged into a 30 L cylindrical aluminum pot, and cooked at 90−95 °C for 7 h with the addition of 8 L of water to obtain approximately 5 L of broth. The beef and precipitate formed were removed from the broth, and the residual extract was then refrigerated at 4 °C overnight. The fats and precipitate formed during refrigeration were further removed through a 60-mesh sieve. Further, the fine precipitate and solids were removed by refrigerated centrifugation (3000*g* × 10 min). The brown transparent supernatant obtained by centrifugation was lyophilized.

Analysis of Components. The components in beef broth were determined by standard methods as follows. The contents of amino acids and carnosine were determined with an amino acid analyzer (Hitachi L-8500 system). Nucleotides were analyzed by HPLC equipped with a Hitachi #3013 column with detection at 254 nm. Creatine and creatinine were determined by the Jaffé reaction (Jaffé, 1886). Sugars were analyzed by HPLC equipped with an anion exchange column with detection at 425 nm after coloring with the orcinol−sulfuric acid reagent (Georg and Christian, 1986). Organic acids were determined with a carbonic acid analyzer (Tokyo Rika S-3000 system), equipped with an anion exchange column with detection at 530 nm after coloring with a solution comprised of EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride), ONPH (2-nitrophenyl hydrazine), and NaOH. Sodium, potassium, magnesium, and calcium were determined with an atomic absorption spectrometer (Seiko Instruments Inc., SAS 760 system). Phosphate was determined by HPLC equipped with an anion exchange column with detection by electric conductivity.

Preparation of the Mixed Reference Solution. The mixed reference solution was prepared from 36 chemicals on the basis of these analytical data, coinciding with the concentration of beef broth. The pH was adjusted to 6.0 with 1 N NaOH. The composition of the mixed reference solution is shown in Table 1.

Sensory Evaluation of Beef Broth and Mixed Reference Solution. The tastes of both solutions were compared at room temperature (20 to ∼25 °C) by 6 panelists that studied condiment in our institute. Cups containing around 50 mL of the broth or the reference solution were presented, and panelists noted the taste characteristics of each, and the language that could have the taste focused attention on in common was selected from mentions of panelists.

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Table 1. Composition of the Mixed Reference Solution Simulating Beef Broth (mg/100 mL)

amino acid		sugar		salt	
threonine	6.8	ribose	0.4	NaCl	85.4
serine	3.8	mannose	0.8	KCl	394.0
glutamic acid·Na·H_2O	6.4	fructose	2.0	$MgCl_2·6H_2O$	110.4
proline	1.6	glucose	4.0	$CaCl_2·2H_2O$	1.4
glycine	5.6				
alanine	19.2				
valine	3.4	nucleotide		others	
methionine	2.0	IMP·2Na·$5H_2O$	42.4	H_3PO_4	155.8
isoleucine	1.6	GMP·2Na·$7H_2O$	0.8	carnosine	91.2
leucine	2.8	AMP	6.0	creatine	110.6
tyrosine	3.8			creatinine	57.2
phenylalanine	2.6				
lysine·HCl	4.4	carbonic acid			
histidine	3.8	pyroglutamic acid	129.6		
arginine	3.2	lactic acid (90% w/v)	356.7		
taurine	24.8	succinic acid	9.8		

Isolation of the Brothy Taste Compound. The selection of fractions giving the brothy taste after each operation was carried out by sensory evaluation. The lyophilized powder of the broth was dissolved in water to a concentration of 8 g/100 mL, and this, contained in cellulose tubes, was dialyzed against distilled water. The dialysis was judged to be complete when no trace of color was visible outside the tube after dialysis against freshwater; this took about 3 days. The diffusates obtained were combined and concentrated in a rotary evaporator at 40 °C. The dialysate (the low molecular weight fraction solution) thus obtained was treated with an electrodialysis membrane (Microdialyzer G3 manufactured by Asahi Chemical Industry Co., Ltd.; membrane pore diameter molecular weight = approximately 1000), and inner (more than MW1000) and outer (less than MW1000) solutions were obtained. Further, the outer solution thus obtained (less than MW1000) was treated with an electrodialysis membrane (membrane pore diameter molecular weight = approximately 100) to obtain the inner solution (MW100 to 1000) and outer solution (less than MW100). On treatment with the electrodialysis membrane, the point at which the conductivity and the current value of the dialysate became zero was defined as the final point. The resulting solutions obtained by electrodialysis were evaluated by dissolving in distilled water after lyophilization.

Fractions of beef broth were subjected to various procedures to collect the substance conferring the brothy taste. The fraction obtained with electrodialysis was divided into five peaks from *P1* to *P5* by gel filtration chromatography (HPLC, Asahipak GS-320 column, 21.5 × 300 mm, manufactured by Showa Denko Co., Ltd.; eluent, 0.1 M ammonium acetate; detection, UV214 nm; flow rate, 5.0 mL/min). The fractions obtained were evaluated by dissolving in distilled water after removing ammonium acetate by vacuum concentration and lyophilization repeatedly. The fraction obtained by gel filtration chromatography was separated into four frac-tions from *A* to *D* by affinity chromatography (HPLC, TSKgel Chelate 5PW column, 21.5 × 150 mm, manufactured by Tosoh Co., Ltd.; eluent, 50 mM phosphate buffer + 500 mM sodium chloride, pH 8.0, to 50 mM phosphate buffer + 500 mM sodium chloride, pH 3.0, gradient; detection, UV214 nm; flow rate, 5.0 mL/min). The fractions obtained were evaluated by dissolving in the reference solution after removing phosphate and sodium chloride by electrodialysis and lyophilized repeatedly. The fraction obtained by affinity chromatography was divided into 14 peaks from *A1* to *A14* by partition chromatography (HPLC, TSKgel Carbon 500 column, 21.5 × 150 mm, manufactured by Tosoh Co., Ltd.; eluent, 0.05% TFA in water → 0.05% TFA in 50% acetonitrile, gradient; detection, UV214 nm; flow rate, 5.0 mL/min). The fractions obtained by partition chromatography were evaluated by dissolving in the reference solution after removing TFA and acetonitrile by vacuum concentration and lyophilization repeatedly. The evaluation solutions were prepared so that the concentrations of each sample were equivalent to those in the original broth by means of estimating from the amount and the concentrations of a sample used with each fractionation roughly.

The purity of the collected substance was measured by reverse-phase chromatography (HPLC, Ultron VX-ODS column, 4.6 × 250 mm, manufactured by Shinwa Kako K. K.; eluent, 0.05% TFA in water → 0.05% TFA in 50% acetonitrile, gradient; detection, UV214 nm; flow rate, 0.8 mL/min). The substance was found to be a single molecular species.

Mass and NMR Spectral Analyses. FAB-MS spectra were obtained on a JMS-HX110/HX110 tandem mass spectrometer (manufactured by JEOL Co., Ltd.), using xenon (6 keV, 20 mA) for ionization and glycerol and thioglycerol as the matrix, with low-resolution scan (2000). HRFAB-MS spectra were obtained with the same system using poly(ethylene glycol) 200 as the matrix with a high-resolution scan (15 000). 600 MHz 1H and 150 MHz ^{13}C NMR spectra were recorded on a Bruker AMX-600 spectrometer using D_2O as the solvent. Chemical shifts were expressed in parts per million (δ) with TMS as a reference. Resonance assignments together with structural information were obtained using the ^1H-detected heteronuclear multiple-bond correlation (HMBC) technique (Kogler et al., 1983; Bax and Marion, 1988) which gives long-range coupling correlations between ^{13}C and ^1H atoms through two to four chemical bonds: aquisition time in the t2 dimension, 102 ms; sine bell filter in t2; 60° shifted sine bell in t1, $\Delta1 = 0$, $\Delta2 = 33$ ms; recycle delay1 s. ^1H and ^{13}C chemical shifts were expressed in parts per million (ppm; δ) with internal DSS and external TMS as the respective references.

RESULTS AND DISCUSSION

Evaluation of Beef Broth and Mixed Reference Solution. Extraction yielded 147 g of dried beef broth from 6 kg of beef shank. The taste characteristics of the mixed reference solution constructed with the analyzed components were compared with those of the broth by sensory evaluation. The taste of the broth was rich and complex. On the other hand, the mixed reference solution had a taste centering around saltiness and umami, and was not complex. Furthermore, there was a large difference with respect to the sour taste in both. Although the reference solution did not have any sour taste, the broth had the characteristic sour taste and thickness. Generally, organic and inorganic acids (for example, citric acid, succinic acid, lactic acid, and phosphoric acid) have a sour taste in the acid pH region, and sharply stimulate the tongue. However, these acids do not exhibit this taste at neutral pH. As the pH of beef broth was around 6, the sour taste was not attributable to organic or inorganic acids and was thought to be due to an unknown compound.

This taste is one of the characteristic flavors of beef bouillon and was defined here as the "brothy taste." The component which imparted this taste was isolated and analyzed.

Isolation of the Component Which Gave the "Brothy Taste". The purification procedure is shown in Figure 1. The low molecular weight fraction solution prepared by dialysis exhibited the "brothy taste." The fractions which gave the "brothy taste" were selected at each procedure by means of sensory evaluation as follows: MW100 to 1000 by electrodialysis, *P1* by gel filtration chromatography, *A* by affinity chromatography, and *A5*, *A6*, and *A8* by partition chromatography (Figure 2).

Examination of the Purity of the Components Which Gave the "Brothy Taste". *A5*, *A6*, and *A8* were applied to an ULTRON VX-ODS column to examine the purity of their components. Chromatograms are shown in Figure 3. Many components were still mixed

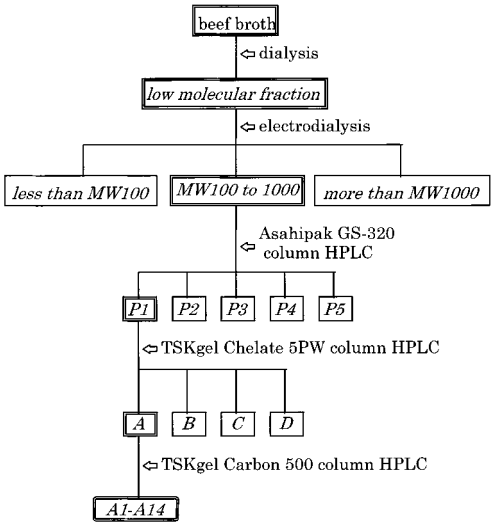

Figure 1. Purification procedure of fractions which gave the "brothy taste" from beef broth.

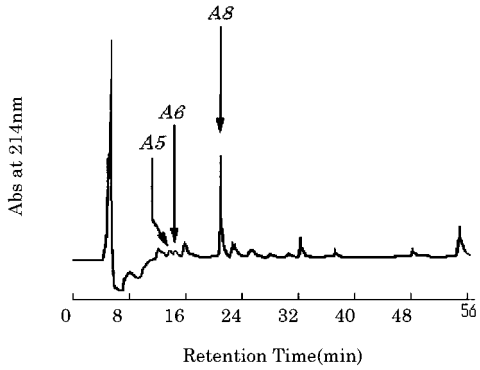

Figure 2. Separation of fraction *A* by Carbon-HPLC. Fraction *A* was separated by Carbon-HPLC with a linear gradient of acetonitrile in 60 min from 0 to 50% in 0.05% TFA at flow rate of 5.0 mL/min.

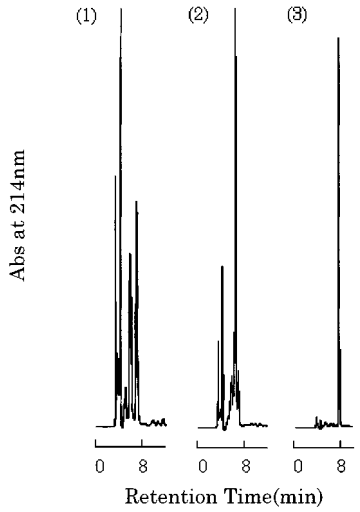

Figure 3. HPLC analysis of three active fractions obtained from fraction *A*. Three fractions were separated by RP-HPLC using an Ultron VX-ODS column with a linear gradient of acetonitrile in 10 min from 0 to 10% in 0.05% TFA at a flow rate of 0.8 mL/min. (1) *A5*; (2) *A6*; (3) *A8*.

in *A5* and *A6*, but *A8* contained only a single component. In comparison with *A5* and *A6*, *A8* contained a large amount of the component, so structural analysis of the main compound of *A8* was carried out.

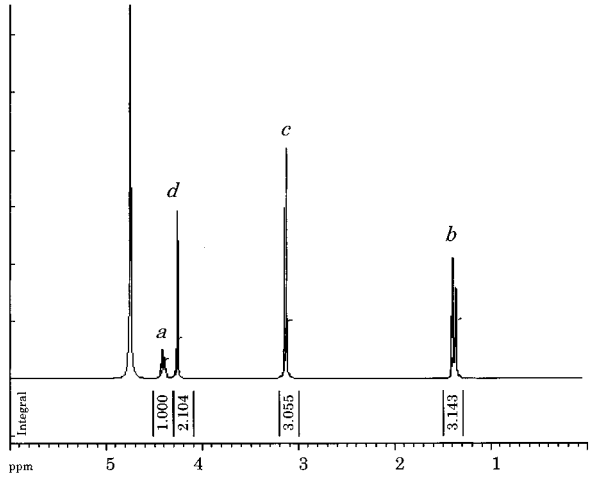

Figure 4. ^{1}H NMR spectrum for the main compound of *A8*.

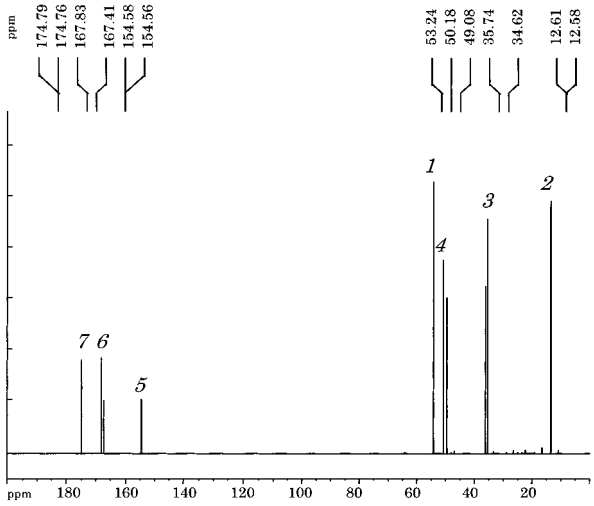

Figure 5. ^{13}C NMR spectrum for the main compound of *A8*.

Structural Analysis of *A8*. Amino acid analysis showed the main compound of *A8* to be a ninhydrin-positive compound, which showed absorption at 440 nm. Amino acid analysis of the hydrolysate of this showed alanine and 1-methylglycine (sarcosine). A peptide sequencer (Shimadzu Corp. Co., Ltd.) was used for the analysis of the amino acid sequence of this compound, but the sequence could not be determined. Moreover, sarcosine does not exist in native proteins. Therefore, this compound was thought to be formed by a nonenzymatic reaction on heating. The peak of MH^{+} was detected at 186.1 in *m/z* by FAB-MS. Therefore, the molecular weight of this compound was determined to be 185 amu. HRFAB-MS was carried out to estimate its elemental composition. A peak was detected at 186.0905 in *m/z*, and the empirical formula was calculated as $C_7H_{11}O_3N_3$.

The results of ^{1}H NMR (Figure 4) and ^{13}C NMR (Figure 5) indicated that this compound contained two types of components, one of which showed superior peak intensities as compared with the other. Moreover, as the numbers of peaks were the same, it was suggested these were structural isomers.

The following structural elements were found in the ^{1}H NMR spectrum: $-CH-CH_3$, $-N-CH_3$, $-CH_2-$. The following carbon signals were also detected: methylene (C^1) at 53.2 ppm, methyl (C^2) at 12.6 ppm, methyl (C^3) at 35 ppm, methylene (C^4) at 50 ppm, and three quaternary carbons (C^5, C^6, C^7) at 154.6, 167.6, and

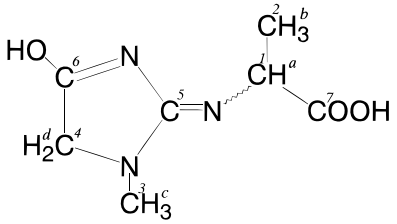

Figure 6. Identified structure of the main compound of *A8*.

174.8 ppm. The signal of C^7 should be assigned to a carboxyl group.

Furthermore, judging from the heteronuclear multiple-bond correlation (HMBC) results, C^5 was attached to a nitrogen situated next to C^3, and C^6 was C=O or =C−OH due to the signal at 167.6 ppm, and was connected to C^4. The remaining two nitrogens were both attached between C^5 and C^6 and between C^1 and C^5. Finally, the structure of this compound was concluded to be *N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine (Figure 6). The structure shown Figure 6 was of the enol type, but it was thought that this was a tautomer, and that the structure of the keto type also existed. Moreover, the above-mentioned isomers were considered to be due to syn-anti isomerism at the $N-C^1$ bond.

This structure estimated by NMR has the frames of alanine and creatinine, and the elemental composition was consistent with the HRFAB-MS results. Furthermore, it is reasonable that this compound could be formed through dehydration between creatinine and lactic acid or deamination between creatinine and alanine upon condensation by heating because these components are abundant in the broth.

Several investigations of the "delicious peptide" and oligopeptides have been carried out in the searches for the compounds responsible for the tastes of beef broth. So far, these studies have been performed on umami-like taste or an ambiguous taste represented only by the "meaty taste," but there have been no studies of the sour taste of beef broth. Moreover, the tastes have been revealed to be mainly due to peptides and not a non-peptide-type compound. Furthermore, there have been few studies of the taste-related materials formed on heating. In this study, we successfully found a novel compound by the investigation of different aspects from previous studies as mentioned above. However, we only show the possibility of the existence of the novel compound in beef broth in this study, and we have confirmed both its structure and its taste characteristics by synthesis.

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