Comprehensive Study of the "Beefy Meaty Peptide"

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The octapeptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, also called "beefy meaty peptide" (BMP), is supposed in the literature to play a key role in the taste of meat. A procedure allowing its detection in beef extract down to a level of 1 ppm was developed using sample enrichment by solid-phase extraction and on-line detection by electrospray LC/MS. It is shown that detectable levels of BMP do not occur naturally, in beef digests, or in cooked or grilled beef. Furthermore, a thorough sensory evaluation showed that BMP, when tasted in water, is described as having a strong acid and astringent taste. Tasting in beef stock, both with and without added NaCl or monosodium glutamate, revealed that the compound has little or no detectable flavor or flavor enhancing properties. Therefore, BMP cannot be considered as a flavor carrier or a potential flavor enhancer.

Keywords: *Peptide; flavor; meat; taste; monosodium glutamate; beefy meaty peptide; liquid chromatography; mass spectrometry*

INTRODUCTION

Attempts have often been made to characterize and identify nonvolatile key compounds contributing to the flavor of meat (*e.g.*, Warendorf and Belitz, 1992; Spanier *et al.*, 1992; Spanier and Miller, 1993). In 1978, Yamasaki and Maekawa analyzed beef extract treated with papain and isolated a peptide fraction that was reported to give a "strong delicious taste" (Yamasaki and Maekawa, 1978). Its main component was the octapeptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (KGDEESLA), whose sequence was further verified by synthesis of the compound (Yamasaki and Maekawa, 1980).

The flavoring properties of this peptide and some of its amino acid subsequences were further investigated by Tamura *et al.* (1989) who reported an *umami* and a sour taste. Due to its flavoring taste, Spanier *et al.* (1992) called it "beef meaty peptide", BMP, and recently renamed it "savory taste-enhancing peptide", STEP (Spanier and Miller, 1995). These authors reported it "to be similar to monosodium glutamate (MSG) in its ability to enhance the flavor of a beef gravy yet it did not present the salty taste of MSG". Nakata *et al.* (1995) reported an *umami* and a salty taste for this peptide and found a variation in taste depending on the concentration of Na^+ or K^+ present in the peptide solution. On the other hand, a publication from van Wassenaar *et al.* (1995) states that it "did not have any umami or other taste", thus there is obviously some disagreement about the flavoring properties of BMP.

BMP and related peptides are the subjects of several investigations (*e.g.*, Cutts *et al.*, 1996). Surprisingly, only the very first publication about BMP describes investigations with "natural" BMP; Yamasaki and Maekawa (1978) reported a yield of "about 40 mg from 100 g beef meat", isolated from papain-treated beef that was not roasted. In all later analytical and/or sensory studies, synthetic BMP was used. In addition, there is no unambiguous report in the literature verifying the presence of BMP in natural, untreated beef. It may be formed by enzymatic digestion, as the very first publication (Yamasaki and Maekawa, 1978) refers to papain-treated beef. Spanier and Miller (1993) state that it occurs naturally in beef, but experimental details were not published.

Moreover, conventional peptide analysis techniques involve separations by liquid chromatography (HPLC) or by capillary electrophoresis (CE) coupled with UV detection. However, UV detection seriously suffers from its lack of specificity. Especially in complex matrices like beef extract, several authors do not verify the identity of the target compound, either by applying standards or at least one additional analytical technique (ACS, 1980).

To investigate both the flavoring properties and the natural occurrence of BMP, we have developed a straightforward technique for its unambiguous detection and identification in meat extract. The samples were separated by reversed-phase HPLC and detected by mass spectrometry using electrospray ionization. Furthermore, a thorough sensory evaluation both in water and in beef stock was performed.

MATERIALS AND METHODS

Chemicals. BMP was purchased from Peninsula Laboratories (Belmont, CA) and used "as received". Its identity was verified using LC/MS and MS/MS. Water was purified in-house using a Millipore Milli-Q water purification system (Millipore, Volketswil, Switzerland). All organic solvents were obtained in "p.A." or HPLC grade qualities from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased in 1-mL vials from Pierce (Rockford, IL), and papain was from Fluka (Buchs, Switzerland; 3.0 units/mg).

Preparation of Beef Extract. Several sets of samples were prepared from finely ground beef. Some were prepared from fresh beef (within 48 h after slaughtering), while others were obtained after storing the beef under "household conditions" (refrigerator, +7 °C) for about 1 week. As the sample extraction procedure was further refined, different cleanup procedures were followed. For the extraction of raw meat, ca. 50 g of beef was added to ca. 100 mL of water. The suspension was stirred on a magnetic stirrer at ca. 40 °C overnight (300 rpm). The gelatinous residue was removed by centrifugation (20 min at 4000 rpm) and washed with water. The liquid phase was then lyophilized. To achieve an enzymatic digest, ca. 10 mg of papain was diluted in 10 mL of water and added to the mixture before stirring overnight as described above.

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The pH of the different solutions was controlled using a Metrohm Type 604 pH-meter with electrode 6.0204.100 (Metrohm, Herisau, Switzerland) and was between pH 6.1 and 6.5 throughout the extraction process. On average, 4.3 g of dry residue was obtained from 100 g of beef. It should be noted that especially the enzymatic digest smelled disgusting. Prior to analysis, ca. 100 mg of lyophilisate was dissolved in 25 mL of water (ultrasonic bath). Fat was removed by extracting twice with 10 mL of hexane. The solution was then filtered through 0.45 μm Nalgene filters and made up to 50 mL in a volumetric flask.

For the grilled beef samples, fresh beef was ground and grilled in a frying pan without any addition of fat or spices. Circa 100 g of this beef and 350 mL of water were cooked for 3 h under nitrogen in a standard reaction vessel. The mixture was centrifuged at 5000 rpm for 15 min, followed by two washing steps with ca. 80 mL of water and repeated centrifugation. Fat was removed by extraction with 3 × 50 mL of heptane. Solid matter was removed by filtration through paper filters and subsequently through 0.45 μm Nalgene filters. The sample volume was then adjusted to 500 mL in a volumetric flask. An aliquot was analyzed directly, while the rest was lyophilized. The average yield was 6.3 g of extract from 100 g of beef.

A BMP-spiked extract of grilled beef was prepared in the same way; however, before cooking the sample, a solution of BMP in water was added, giving a final concentration of ca. 0.5 ng/μL. This corresponds to ca. 2.5 mg of BMP per kg of beef, *i.e.*, about 2 orders of magnitude inferior to the concentration given by Yamasaki and Maekawa (1978).

Solid Phase Extraction (SPE). Sample cleanup and enrichment were performed on Waters C-18 SPE cartridges (WAT 051910, "classic"; Millipore, Basel, Switzerland). All solvents and samples were manually applied using plastic syringes. Following a series of trials, the following procedure was established: (1) condition SPE cartridge with 3 mL of acetonitrile containing 2% water and 0.1% TFA; (2) condition with 5 mL of water containing 0.1% TFA; (3) load 2.0 mL of beef extract (or 10.0 mL of aqueous BMP standards); (4) wash with 10 mL of water containing 0.1% TFA; (5) elute with 2.0 mL of acetonitrile (15% in water, no TFA); (6) evaporate to dryness under nitrogen; (7) use 300 μL of "solvent A" (see below) to dissolve and transfer the sample into polyethylene autosampler vials. This procedure gave an overall recovery of ca. 67%, as calculated from the BMP-spiked beef standard. BMP calibration solutions were prepared in water; all calibration points were determined at least four times, while samples were each measured twice.

Liquid Chromatography. The separations were performed using a Waters HPLC system, consisting of a type 757 autosampler, a 600-MS pump with system controller and a type 486-MS UV detector. 20 μL of sample was injected onto a C18 5 μm reversed-phase column (250 × 2.1 mm with precolumn 20 × 2.1 mm, Vydac 218TP52, Vydac, Hesperia, CA). UV absorption at 214 nm was recorded using an analogue input to the mass spectrometer's data system. Solvent A was 2% (v/v) acetonitrile in water containing 0.1% acetic acid, and solvent B consisted of 2% (v/v) water in acetonitrile with 0.1% acetic acid. Using a flow rate of 0.3 mL/min, a linear gradient was set up starting at 100% A and reaching 25% B at 21 min. Over the next 6.5 min, the solvent composition was changed to 100% B and held constant for 2.5 min to flush the column. Solvent was changed back to the initial conditions within 8 min, followed by a 12 min hold to equilibrate the column. Data were acquired from injection time to 25 min. The HPLC eluent was split at a ratio of 1:10 using an AcuRate flow splitter (LC packings, via Omnilab, Mettmenstetten, Switzerland) so that approximately 30 μL min^{-1} entered the ion source of the mass spectrometer.

Mass Spectrometry. The mass spectrometer was a Finnigan TSQ 700 triple-quadrupole MS equipped with a Finnigan ESI II electrospray ion source. Data acquisition was performed on a DECstation 2100 running under Ultrix 4.2A (Digital Equipment) using the Finnigan software package ICIS2, Ver. 7.0. The electrospray voltage was set to 4.2 kV, the transfer capillary voltage to 20 V and its temperature to 200 °C. Prior to each series of analyses, the ion source was re-tuned to the $[M + H]^+$ ion of BMP using continuous sample infusion via a Harvard "22" syringe pump. MS/MS data were obtained at a collision energy of −40 eV in the laboratory frame using argon at a pressure of 0.4 Pa (3 mTorr) as the collision gas. Mass spectra were acquired by scanning from m/z 150 to m/z 1000 in 3 s. Quantification data were acquired in single ion monitoring mode (SIM) using a cycle time of 0.33 s and recording the intensities of the protonated molecular ion of BMP (m/z 848.4), its first ^{13}C isotope peak (m/z 849.4), and the doubly charged molecular ion (m/z 424.7). To reduce the abundance of the latter during quantitative determinations, the ion source was operated under "in-source collision" conditions (20 V) as will be discussed below.

Sensory Characterization. For the tests in beef stock, a commercially available brand ("Maggi") was chosen but was obtained without any NaCl or MSG; the two compounds were added in various amounts during the study. The concentration of all other ingredients was kept constant, so that variations could be clearly attributed to the influence of NaCl and MSG, respectively. For sample preparation, Volvic mineral water (Société des Eaux de Volvic, Volvic, France) was used. By using this slightly mineralized water (total mineral content 109 mg/L, pH 7) instead of distilled or tap water, a buffering effect as in "real" food was achieved, avoiding side effects both from high mineral contents and from the artificial taste of distilled water. Beef stock samples were dissolved in boiling water; however, all tests were performed with the samples at ambient temperature. For determination of the perception threshold for BMP in water, three tasting sessions using BMP concentrations between 0.1 and 1.0 mM in water were performed. The range of concentrations used in a certain session was adapted according to the results obtained in the previous session.

A sensory panel of 18 internal collaborators was selected according to their ability to detect MSG at a concentration of 1.5 mM. The same panelists performed the sensory evaluations throughout the whole study. The panelists tasted the samples, spit, and rinsed with Volvic water after each test.

Evaluation of Sensory Data. The perception threshold, as well as the influence of MSG and NaCl, were determined by using a signal detection test (triangle test) presentation: three series of three samples, coded by random three-digit numbers, were presented in a balanced design to the panelists. One contained the stimulus (*i.e.*, BMP in water or beef stock at variable concentrations), and the other two contained the reference (*i.e.*, water or beef stock). In each session, the panelists were presented with three sets of samples without time control between each test. The panelists were instructed to indicate which of the samples was different from the others. This was a "forced choice". They could add comments to justify their choice or to describe their impression about the difference.

In a "forced choice" test, the panelists are *forced* to select a sample that is "different" from the other two samples and are advised to make their best guess if they cannot detect an obvious difference. Therefore, some of the correct answers could be made by chance. The theoretical percentages of BMP perception resulting from the percentage of correct answers and, subsequently, the perception threshold were calculated as described by Voirol and Daget (1986).

Database Search. We accessed the SwissProt (Release 31.0) database via the ExPASy World Wide Web molecular biology server of the Geneva University Hospital and the University of Geneva (http://expasy.hcuge.ch/sprot/sprot-top.html). For the similarity searches, *Blitz*, an automatic electronic mail server for the MPsrch program of Shane Sturrock and John Collins, Biocomputing Research Unit, University of Edinburgh, Scotland, was used (http://www.ebi.ac.uk/searches/blitz.html). All searches were performed in August and September 1995.

RESULTS

Mass Spectrometry of BMP. Initial studies were performed with a solution of BMP in methanol/water/acetic acid (40:40:1, v/v/v). The peptide shows the singly charged protonated molecular ion at m/z 848.4 as well as the doubly charged ion ($[M + 2H]^{2+}$, m/z 424.7) (Figure 1). It should be observed that the spectrum

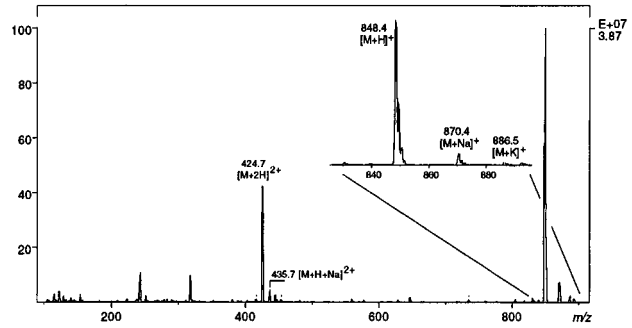

Figure 1. Electrospray mass spectrum of BMP showing the protonated molecular ion at *m*/*z* 848.4 and the doubly charged ion at *m*/*z* 424.7. The sodium and potassium clusters originate from the peptide preparation.

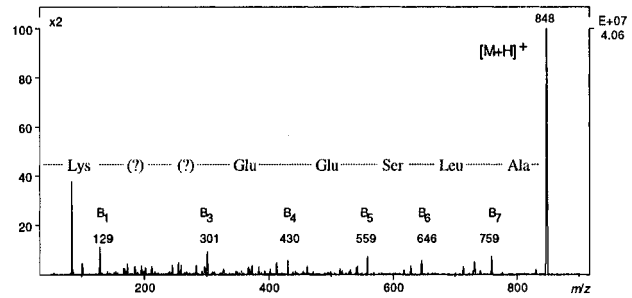

Figure 2. MS/MS daughter ion spectrum of the protonated molecular ion of BMP, obtained at a collision energy of −40 eV and by continuous sample infusion with a syringe pump. Almost all ions of the "B-series" are observed. The "missing" subsequence, indicated by question marks, is Gly-Asp.

given here shows the correct isotope pattern for the BMP signal (43% relative intensity for the ^{13}C isotope peak). Previously, spectra have been published where a distorted isotope pattern was recorded (Spanier *et al.*, 1995).

Upon MS/MS experiments, we could obtain a daughter ion spectrum (Figure 2) by continuous infusion and using collision induced dissociation. At this point, it would have been desirable to determine the presence of BMP by an on-line LC/MS/MS experiment as this would have significantly increased the selectivity and therefore, the specificity of the detection. However, the collision efficiency (and subsequently the signal-to-noise ratio) observed in preliminary experiments was much too low to allow unambiguous identification by LC/MS/MS.

Therefore, all further investigations were performed using LC/MS only working in SIM mode. The presence of BMP was tracked by observing the protonated molecular ion at *m*/*z* 848.4, its first ^{13}C isotope peak (*m*/*z* 849.4) and the doubly charged molecular ion (*m*/*z* 424.7). It should be noted that the abundance of the signal of the doubly charged ion varies widely with the operating conditions; at low pH, it may be one order of magnitude more abundant than the $[M + H]^+$ signal. On the other hand, under in-source CID conditions, the ion "strips off" one proton, leading to a signal decrease of the doubly charged peak. As a consequence, the signal at *m*/*z* 424.7 cannot be used for quantification. However, its presence together with the $[M + H]^+$ signal provides another independent signal to trace the presence of BMP, thereby minimizing the risk of data misinterpretation due to interference from other compounds. Our experiments indicate that information about retention time *and* three masses *and* comparison to the behavior of a known standard is sufficient for unambiguous identification of BMP.

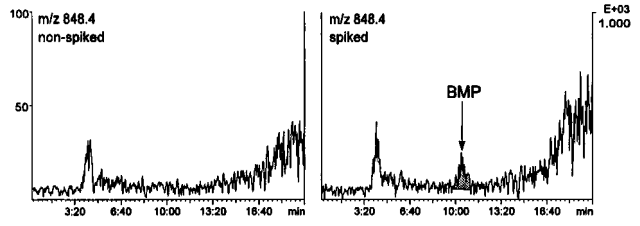

Figure 3. Analysis of beef extract without any sample enrichment. Although the signal is rather noisy, the total amount of BMP found in the spiked sample fits to the calculated value within a factor of 1.6 (464 pg/μL calculated, 740 pg/μL found).

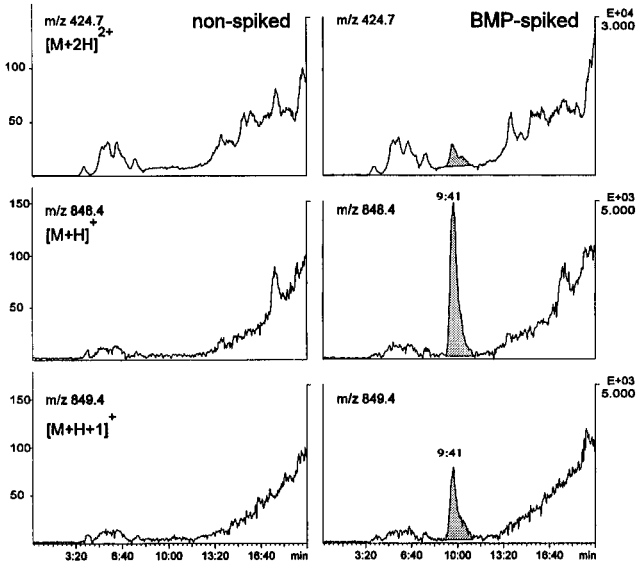

Figure 4. Analysis of beef extract using sample purification by SPE. The samples were prepared from the same batch as in Figure 3. The chromatograms show the doubly charged ion (upper traces), the protonated molecular ion (middle), and its ^{13}C isotope peak (lower). Mass chromatograms showing the same *m*/*z* are normalized to the same intensities. The spiked sample (right column) contains 464 pg/μL of BMP, corresponding to 2.3 mg per kg of roasted beef. The nonspiked beef extract (left column) does not show any indication of the presence of BMP.

Quantification. Quantification using the peak areas of the $[M + H]^+$ ion gave a linear calibration plot over 3 orders of magnitude with a correlation coefficient $R = 0.9996$. The limit of detection is 0.3 pmol/μL (S/N = 3), the limit of quantification is ca. 1 pmol/μL. Corresponding "blank" runs with water as sample showed neither background contributions nor interferences in any of the three mass traces.

BMP in Beef Extract. Initial experiments with spiked samples showed that BMP could be detected in beef extract down to ca. 0.5 ng/μL (0.6 pmol/μL) without any sample clean-up (Figure 3). However, these data show matrix interference, and further samples were prepared using SPE as described above. The increase in detection efficiency by applying SPE is clearly visible by comparing Figures 3 and 4.

From Figure 4 it can be seen that an abundant signal is obtained from a spiked sample containing 2.3 mg of BMP per kg of beef, thus the limit of detection using SPE in beef matrix is clearly below 1 mg/kg of beef (1 ppm). However, comparison with the nonspiked beef extract shows no indication about the presence of BMP. In fact, using SPE/LC/MS, no BMP could be detected in any extract investigated here, neither from enzymatic digests nor from grilled meat.

Database Searches. To elucidate the possible biological origin of BMP, we have searched its amino acid

Table 1. Results of a Search for BMP and Related Sequences in the SwissProt Database[a]

sequence	hits with 6 AA	hits with 7 AA	hits with 8 AA
KGDEESLA	3	0	0
KGNEESLA	4	0	0
KGDQESLA	0	0	0
KGDEQSLA	2	0	0
KGDQQSLA	2	0	0
KGNQESLA	1	0	0
KGNEQSLA	6	0	0
KGNQQSLA	2	3	0

[a] AA, amino acid.

sequence in protein databases. Spanier and Miller (1993) reported that they found only one peptide matching BMP for at least four of its eight amino acids. As these results were published in 1993, we have performed an actual search in the SwissProt database.

However, it cannot be excluded that changes in amino acid composition occur during meat preparation. Looking at the amino acid composition of BMP, it may be particularly likely that a "precursor" contains asparagine instead of aspartic acid and/or glutamine instead of glutamic acid. Thus, all sequences containing these potential precursors were included in the search. The results are listed in Table 1. The list contains only peptides where at least six amino acids in series were matching, *i.e.*, without "missing" amino acids.

Only the last sequence containing two "unoxidized" amino acid residues (KGNQQSLA) was attributable to muscle tissue. The full sequence of all eight amino acids constituting BMP or its "precursors" was not found in any peptide nor protein.

It has to be noted that only known and published data have been addressed. Furthermore, "negative" results of database searches of this kind have to be regarded with care: Up to now, most peptide/protein databases were created in (and for) molecular biology and similar fields of research, *i.e.*, they mostly contain compounds that are of some "biological interest" but not necessarily of interest for food science. Therefore, the result "not found in database" does not necessarily mean "the compound does not exist".

Sensory Characterization of BMP. Besides defining the taste of BMP, the main objectives were to determine the perception threshold of BMP in beef stock and to assess the influence of dose and interaction with other food ingredients, such as monosodium glutamate (MSG) and salt.

BMP in Water. The response curve for the perception of BMP in water is given in Figure 5. The perception threshold in water was found at 0.5 ± 0.15 mM, corresponding to 440 ± 130 mg/L (significance level $p < 0.01$). The panelists described the taste of pure BMP in water as "acid, then astringent", "sweet", "salty", "bitter", "acid", and even "pungent". Remarkably, none of the panelists noted any kind of taste or flavor that could be reasonably attributed to "meat" or "beef".

BMP in Beef Stock. Interaction of BMP with NaCl. Based on the perception threshold value for BMP in water, the following studies covered BMP concentrations above and below the threshold: 0.45, 0.50, and 0.55 mM. They were prepared in beef stock containing NaCl concentrations of 8.6, 1.2, and 0.0 g/L.

In beef stock without NaCl, no significant effect of BMP was detected. Higher concentrations of NaCl in beef stock increase BMP perception; at 8.6 g/L of NaCl (Figure 6) the sensation of beef stock is significantly different ($p < 0.05$), but only if BMP concentrations of 0.5 mM and above are used.

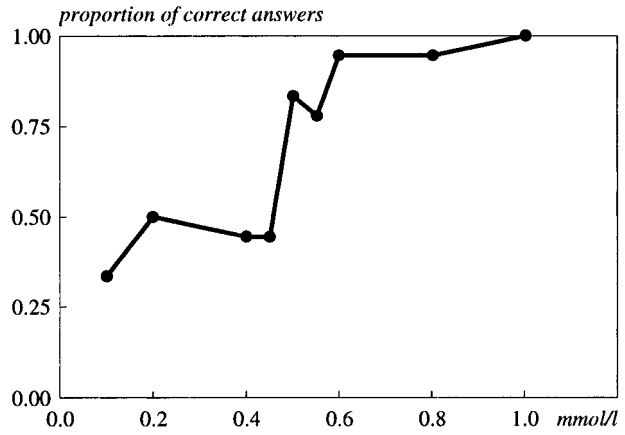

Figure 5. Response curve for the perception of BMP in water, determined with 18 panelists. The perception threshold of BMP is 0.51 ± 0.15 mM.

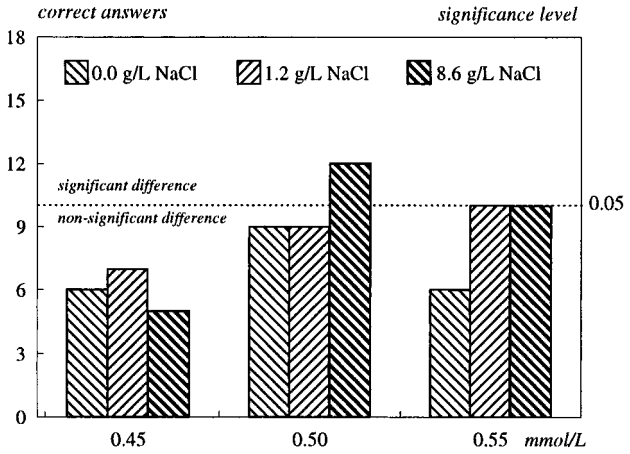

Figure 6. Results of the sensory evaluation of BMP in beef stock at various NaCl concentrations, without MSG.

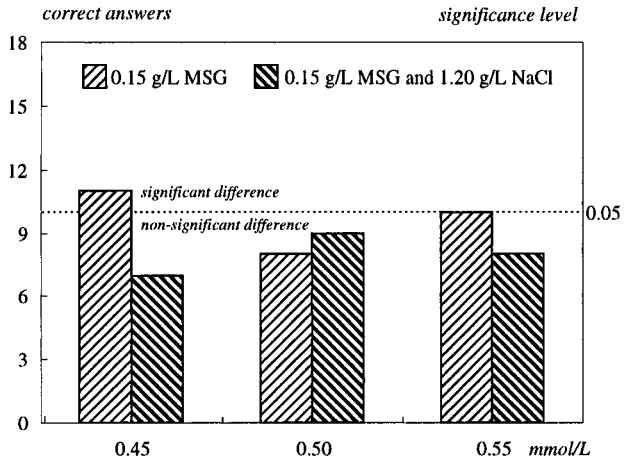

Figure 7. Results of the sensory evaluation of BMP in beef stock with MSG and NaCl.

The tasters who detected a difference described the taste in the solution containing 1.2 g/L of NaCl as "more salty" and "more stock and meaty taste". At 8.6 g/L of NaCl the attributes included "more salty", but also "acid", "less 'round' and less 'balanced'", "pungent", and "astringent".

Interaction with Monosodium Glutamate. To determine whether there is an interaction between BMP and MSG, a similar test as described above was carried out using beef stock without NaCl, but containing 0.15 g/L of MSG. The result is given in Figure 7, suggesting that the perception threshold of BMP in beef stock containing MSG is also about the same as in water or in beef stock

containing NaCl. The main comments collected described the BMP flavor in the presence of MSG as "more stock flavor", "more 'round'", "more acid", "slightly more salty", and "more bland".

Interaction with Monosodium Glutamate and NaCl. To evaluate the influence of both NaCl and MSG, another assessment used beef stock containing both NaCl (1.2 g/L) and MSG (0.15 g/L). Both concentrations reflect the NaCl and MSG proportions that were found to have the highest number of correct answers in the previous tests. The results (Figure 7) show that BMP was not detected by the panelists ($p > 0.05$) when used in combination with NaCl and MSG.

DISCUSSION

In the context of analyzing flavoring peptides, the octapeptide BMP could not be detected in beef extracts, at least not down to a level of less than 1 mg per kg of roasted beef (1 ppm). With regard to the fact that Yamasaki and Maekawa (1978) reported a preparative yield of "about 40 mg from 100 g beef meat", this result is contradictory to almost all previous publications stating a natural occurrence of this peptide. This is underlined by the fact that a peptide database search did not give any indication of a peptide or protein containing the sequence of BMP or of possible "precursor" peptides. It can be concluded that BMP does not occur naturally, at least not in "detectable" amounts. Therefore, the identity of the peptide originally described by Yamasaki and Maekawa (1978, 1980) remains unclear.

The sensory evaluation shows that the taste of BMP on its own can be described as "acid" and "astringent". This does not fit well with the terms "delicious" (Yamasaki and Maekawa, 1978), "simple *umami*", or "slightly sour" as described in the literature (Tamura *et al.*, 1989). On the other hand, our data correspond well to those reported by van Wassenaar *et al.* (1995) who could not find any *umami* or other taste. These authors showed that the presence of impurities influencing the taste of BMP can be excluded, which is further underlined by Wang *et al.* (1995) who demonstrated the stability of BMP against heat treatment.

The perception threshold in water of 0.5 ± 0.15 mM indicates that our panel was more sensitive than that of Tamura *et al.* (1989) who reported a value of 1.41 mM. Furthermore, our result corresponds to the data reported recently by Wang *et al.* (1996). The threshold in Maggi beef stock containing NaCl alone or MSG alone is almost identical to the value in water; as a consequence, there is no convincing evidence for a "taste-enhancing" effect of BMP in beef stock at the concentrations used here.

Further, BMP has little flavoring action. Its perception seems to mainly depend on the individual sensitivity and preferences of the tasters, as it was either described as a flavor enhancer ("more salty, more stock and meaty taste, stronger in taste") or having its own taste ("bitter, acid, astringent, pungent, sweet"). A possible synergistic effect ("more stock and meaty taste", "more salty") is observed when BMP was used together with 1.2 g/L of NaCl. In a mixture with flavor enhancers such as NaCl and MSG, BMP was not perceived at concentrations in the 0.5 mM range, corresponding to 450 mg/L. This may be due to the combined action of NaCl and MSG, which could cover flavoring properties of BMP. Wang *et al.* (1996) reported that diluted beef extracts with 2 mM of BMP could be differentiated from non-spiked extract; however, it should be noted that this concentration is 4-fold higher than the perception threshold found in water or in beef soup.

In summary, there is no evidence that BMP exists naturally nor that it has any flavoring properties. Therefore, BMP cannot be considered as a flavor carrier or a potential flavor enhancer.

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