Taste of "Delicious" Beefy Meaty Peptide. Revised

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An octapeptide, previously isolated and named by others as beefy meaty peptide and described as a beef flavor enhancer having a delicious taste, was synthesized. Using Fmoc-amino acids and HBTU reagent, the peptide was synthesized and recovered in high yield. The peptide was characterized by biochemical methods and evaluated for its alleged umami taste properties similar to the taste of monosodium glutamate (MSG). The peptide was shown to be homogeneous by high-performance chromatography, amino acid composition analysis, and amino acid sequence analysis. Electrospray mass spectrometry confirmed the sequence analysis. Stability of the peptide in solution was also evaluated. Taste evaluation by a trained flavor panel showed that the synthesized octapeptide and some peptide fragments did not have any umami or other taste. Suggestions are given to explain the observed difference between our results and those reported by others.

INTRODUCTION

In 1978 Yamasaki and Maekawa (1978) reported the isolation of a "delicious" peptide from beef broth. It was proposed that it was an octapeptide, having the primary structure Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala. The synthesis of this peptide was reported later (Yamasaki, 1980), and the taste of the "beefy meaty peptide" (BMP) was then described as meaty; others described BMP as tasting like beef soup, savory, or umami (Tamura et al., 1989; Kuramitsu et al., 1993; Spanier and Miller, 1993), similar to the taste of monosodium glutamate (MSG). The threshold concentration for BMP was reported to be 1.41 mM, while that for MSG is 1.56 mM (Tamura et al., 1989). The history of BMP has been reviewed recently by Spanier and Miller (1993), while its synthesis has also been discussed in detail at a recent symposium (Kuramitsu et al., 1993). Umami may be considered as a basic taste sensation, the fifth one besides the classic four of salt, sweet, sour, and bitter (Yamaguchi, 1987; Brand and Bryant, 1994). Compounds that arouse the umami sensation are the well-known flavor potentiators monosodium L-glutamate and the ribonucleotides inosine 5'-monophosphate and guanosine 5'-monophosphate. Quite some information is available with regard to the structure–flavor relationships of these purine ribonucleoside monophosphates (Maga, 1983). With regard to monosodium L-glutamate, such a relationship has been studied in little detail and the results are fragmented, but the published data show a high specificity for the umami effect triggered by this amino acid. First of all, glutamic acid is the only amino acid that can arouse a umami effect (Brand and Bryant, 1994). It is the L-glutamic acid that produces the effect, whereas the D-form does not, nor does L-glutamine (Maga, 1983). Moreover, the electronic configuration (dissociation form) of L-glutamic acid in solution deter-

mines the umami effect, which supports the specificity of the effect. Compounds having some structural relationship with glutamic acid, e.g. ibotenic acid and tricholomic acid, are said to give a umami sensation (Maga, 1983). Further, several amino acid derivatives, especially derivatives of L-glutamic acid, were reported to give a flavor sensation similar to that of L-glutamic acid itself; however, the effect was never stronger (Maga, 1983). Other derivatives of glutamic acid that were tested had lost the ability to give a umami effect. The taste sensation given by L-glutamyl oligopeptides has also been reported (Arai et al., 1973). The perceived tastes were flat, bitter, sweet, sour, and brothy (Arai et al., 1973; Maga, 1983). The taste of some of these small peptides has also been described as MSG-like by other authors (Noguchi et al., 1975). Hence there seems to be some confusion in describing the nature of the perceived taste. We therefore were interested in the taste sensation given by the BMP. Confirmation of the reported effects might help in our understanding of the intriguing structure–activity relationship for the umami effect. The present work was prompted by the availability of novel methods for efficient solid phase peptide synthesis (Fields et al., 1991). This technique was applied to the preparation of a quantity of the "delicious" octapeptide, sufficient for sensory evaluation.

BMP is composed of eight amino acids in the sequence Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala and has a calculated monoisotopic molecular mass of 847.39 Da. Because of this composition, it has a calculated acidic pI (3.7) and shows only UV absorbance near 200–220 nm due to peptide bonds. The peptide has a charged, polar N-terminal part and a neutral, apolar C-terminal part. The calculated hydrophobicity is 790 kcal mol^{-1}. In the pH region (5–7) where taste evaluation is performed, the peptide has an overall negative charge. The octapeptide is also available commercially from Peninsula Laboratories, Inc., Belmont, CA. We purchased BMP from this supplier for organoleptic testing (product no. 8769).

MATERIALS AND METHODS

Synthesis of BMP. *Materials.* N-Methylpyrrolidone (NMP, peptide synthesis grade), piperidine, and 2-(1H-benzotriazol-

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1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Perkin-Elmer (Warrington, U.K.). Diisopropylethylamine (DIEA), trifluoroacetic acid, and diethyl ether were of pro-analysis grade and were purchased from Merck (Darmstadt, Germany); diethyl ether was purified over a column of activated basic aluminum oxide, and DIEA was distilled twice over ninhydrin and potassium hydroxide before use. Fluoren-9-ylmethoxycarbonyl (Fmoc) amino acids and Fmoc-Ala-HMP resin (hydroxymethylphenoxymethyl resin; Wang, 1973) were purchased form Saxon Biochemicals GmbH (Hannover, Germany).

Peptide Synthesis and Purification. The synthesis was carried out in a 50 mL glass vessel with a glass filter at the bottom. Washings, Fmoc deprotection, and coupling steps were performed by hand. After 2 h of swelling in NMP, Fmoc-Ala-HMP resin (0.54 mequiv/g, 2.25 g) was washed with NMP. Fmoc-Leu, Fmoc-Ser(tBu), Fmoc-Glu(OtBu), Fmoc-Glu(OtBu), Fmoc-Asp(OtBu), Fmoc-Gly, and Fmoc-Lys(Boc) were successively coupled using the following coupling cycles: 1, NMP wash (2 × 2 min); 2, 30% (v/v) piperidine/NMP (2 min); 3, 30% (v/v) piperidine/NMP (20 min); 4, NMP wash (5 × 2 min); 5, coupling step, 60 min); 6, NMP wash (2 × 2 min).

(a) Coupling Step (Fields et al., 1991). Fmoc-amino acid (4 mmol) was dissolved in 10 mL of NMP, activated with 4 mmol of 0.45 M HBTU/0.45 M 1-hydroxybenzotriazole (HOBt) in DMF, and 8 mmol of 2 M DIEA in NMP, and transferred into the reaction vessel. The vessel was shaken for 60 min.

After cleavage of the last Fmoc group with piperidine, the resin was washed with NMP (two times) and ethanol (four times) and then carefully dried under vacuum for a yield of 3.16 g (96%). The peptide was cleaved and deprotected by stirring the peptide resin with a mixture of 30 mL of TFA and 1.5 mL of water for 1.5 h. The mixture was filtered and the resin washed twice with 3 mL of TFA. The combined filtrates were mixed directly with 150 mL of cold ether. The peptide was allowed to precipitate completely in 2 h at −20 °C and was collected by centrifugation. The product was again washed with cold ether and collected by centrifugation twice and then thoroughly dried. The crude product was then dissolved in 50% acetonitrile/water and lyophilized for a yield of 1.36 g (133%, based on starting resin).

The purification was carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cartridges plus a guard cartridge (40 mm × 210 mm) filled with Delta-Pak C$_{18}$ (15 µm, 100 Å) material. The peptides were detected at 215 nm using a Waters 486 spectrophotometer with a preparative cell. About 335 mg of crude peptide in 3 mL of water and a few drops of trifluoroacetic acid was injected and run in a linear gradient from 4% B to 13% B in 30 min at a flow rate of 100 mL/min (eluent A, water with 0.1% TFA; eluent B, acetonitrile with 0.1% TFA). The main fraction was collected after about 15 min and lyophilized four times from water for a yield in four runs of 875 mg (86%, based on the starting resin and not corrected for peptide content).

Characterization of Synthesized Beefy Meaty Peptide. *High-Performance Liquid Chromatography.* For analytical HPLC, we used two Waters Model 510 pumps, a Waters Model 680 gradient controller, a Waters Model WISP 712 autoinjector, and a Waters Model 911 photodiode array detector. The product was analyzed in a linear gradient from water with 0.1% TFA to 60% acetonitrile in water with 0.1% TFA in 60 min on a Waters-Delta Pak C$_{18}$ 100 Å (3.9 × 150 mm, 5 µm) column at 1 mL/min.

Amino Acid Analysis. Determination of the amino acid composition of the peptide was achieved after hydrolysis in a Pico-Tag work station using 6 N HCl at 150 °C for 1 h. The analysis was run after derivatization with phenyl isothiocyanate (PITC) using the Waters Pico-Tag system.

Amino Acid Sequence Analysis. Determination of the amino acid sequence of BMP and peptides was carried out using an Applied Biosystems Model 470A sequencer equipped with an on-line Model 120A PTH analyzer. A 1 nmol solution of the peptide in distilled water was used for sequence analysis of BMP.

Mass Spectrometry. Molecular mass determination was performed on a VG-Trio-3 triple quad mass spectrometer equipped with a microflow ESP source. The scan range was 100−1500 Da at a rate of 10 s scan^{-1}. The sample was dissolved in water/methanol (1:1 v/v) at a concentration of 10 pmol L^{-1}. The injection volume was 10 µL. Peptides recovered from an enzymatic digest were characterized by detection of the molecular masses by MALDI-TOF (Fisons TofSpec) using α-cyano-4-hydroxy-cinnamic acid as matrix and FAB-MS−MS (VG-Trio-3) using glycerol/thioglycerol (75/25) as matrix.

Enzymatic Hydrolysis. An enzymatic digest of BMP was prepared by incubation of BMP with a proteolytic enzyme EndoGlu (Boehringer, Mannheim, Germany) that cleaves after glutamic acid (E) and/or aspartic acid (D). Incubation was done at 37 °C overnight using 0.1 M ammonium bicarbonate buffer, pH 8.0, at a substrate/enzyme ratio of 100:1. After incubation, the material was lyophilized and analyzed by RP-HPLC using a Vydac column (218TP5415). Separation was achieved using a linear gradient of acetonitrile in water containing 0.06% trifluoroacetic acid. Detection was done at 214 nm, and peaks were collected for further characterization by N-terminal sequence analysis and mass spectrometry.

Stability in Solution. Aqueous solutions of BMP at a concentration of 4 mM at pH 4−7 were incubated at 37 °C. During the incubation time, samples were taken and characterized by RP-HPLC as described for the enzymatic hydrolysis.

Taste Evaluation. The octapeptides were dissolved in purified water (Milli-Q, Millipore), and the pH, originally being 3.40, was raised to 6.70 with NaOH (0.5 N solution). The peptide resulting from our own synthesis was offered to a panel at concentrations (corrected for the peptide content) of 2.67 and 5.34 mM, well above the reported threshold level (1.41 mM). This peptide was also offered to the panel at 2.67 mM at pH 3.40. The panel consisted of eight persons, six males (five being 27−31 years old, one being 55 years old) and two females (25 and 28 years old). Seven of them were members of the Flavour Department of the Unilever Research Laboratory, experienced in organoleptic testing of flavors in model systems and foods and selected for their ability to evaluate and describe taste. One panel member (male, 31 years) was Japanese, visiting our laboratory for training purposes. The octapeptide bought from Peninsula Laboratories was offered to the three most experienced tasters from the panel at 2.67 mM, both at pH 3.40 and at pH 6.70. In all taste sessions 2.67 and 5.34 mM solutions of L-glutamic acid made up in Milli-Q water, at both pH 3.40 and 6.70, were also offered to the panel. Similar solutions were prepared from monosodium L-glutamate. In between the tastings sufficiently long pauses (>3 min) were taken to neutralize any taste effect in the mouth. The panelists were offered purified water to rinse their mouths and to "taste" the control solution. All solutions were prepared and offered at room temperature.

RESULTS AND DISCUSSION

Peptide Synthesis and Characterization. As can be seen from the yield data on synthesis, the recovery of the product amounts to 86%. From this and the data from the product characterization it can be concluded that synthesis of BMP was very efficient and successful.

HPLC. RP-HPLC analysis of the purified BMP shows that it is a single peak with a retention time of 17 min, having a purity of >98% as estimated from the UV trace at 215 nm (see Figure 1). The UV spectrum of the peptide shows the typical peptide bond absorption at the lower nanometer range and the absence of aromatic residues at 280 nm.

Amino Acid Analysis. The results of the amino acid analysis after hydrolysis of the material are given in Table 1. From these data it can be deduced that the composition is in full agreement with the expected data of BMP. The peptide content in the product is about 70% on the basis of this analysis, the remaining being moisture.
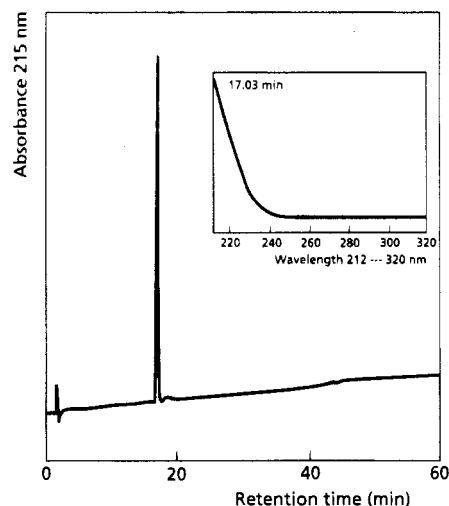
Figure 1. HPLC trace of synthesized and purified BMP.

Table 1. Amino Acid Analysis[a] of Synthesized BMP

amino acid	observed value, residues/mol	expected value, residues/mol
Asp	0.94	1
Glu	2.13	2
Ser	0.89	1
Gly	1.03	1
Ala	1.04	1
Leu	0.98	1
Lys	0.99	1

[a] No other amino acids could be observed in the hydrolysate.

Table 2. Amino Acid Sequence Determination[a] of Synthesized BMP

Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala

[a] Initial sequencing yield: 43%.

Amino Acid Sequence Analysis. The results of the amino acid sequence analysis of BMP are given in Table 2. The observed sequence is in full agreement with the expected sequence of BMP. No contaminating signals of any other amino acids were detected.

Mass Spectrometry. The theoretical molecular mass of the peptide of 847.39 Da was confirmed by electrospray mass spectrometry because of the presence of the $[M + H]^+$ ion m/z 848.36. Two fragment ions, m/z 759 and 646, represent the Y'''7 and Y''6 C-terminal fragment ions, respectively. The spectrum is displayed in Figure 2.

Enzymatic Hydrolysis. RP-HPLC analysis of the endo-Glu hydrolysate of BMP yielded three components that were characterized by N-terminal sequence analysis and mass spectrometry and appeared to be the peptides KGDEE, SLA, and KGDEESLA, respectively.

Stability in Solution. To establish that BMP in solution does not degrade into smaller peptides (due to, e.g., cleavage at the somewhat labile Asp-Glu peptide bond), which could be responsible for any taste during the taste evaluation, analysis by RP-HPLC was carried out. During a 24 h incubation period at any pH, no degradation of BMP was observed.

Taste Evaluation. All panel members could perceive and describe the typical taste sensation brought about by L-glutamic acid at pH 6.70, at a concentration as low as 2.67 mM. At 5.34 mM the taste was more intense. MSG at the same pH and concentrations gave the same sensation to the panelists. The solutions of L-glutamic acid and MSG at pH 3.40 were described as sour. The solutions of the octapeptides at pH 6.70 were described
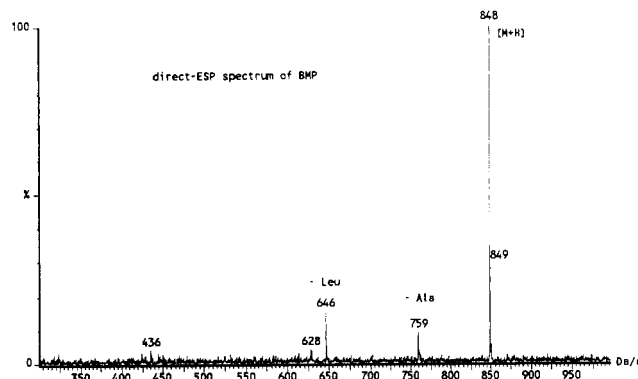

Figure 2. ES spectrum of synthesized and purified BMP.

as neutral, with no reminiscence of umami or broth at all. None of the panel members perceived saltiness. Two panel members noted a "slightly musty" taste. The octapeptide from Peninsula Laboratories was found to have a slight off-flavor, which could be traced back to the rubber stopper of the glass packaging. At pH 3.40 both BMP solutions were described as slightly acidic.

There was a high degree of agreement among the panel members in their description of the perceived tastes. The umami perception of the Japanese panel member was in no way different from that of the Dutch and German panel members. The smaller peptides released from the octapeptide by treatment with endo-Glu protease also failed to give any taste sensation when tested above the threshold concentration given for the octapeptide. Additional experiments using synthesized peptides SLA and KG, tasted as such and in mixtures with BMP, well above the claimed levels, also failed to give any taste sensation (Kuramitsu et al., 1993).

Discussion. Peptides having a pronounced taste are well-known; e.g., bitter peptides are notorious in cheese, and monellin is intensely sweet, as is aspartame (Asp-Phe methyl ester). The existence of a peptide with an interesting "delicious" taste thus seemed to be a real possibility. Its isolation and synthesis have been well documented. However, in the description of the tastes of small peptides there is quite some confusion. In view of the specificity of the umami effect of L-glutamic acid, the existence of umami peptides should be approached very carefully. On the other hand, ribonucleotides having a structure quite different from that of glutamate also provide a umami sensation. Nevertheless, the description of the beefy meaty octapeptide as being umami would need confirmation. The alleged existence of salty peptides (Tada et al., 1984) could not be confirmed (Huynh-ba and Philippossian, 1987), emphasizing this need. Having the ability to prepare BMP on a gram scale, we therefore considered it worthwhile to re-evaluate the taste of the beefy meaty peptide. Yamasaki and Maekawa (1980) used mixed anhydrides from Boc-amino acids for their solution synthesis of BMP. In the last step they coupled Lys-Gly as protected segment. Elemental analysis of the protected peptide shows the expected values; however, amino acid analysis of the deprotected peptide shows a very low lysine content (0.56). The octapeptide was identical to the original isolated sequence according to thin layer chromatography, paper electrophoresis, and sensory experiments. Also, the mass fragmentation pattern was similar to the pattern of the isolated material; however, no mass peak was found in either case. A second synthesis in solution of BMP was described by Tamura et al. (1989). This synthesis is rather complex, probably

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because the authors wanted to isolate simultaneously intermediate segments of the peptide. The use of Boc-Ser without side-chain protection in a coupling step of 18 h mediated by carbodiimide, followed by four overnight mixed anhydride couplings, may lead to numerous unwanted side products. The use of unprotected serine has been described in the literature only successfully in combination with the mild azide or active ester couplings. Single mixed anhydride or carbodiimide couplings with unprotected serine have been used occasionally in the synthesis of small di- or tripeptides. The use of only 1 equiv of activated amino acid in these coupling steps may suppress the formation of side products but also decreases the yield of the coupling reaction. The yields for one coupling described by Tamura et al. (1989) range from 66% to 87%, which means that the purification of the intermediates by extraction or selective precipitation has to be very efficient. However, Tamura et al. (1989) describe the HPLC chromatogram of the deprotected product as showing only one peak, and the results from the amino acid analysis show the expected data. Recently, Spanier et al. (1995) briefly described a third synthesis using an automated peptide synthesizer. They used Fmoc chemistry and probably carbodiimide as coupling reagent. The crude peptide, which contained pyridine after precipitation, was purified by ion exchange chromatography in ammonium carbonate buffer.

Our synthesis was a straightforward solid phase synthesis starting with a Fmoc-Ala-HMP resin. Fmoc-amino acids were coupled using the HBTU reagent. HBTU (Knorr et al., 1989) belongs to a new generation of coupling reagents in peptide synthesis that combine high coupling yields (>99%) with low racemization. Although we cannot compare the published synthesis or the purity of the octapeptides with our results, we cannot exclude the possibility that impurities in the product are responsible for the reported umami taste. These impurities may originate, for instance, from side reactions with serine, racemization during the mixed anhydride coupling, or truncated sequences. This, however, is in contradiction with the observation that the peptide, originally isolated from a beef extract, was also shown to possess the umami taste. Such a natural peptide is unlikely to contain a similar modification or artifact or other than L-amino acids.

The octapeptide synthesized as described in this paper was shown to possess the sequence as reported by Yamasaki and Maekawa (1978) and to be of high purity. It did not bring about the typical umami taste, or any other taste sensation, when tasted well above the reported threshold concentration. The same octapeptide, obtained from a commercial source, failed to give any taste effect. The persons involved in the tasting sessions were experienced tasters and flavorists, quite able to perceive and describe umami. On the basis of our findings we must conclude, therefore, that the octapeptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala is neither delicious nor meaty nor umami. Spanier et al. (1995) did not specifically confirm the umami taste per se of the BMP they synthesized; they only describe a "flavor enhancing activity" of BMP in a beef-flavored gravy. Unfortunately, these authors did not specify the composition of the gravy; reproducing their findings is not possible, therefore.

The discrepancy between our results and those reported by Yamasaki and Maekawa (1978, 1980), Tamura et al. (1989), or Spanier et al. (1995) is difficult to explain. If the commercial delicious octapeptide sold by Peninsula Laboratories is the same material as that synthesized by Yamasaki and Maekawa (1978), to whom Peninsula Laboratories refer, then the purity of the peptide is not a likely explanation. It may be assumed, however, that in the synthesis carried out by Yamasaki and Maekawa (1978), Tamura et al. (1989), or Spanier et al. (1995) some racemization of the composing amino acids may have occurred. The stability of the peptide could be a further clue to the origin of this discrepancy; some composing peptides were also reported to have a umami taste (Tamura et al., 1989). We did not find any enzymatic breakdown of the octapeptide, which might result in several small peptides, responsible for any taste. Neither did we observe any aspecific hydrolysis of BMP that might be responsible for any taste as claimed by the original authors.

ACKNOWLEDGMENT

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