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Analysis of Taste-Active Compounds in an Enzymatic Hydrolysate of Deamidated Wheat Gluten

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Hydrolyzed plant proteins are widely used as ingredients in culinary products for their glutamate-like ("umami") taste. Three hydrolysates were prepared from wheat gluten using different enzymatic approaches. Comparison of their taste profiles revealed the enzymatic hydrolysate of an acid-deamidated wheat gluten (WGH-3) to be the least bitter of all and to elicit an intense glutamate-like taste. Its umami taste intensity was similar to that of an enzymatic hydrolysate in which glutaminase had been employed to convert free glutamine to glutamic acid and which had a 3-fold higher concentration of free glutamate. Reconstitution studies based on the results of the chemical analysis of WGH-3 and sensory comparison of the model solution and WGH-3 indicated that other components in addition to glutamate and organic acids contribute to its glutamate-like taste. WGH-3 was fractionated by gel permeation chromatography and reversed phase high-performance liquid chromatography, and two fractions with a pronounced glutamate-like taste were obtained. In one of them four pyroglutamyl peptides were tentatively identified: pGlu-Pro-Ser, pGlu-Pro, pGlu-Pro-Glu, and pGlu-Pro-Gln. Apparently, these peptides were formed by cyclization of the N-terminal glutamine residues during the preparation of the hydrolysates.

KEYWORDS: Wheat gluten; umami; taste; peptides; taste profile; taste-active compounds; gel permeation chromatography; HPLC; LC-MS; deamidation

INTRODUCTION

Proteolytic reactions play an important role in the development of flavor in protein-rich foods such as cheese, meat, sausage, and fermented soy products. During proteolysis in fermentative or pure enzymatic processes free amino acids and peptides are formed. Hydrolyzed vegetable proteins, for example, soy sauce, are widely used as savory ingredients in a variety of foods because of their "umami" taste properties. The Japanese word "umami" means delicious and is used as a synonym for the characteristic sensory properties of monosodium glutamate (MSG) and certain purine-5′-nucleotides such as inosine-5′-monophosphate (5′-IMP) or guanosine-5′-monophosphate (5′-GMP). A particularly important characteristic of the umami substances is their ability to enhance the flavor (aroma and taste) and mouthfeel of savory dishes. Another peculiar property of umami compounds is their mutual taste synergism. The synergistic effects especially between MSG and purine-5′-nucleotides have been studied intensively and systematically (*1, 2*).

The savory flavor of protein hydrolysates is assumed to be caused by a high content of free amino acids, especially glutamic acid, low molecular weight peptides, salt, and organic acids (*3*). The taste quality produced by amino acids and peptides was also described as "kokumi" (*4, 5*), which was translated as "rich, thick taste". On the other hand, peptides have also been described as being responsible for the undesirable bitter tastes of cheese and enzymatically hydrolyzed fish, soy, and wheat proteins (*6*−*13*). The bitterness is due to low molecular weight peptides composed of hydrophobic amino acids (*14*, *15*).

Savory peptides are discussed controversially in the literature. A tetrapeptide from a yeast seasoning consisting of four aspartic acid residues was described as eliciting glutamate-like and bouillon-like taste qualities and as masking the bitter note of the yeast extract (*16*). Other acidic peptides were found in a fraction of an enzymatic fish protein hydrolysate; for example, Glu-Asp-Glu, Asp-Glu-Ser, Thr-Glu, and Ser-Glu-Glu and were reported to have sensory properties similar to those of sodium glutamate (*13*). Arai et al. (*7*, *17*) concluded that the glutamate-like taste of peptides is caused by high molar contents of glutamic acid and hydrophilic amino acid residues. In contrast, some of these peptides were reported as being neutral or even slightly bitter by van den Oord and van Wassenaar (*18*), who generally questioned the existence of umami peptides. A study about the effect of certain alleged umami peptides on the metabotropic glutamate receptor 4a in rat brain (mGluR4a) revealed that several of them were glutamate agonists but showed only a weak mouthfeel effect in the sensory evaluation (*19*). The authors concluded that the strong umami effect of

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L-glutamic acid has been lost in Glu-X di- and tripeptides. Only the glutamic acid derivative *N*-lactoylglutamic acid still possessed umami taste properties. Recently, a truncated form of the mGluR4a has been identified in the taste buds on rat tongue, and the authors suggested the truncated form as umami taste receptor (*20*).

Systematic approaches are necessary to evaluate the taste compounds of foods. Recently, the "taste dilution analysis" was proposed to rank HPLC fractions from a Maillard reaction mixture according to their relative taste impact in order to screen taste-active molecules (*21*). The method, which is based on the "dilution indices methodology" developed by Tilgner (*22*), implies the sensory evaluation of serial dilutions of the HPLC fractions. The dilution at which a taste can just be detected is defined as the "taste dilution factor" of the fraction. The "taste activity value" (TAV) concept is another approach and an objective screening tool to separate single taste-active compounds of a food from other constituents and was successfully applied on Emmental cheese and stewed beef juice (*23*, *24*). In analogy to the "aroma value" (*25*), the TAV is defined as the quotient of the concentration and the taste threshold concentration of a compound, and it is a means to estimate the contribution of a compound to the taste of a food.

To date, knowledge about taste compounds in hydrolyzed plant proteins is limited, and the contribution of peptides to their savory taste is discussed controversially. The objective of this study was to investigate the chemical composition and the sensory qualities of enzymatic wheat gluten hydrolysates. The taste activity concept as a combination of instrumental and sensory analysis was employed to elucidate the contribution of the constituents to the flavor of the hydrolysates, particularly their impact on the umami taste. Special attention was given to low molecular weight peptides.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals used were of analytical or HPLC grade and were purchased from Sigma (St. Louis, MO), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), and Amersham Pharmacia Biotech (Uppsala, Sweden). Flavourzyme L1000 (activity = 1000 LAPU/g) was from Novozymes (Bagsvaerd, Denmark), and glutaminase C 100 (activity = 100 GTU/g) was from Valley Research (South Bend, IN).

Wheat Gluten Hydrolysates. The wheat gluten hydrolysates were produced from wheat gluten [Viten, minimum protein content = 76% (N × 6.25), Roquette, Lestrem, France] by enzymatic hydrolysis using Flavourzyme alone (4.0 mg/100 g; WGH-1) or in combination with glutaminase C 100 (0.75 mg/100 g) to convert released glutamine into free glutamic acid (WGH-2). The hydrolysis was carried out in aqueous suspension (24% dry matter) at pH 6.0 and 55 °C during 16 h. The enzymes were deactivated at 95 °C for 10 min according to the manufacturer's recommendations. After filtration, the obtained solutions were spray-dried (inlet temperature = 145 °C, outlet temperature = 88 °C, Production Minor spray dryer, Niro Inc., Copenhagen, Denmark) to yield beige powders. WGH-3 was obtained by enzymatic hydrolysis of deamidated wheat gluten with Flavourzyme (4.0 mg/100 g) and subsequent enzyme deactivation and spray-drying yielding a yellowish powder. Deamidated wheat gluten was obtained by treatment with hydrochloric acid in aqueous suspension (24% dry matter) at pH 1.0 and 65 °C for 24 h. The pH was adjusted to 6.0 with sodium hydroxide solution prior to the enzymatic hydrolysis.

Chemical Analysis. Organic acids and ammonia were determined using enzymatic test kits (Roche Diagnostics, Rotkreuz, Switzerland). Chloride was measured photometrically using a test kit from Sigma (St. Louis, MO). Inorganic cations were quantified by inductively coupled plasma combined with atomic emission spectroscopy (ICP-AES). Free amino acids were analyzed in duplicates after precolumn derivatization with phenyl isothiocyanate (*26*). Total amino acids were determined in duplicates after hydrolysis at 110 °C for 24 h with hydrochloric acid (6 mol/L) prior to the derivatization with phenyl isothiocyanate. Total nitrogen was analyzed using the Kjeldahl method, and α-amino nitrogen was analyzed according to the method described in ref *27*. Ammonia nitrogen was determined titrimetrically after distillation of the ammonia under alkaline conditions.

Gel Permeation Chromatography (GPC). The FPLC system comprised a LCC-501 Plus controller, a P-500 pump, a Frac-100 fraction collector (Amersham Pharmacia Biotech, Uppsala, Sweden), and a microprocessor conductivity meter LF 537 (Gerber Instruments K. Schneider, Effretikon, Switzerland) using the software FPLCdirector version 1.10 (Amersham Pharmacia Biotech). An ultrafiltered solution of WGH-3 in deionized water (200 g/L; M_r < 3000; 10 mL) was applied onto a water-cooled Sephadex G10 column (2.6 × 64 cm) and eluted with a solution of ethanol in deionized water (15%, v/v; 39 mL/h). The effluent was monitored for its UV absorbance at 280 nm and for its electrical conductivity and separated into seven fractions. The peptide-bond specific wavelength of 214 nm could not be used because the solvent contained 15% ethanol to limit matrix interactions of aromatic compounds present in the sample. The fractions were freeze-dried and stored at −20 °C prior to use.

Reversed Phase High-Performance Liquid Chromatography. RP-HPLC was performed on a LaChrom HPLC system (Merck, Darmstadt, Germany) equipped with a diode array detector (L-4750) and a column thermostat (L-7360) set to 50 °C. Analytical chromatography was carried out using an ODS-AQ RP-18 column (3 μm, 4 × 250 mm, guard column 4 × 20 mm, YMC, Kyoto, Japan). Semipreparative chromatography was performed with a 201SP510 RP-18 column (5 μm, 10 × 250 mm, Vydac, Hesperia, CA). The mobile phase consisted of an aqueous solution of ammonium acetate (10 mmol/L, pH 6.0, solvent A) and a solution of ammonium acetate in 60% aqueous methanol (10 mmol/L, pH 6.0, solvent B). The gradient was as follows: 0−70% B from 7 to 30 min; 70−100% B from 30 to 35 min; 100% B from 35 to 43 min. The flow was 0.8 mL/min for analytical RP-HPLC and 3.1 mL/min for semipreparative RP-HPLC. Nine fractions (5 min each) were collected and freeze-dried.

RP-HPLC fraction F2-2 was rechromatographed using the analytical RP-HPLC system described above and the following mobile phases: aqueous formic acid (10 mmol/L, solvent A) and a solution of formic acid in 60% aqueous acetonitrile (10 mmol/L, solvent B). The gradient was 0−30% B from 10 to 50 min. The effluents corresponding to single peaks were collected, freeze-dried, and used for peptide analysis.

Peptide Analysis. Peptides were characterized by their amino acid compositions and by electrospray ionization−tandem mass spectrometry (ESI-MS/MS) using an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). The ESI source was set to 4.5 kV and the interface capillary heater to 200 °C. The MS and MS/MS spectra were obtained in the positive ionization mode using nitrogen as sheath and auxiliary gas. Automated MS/MS spectra were acquired with a relative collision energy for collision-induced dissociation (CID) preset at 35%. The scan range was *m*/*z* 50−1000. The samples were infused directly into the mass spectrometer by a syringe pump at a flow of 5 μL/min.

Peptide Sequence Analysis. The N-terminal amino acid sequence analysis was performed using Edman degradation in a pulsed liquid-phase sequenator 477A (Applied Biosystems Inc., Foster City, CA) with on-line RP-HPLC analysis of the released amino acids using a program adapted from that in ref *28*.

Sensory Analysis. The panel was composed of eight trained assessors (ages between 23 and 49 years) from the Institute of Food Science of the Swiss Federal Institute of Technology, Zurich, Switzerland. The panel was trained with reference solutions for the taste attributes "sweet" (sucrose solution, 35 mmol/L), "sour" (tartaric acid solution, 1.5 mmol/L), "salty" (sodium chloride solution, 40 mmol/L), "glutamate-like, umami" (MSG, 10 mmol/L), and "bitter" (L-isoleucine, 20 mmol/L), and with mixtures of the reference stimuli.

The wheat gluten hydrolysates were dissolved in deionized water at a concentration of 10 g/L. The freeze-dried fractions obtained by GPC of 2 g of wheat gluten hydrolysate were dissolved in 200 mL of deionized water, and the freeze-dried HPLC fraction 2-1 was redissolved in 200 mL of deionized water. All samples were coded, served in a randomized order, and tasted at 22 ± 2 °C under red light in a room

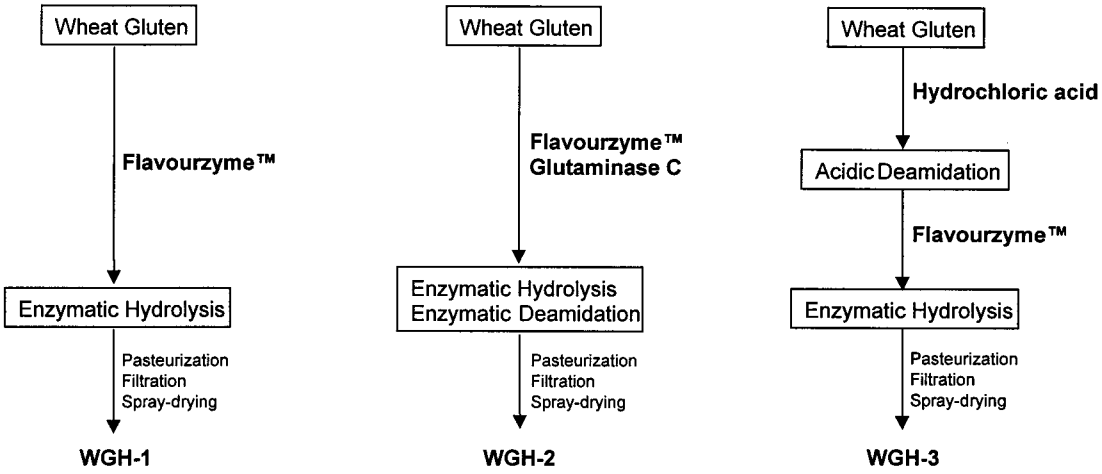

Figure 1. Preparation scheme of wheat gluten hydrolysates.

Table 1. Chemical Composition of Wheat Gluten Hydrolysates

	WGH-1	WGH-2	WGH-3
dry matter (%)	94.3	94.5	97.8
chloride[a] (%)	0.42	0.92	8.4
total nitrogen (%)	12.4	12.4	12.0
α-amino nitrogen (%)	3.9	4.4	3.1
degree of hydrolysis[b] (%)	31.5	35.5	25.8
free amino acids (%)	25.4	27.1	21.2
total amino acids[c] (%)	72.4	79.9	75.3
peptide-bound amino acids[d] (%)	47.0	52.8	54.1
free glutamic acid (%)	0.82	7.6	2.5
free glutamine (%)	5.7	0.6	3.5

[a] Chloride concentration is expressed as sodium chloride. [b] Degree of hydrolysis was calculated as the ratio of α-amino nitrogen to total nitrogen. [c] Amino acid content was determined without tryptophan. [d] Peptide-bound amino acids were calculated as the difference of total and free amino acids.

fitted with 12 separated booths. Prior to the evaluation of the samples, the panelists tasted the reference solutions and were asked to memorize the respective intensities, which had been set in mutual consensus to 50% of the assessment scale. The panelists were asked to judge the intensities of the solutions using a 100-mm line scale. The values given by the panelists were averaged. To evaluate the taste profile of the reconstituted WGH-3, the analyzed compounds were mixed according to their concentrations and dissolved in deionized water, corresponding to 10 g/L WGH-3. The pH was adjusted to pH 5.3 with sodium hydroxide solution (1 mol/L).

RESULTS AND DISCUSSION

Chemical and Sensory Characterization of Wheat Gluten Hydrolysates. The preparation scheme of the three wheat gluten hydrolysates is shown in **Figure 1**. Wheat gluten hydrolysate WGH-1 was produced using only proteases and peptidases (Flavourzyme), whereas a glutaminase was additionally used for the preparation of wheat gluten hydrolysate WGH-2 to catalyze the conversion of released glutamine into free glutamic acid. Wheat gluten hydrolysate WGH-3 was treated with hydrochloric acid prior to the enzymatic hydrolysis to convert protein-bound glutamine into protein-bound glutamic acid. The degrees of hydrolysis were between 25 and 35%, indicating that the majority of the amino acids was present as small peptides (**Table 1**). The contents of peptide-bound and free amino acids were ~50 and ~25% in all three hydrolysates, respectively. About one-third of the total amino acids of wheat gluten consist of glutamine, the amide of glutamic acid, which itself is present only at a low level. Consequently, WGH-1 contains a relatively high concentration of free glutamine (5.7%) and only little free

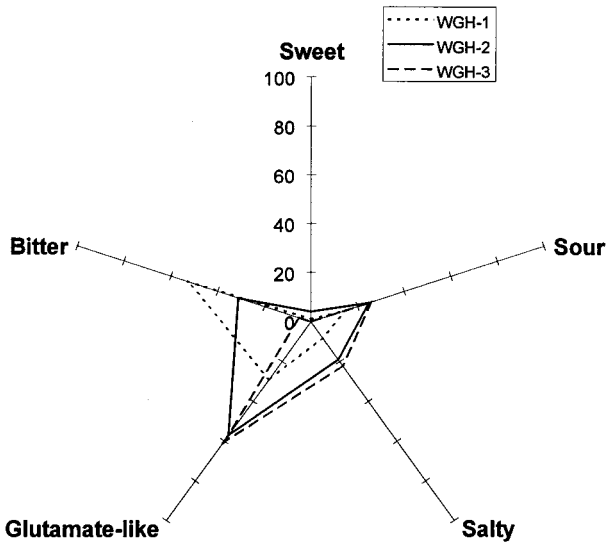

Figure 2. Taste profiles of enzymatic wheat gluten hydrolysates. Each value represents the averaged taste intensity scored on a 100-mm line scale ranging from 0 to 100 by eight panelists.

glutamic acid (0.8%). On the other hand, WGH-2 shows a comparably low content of free glutamine (0.6%) due to the enzymatic conversion into free glutamic acid (7.6%). The free glutamine and glutamic acid concentrations in WGH-3 of 3.5 and 2.5%, respectively, suggest that the hydrochloric acid treatment at pH 1.0 had led to a partial deamidation of the glutamine of the wheat protein. In preliminary studies the extent of deamidation of wheat gluten had been investigated. Hydrolysis at 70 °C for 24 h at pH 1.0 yielded a degree of deamidation of 8.2% (expressed as quotient of ammonia nitrogen and total nitrogen), corresponding to a deamidation of 27% of the glutamine and asparagine residues of wheat gluten. The peptide bonds had not been affected under these conditions (data not shown).

Comparison of the taste profiles shown in **Figure 2** reveals that the three protein hydrolysates differ mainly in the bitter and glutamate-like taste qualities. WGH-1 was mainly bitter but only slightly glutamate-like, sour, and salty. In contrast, WGH-2 had a distinct glutamate-like taste still accompanied by bitterness, but it was notably less bitter than WGH-1. WGH-3 tasted predominantly glutamate-like and exhibited the weakest bitterness of all three hydrolysates. Surprisingly, its glutamate-like taste was evaluated as intense as the one of WGH-2, despite its 3-fold lower glutamic acid concentration. Similar results have

Table 2. Compounds with High Taste Activity Values (TAV) in Enzymatic Wheat Gluten Hydrolysates

	taste threshold (mmol/L)	ref	TAV[a] WGH-1	WGH-2	WGH-3
alanine	8.0	*24*	12.7	13.4	9.8
ammonium	5.0	*23*	152	289	212
aspartic acid	4.0	*28*	8.1	8.3	7.8
chloride	7.5[b]	*30*	9.6	21.0	191
glutamic acid	3.0	*30*	18.5	171	56.0
glutamine	43.0	*28*	9.0	1.0	5.5
isoleucine	11.0	*31*	9.5	10.5	7.8
leucine	12.0	*31*	18.0	18.6	16.1
sodium	7.5[b]	*24*	2.2	13.2	170
succinic acid	0.4	*23*	12.0	9.4	22.1
tyrosine	5.0	*31*	12.8	13.0	10.9

[a] TAV was calculated as the ratio of the concentration and the taste threshold in water. [b] Threshold concentration was determined using sodium chloride.

Table 3. Composition of the Reconstituted Wheat Gluten Hydrolysate WGH-3

compound	concn[a] (mg/L)	compound	concn[a] (mg/L)
amino acids		**amino acids**	
L-alanine	69.8	L-serine	146.4
L-arginine	106.0	L-threonine	78.8
L-asparagine	53.4	L-tryptophan	27.6
L-aspartic acid	41.2	L-tyrosine	98.8
L-cystine	19.2	L-valine	123.8
L-glutamine	347.2	**organic acids**	
L-glutamic acid	247.0	acetic acid	8.8
glycine	38.4	lactic acid	52.0
L-histidine	67.8	succinic acid	10.4
L-isoleucine	112.8	**salts**	
L-leucine	253.4	magnesium chloride hexahydrate	46.8
L-lysine	40.6	potassium chloride	6.0
L-methionine	55.6	potassium dihydrogen phosphate	58.1
L-phenylalanine	121.8		
L-proline	64.0		
L-pyroglutamic acid	267.8	sodium chloride	584.4

[a] Concentrations correspond to those of a solution of WGH-3 (10 g/L) in water.

been reported by Ishii et al. (*9*), who found a reduced bitter and a stronger umami taste in an acid-deamidated peptide fraction of an enzymatic wheat gluten hydrolysate.

The TAVs of the free amino acids including pyroglutamic acid, of inorganic cations (ammonium, calcium, magnesium, potassium, and sodium) and anions (chloride and phosphate), and of lactic, succinic, acetic, and citric acid were calculated for all three hydrolysates. **Table 2** comprises the analyzed compounds with the 10 highest TAVs of each hydrolysate. In WGH-1 and WGH-2 ammonium, glutamic acid, and leucine were the components with the highest values, and for WGH-2 chloride showed a high TAV as well. Unlike the other hydrolysates WGH-3 showed relatively high TAVs for sodium, chloride, and succinic acid. Succinic acid might have been liberated during acidic hydrolysis or might have been formed by halophilic or halotolerant microorganisms during enzymatic hydrolysis. WGH-3 was chosen for further investigation because of its superior glutamate-like taste and lower bitterness compared to the other hydrolysates.

Reconstitution of Wheat Gluten Hydrolysate WGH-3. To examine whether the analyzed substances were sufficient to mimic the taste of WGH-3, the taste profile of a reconstituted model solution containing all analyzed substances (**Table 3**) was compared to the taste profile of the original hydrolysate. The comparison (**Figure 3**) shows that the model was perceived

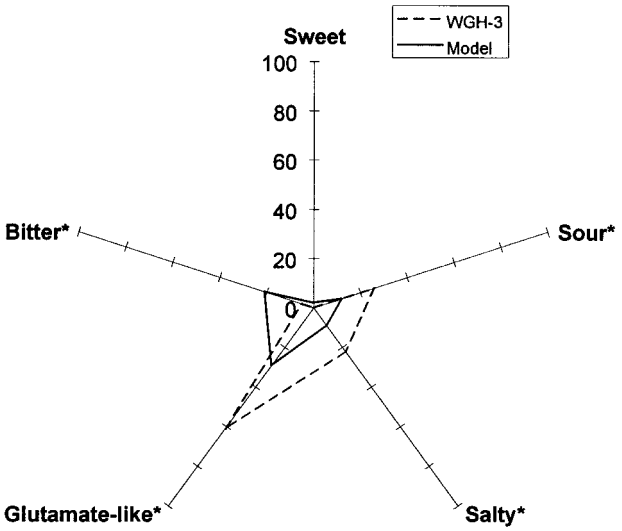

Figure 3. Taste profiles of wheat gluten hydrolysate WGH-3 and a reconstituted model solution (cf. **Table 3**). Each value represents the averaged taste intensity scored on a 100-mm line scale ranging from 0 to 100 by eight panelists. *, $p < 0.05$.

as less sour, less salty, and distinctly less glutamate-like than WGH-3. On the other hand, it had a stronger bitter note than the hydrolysate. All taste differences except for the sweet taste quality were significant ($p < 0.05$). Obviously, other components in addition to the analyzed free glutamic acid and other free amino acids, organic acids, and inorganic salts contribute to the savory taste of WGH-3.

Fractionation by Gel Permeation Chromatography and High-Performance Liquid Chromatography. An ultrafiltered solution of WGH-3 ($M_r < 3000$) was fractionated using GPC (Sephadex G10, cutoff $M_r > 700$) to localize the glutamate-like taste compounds. The column effluent was separated into seven fractions as shown in **Figure 4**. The obtained fractions were dissolved in water corresponding to a concentration of WGH-3 of 10 g/L. The sensory evaluation of the fractions revealed that the savory taste was predominant in fraction F2, followed by F4 and F3 (**Table 4**). F2 showed the most intense glutamate-like taste among all fractions and also exhibited a sour and a salty note. In contrast to the other fractions, F2 was not perceived as bitter. Fraction F3 also revealed a glutamate-like taste apart from a significantly stronger saltiness and bitterness than that of F2. In F4 the bitter taste was the most intense taste quality, although it did elicit a glutamate-like and salty note, too. F1 and F5−F7 showed few other tastes than bitterness. The fractions F2−F7 contain only low molecular weight compounds ($M_r < 700$) and, consequently, these findings indicate that low molecular weight compounds contribute to the glutamate-like taste of WGH-3. Similar results were reported for the umami taste-active constituents of other foods, such as fish protein hydrolysate, cheese, and beef bouillon (*13*, *23*, *30*). The addition of an acid-deamidated peptide fraction ($M_r = 500-1000$) from an enzymatic wheat gluten hydrolysate enhanced the glutamate-like taste of a soup stock based on fish extract (*9*).

The GPC fraction with the most intense glutamate-like taste, F2, was subdivided into nine subfractions on a semipreparative scale by RP-HPLC as shown in **Figure 5**. The subfractions were assessed sensorially to identify the ones with glutamate-like taste. The taste profiles of the HPLC subfractions F2-1−F2-9 are shown in **Table 5**. Subfractions F2-1−F2-3 each had a umami and a sour taste. The other fractions were judged to be

Table 4. Taste Profiles of Fractions Obtained by Gel Permeation Chromatography[a]

taste attribute	F1	F2	F3	F4	F5	F6	F7
sweet	4 ± 3	0 ± 0	0 ± 0	0 ± 0	2 ± 1	1 ± 1	3 ± 3
sour	0 ± 0^{b}	19 ± 9^{c}	0 ± 0^{b}	0 ± 0^{b}	4 ± 3^{b}	1 ± 1^{b}	0 ± 0^{b}
salty	0 ± 0^{b}	19 ± 4^{c}	28 ± 8^{d}	19 ± 4^{c}	1 ± 1^{b}	0 ± 0^{b}	0 ± 0^{b}
glutamate-like	1 ± 1^{b}	46 ± 19^{d}	21 ± 6^{c}	24 ± 12^{c}	6 ± 4^{b}	0 ± 0^{b}	0 ± 0^{b}
bitter	14 ± 7^{c}	0 ± 0^{b}	16 ± 8^{c}	28 ± 11^{d}	16 ± 7^{c}	6 ± 3^{b}	32 ± 15^{d}

[a] Data are expressed as taste intensities (means) scored on a 100-mm line scale (ranging from 0 = absent to 100 = very strong) by eight trained panelists. [b−d] Means within a row showing different letters are significantly different ($p < 0.05$).

Table 5. Taste Profiles of HPLC Subfractions from Wheat Gluten Hydrolysate WGH-3[a]

taste attribute	F2-1	F2-2	F2-3	F2-4	F2-5	F2-6	F2-7	F2-8	F2-9
sweet	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	2 ± 1^{c}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}
sour	29 ± 9^{e}	10 ± 6^{d}	14 ± 6^{d}	$8 \pm 6^{c,d}$	0 ± 0^{b}	$4 \pm 3^{b,c}$	0 ± 0^{b}	2 ± 1^{b}	0 ± 0^{b}
salty	22 ± 14^{c}	3 ± 3^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	1 ± 0^{b}	0 ± 0	0 ± 0^{b}
glutamate-like	49 ± 17^{d}	15 ± 5^{c}	10 ± 5^{c}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	1 ± 1^{b}	0 ± 0^{b}	0 ± 0^{b}
bitter	0 ± 0^{b}	$15 \pm 8^{d,e}$	$5 \pm 3^{b,c}$	$9 \pm 6^{c,d}$	22 ± 10^{e}	20 ± 10^{e}	31 ± 11^{f}	$16 \pm 10^{d,e}$	$8 \pm 4^{b,c,d}$

[a] Data are expressed as taste intensities (means) scored on a 100-mm line scale (ranging from 0 = absent to 100 = very strong) by eight trained panelists. [b−f] Means within a row showing different letters are significantly different ($p < 0.05$).

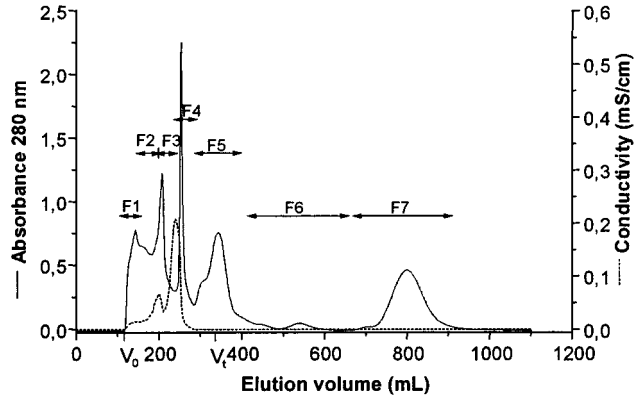

Figure 4. Gel permeation chromatogram of wheat gluten hydrolysate WGH-3 ($M_r < 3000$; 2.0 g). V_0 and V_t are the void and the total volume, respectively, of the packed gel bed.

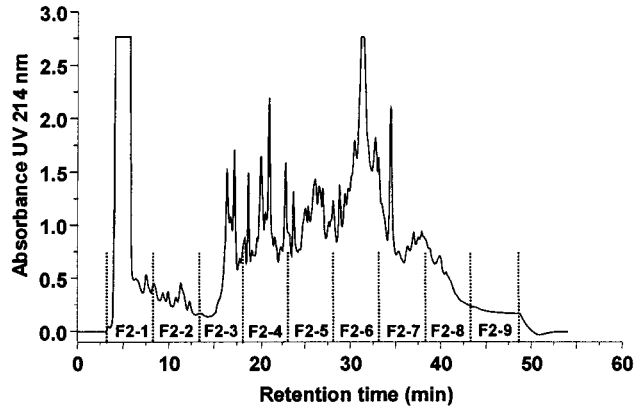

Figure 5. RP-HPLC chromatogram of the semipreparative subfractionation of the taste-active fraction F2.

predominantly bitter. Subfraction F2-1 represents the void volume of the chromatogram and hence contains compounds that are not retained by the reversed phase column (RP-C18), such as polar amino acids. Most of the amino acids in F2-1 were found to be present in their free form, the ratio of free amino acids to peptides being ~3 (data not shown). On the other hand, subfractions F2-2−F2-9 supposedly contain predominantly

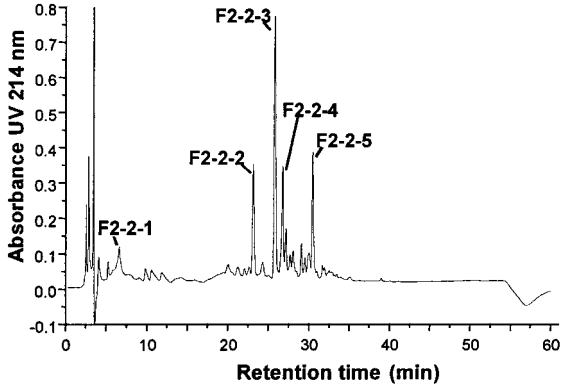

Figure 6. Rechromatography of HPLC subfraction F2-2.

peptides. Further characterization was focused on the peptides in the HPLC fraction F2-2, which has a pronounced glutamate-like taste.

Characterization of Peptides in WGH-3. Fraction F2-2 was further subfractionated by RP-HPLC, and the eluting compounds corresponding to the major peaks were collected (**Figure 6**). The amino acid compositions of the freeze-dried subfractions F2-2-1−F2-2-5 were determined after acidic hydrolysis, and they were analyzed by ESI-MS/MS. The results are shown in **Table 6**. All subfractions contained Glx except for F2-2-1, which was identified as free leucine on the basis of the amino acid analysis and the MS spectra. The peptide sequence analyses of the subfractions F2-2-2−F2-2-5 revealed that the N termini of all peptides were blocked and did not react with phenyl isothiocyanate during Edman degradation, insinuating a pyroglutamyl residue at the N terminus which is known for blocking the Edman degradation (*32*, *33*). These findings are in accordance with the proposed peptide sequences containing an N-terminal pyroglutamyl residue (**Table 6**). The conclusion is further corroborated by the pseudomolecular ions $(M + H)^+$, which correspond to the sum of the constituting amino acid residues minus one water molecule. The product ion spectra of the peptides in F2-2 obtained by ESI-MS/MS are given in **Figure 7**. Interestingly, all peptides display the same product ion *m*/*z* 209. The product ion spectrum of F2-2-2 (**Figure 7A**) shows the fragment ions *m*/*z* 296, 209, and 106 corresponding to the loss of water and to the loss of a C-terminal serine,

Table 6. Amino Acid Composition and Proposed Peptide Sequences of Selected Peptides in HPLC Subfraction F2-2

subfraction[a]	amino acid composition[b]	parent ion (*m*/*z*)[c]	product ions by ESI-MS/MS (*m*/*z*)[c]	proposed peptide sequence[d]
F2-2-1	Leu	132.0	86 (immonium ion $NH_2{=}CHR^+$ of Leu), 44	Leu
F2-2-2	Glx, Ser, Pro	314.0	296 $(M - H_2O)$, 209 $(M - Ser)^+$, 106 $(Ser + H)^+$	pGlu-Pro-Ser
F2-2-3	Glx, Pro	227.0	209 $(M - H_2O)^+$, 181 $(209 - CO)^+$, 116 $(Pro + H)^+$, 70 (immonium ion $NH_2{=}CHR^+$ of Pro)	pGlu-Pro
F2-2-4	$(Glx)_2$, Pro	356.0	338 $(M - H_2O)^+$, 209 $(M - Glu)^+$, 148 $(Glu + H)^+$	pGlu-Pro-Glu
F2-2-5	$(Glx)_2$, Pro	355.1	337 $(M - H_2O)^+$, 209 $(M - Gln)^+$, 147 $(Gln + H)^+$, 130 $(147 - NH_3)^+$	pGlu-Pro-Gln

[a] HPLC fraction F2-2 was rechromatographed and the subfractions representing single peaks were collected manually (numbering refers to **Figure 6**). [b] Amino acid composition results from the amino acid analysis of the fraction after acidic hydrolysis. [c] Measured MS and MS/MS signals, respectively, obtained in the positive mode by electrospray ionization. [d] Peptide sequences were tentatively identified on the basis of the amino acid composition and MS data.

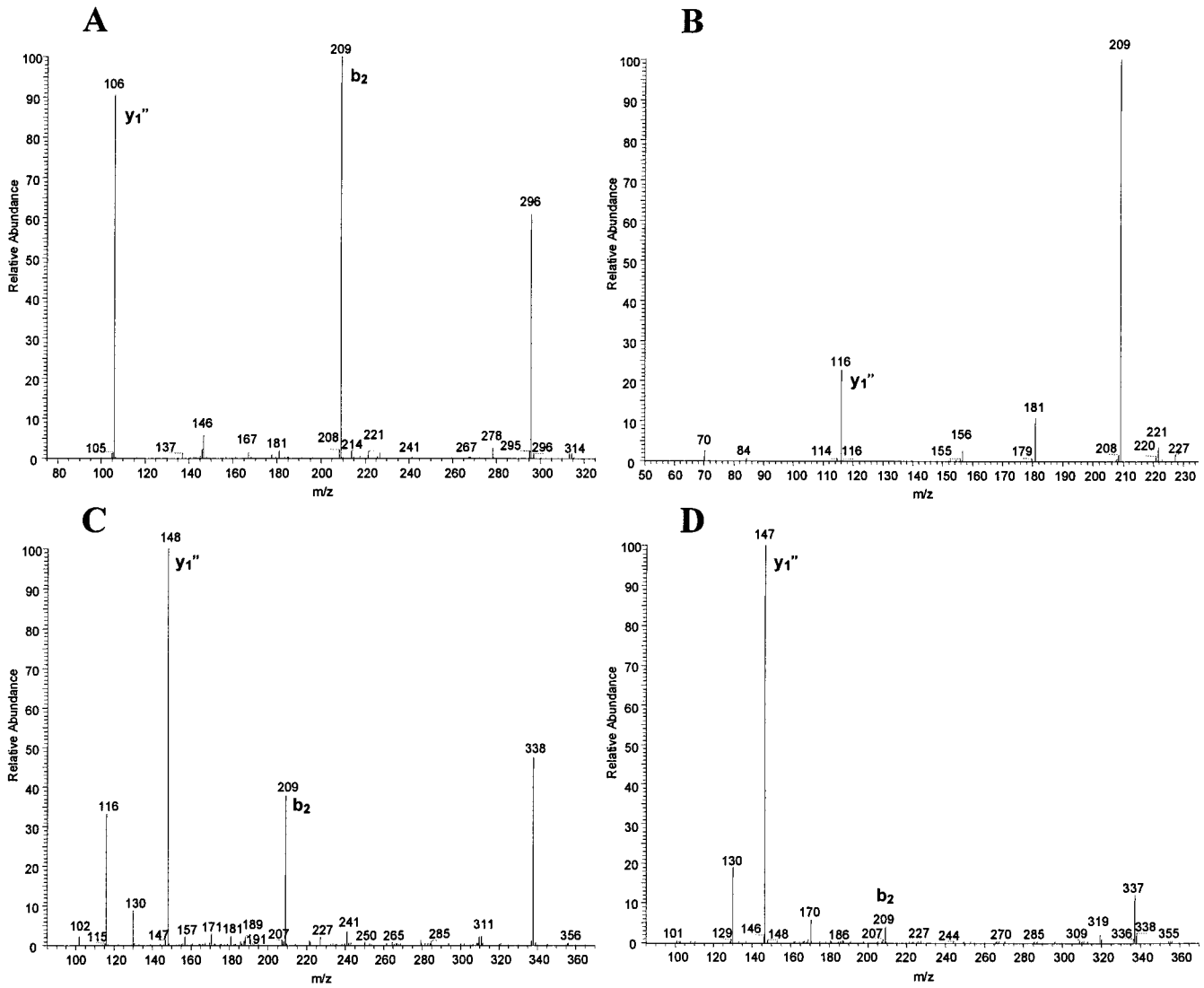

Figure 7. Product ion spectra of the tentatively identified pyroglutamyl peptides in F2-2: pGlu-Pro-Ser (*m*/*z* 314, A), pGlu-Pro (*m*/*z* 227, B), pGlu-Pro-Glu (*m*/*z* 356, C), and pGlu-Pro-Gln (*m*/*z* 355, D). The fragment ion types *b* and *y* are named according to the nomenclature proposed by Roepstorff and Fohlman (*36*).

respectively. On the basis of the amino acid composition and the MS/MS data the peptide sequence for F2-2-2 (*m*/*z* 314) was tentatively identified as pGlu-Pro-Ser. The product ion spectrum of F2-2-3 (**Figure 7B**) is dominated by the intense fragment ion *m*/*z* 209 corresponding to a loss of water from the pseudomolecular ion *m*/*z* 227 and shows further product ions *m*/*z* 116 and 70 corresponding to a C-terminal proline and the immonium ion $(NH_2{=}CHR)^+$ of proline (*34*). Ginz and Engelhardt (*35*) recently examined all 20 proteinaceous amino acids by ESI-MS/MS and found the product ion *m*/*z* 70 only with proline. It is concluded from the MS data and the amino acid analysis that subfraction F2-2-3 is pGlu-Pro.

The peptide sequence of F2-2-4 (*m*/*z* 356) is proposed as pGlu-Pro-Glu (**Figure 7C**). The product ion spectrum shows a predominant signal *m*/*z* 148 corresponding to a C-terminal glutamic acid residue (y_1'' ion) and again the product ion *m*/*z* 209 for the b_2 ion of the tentative peptide sequence. The fragment ions *y* and *b* are named according to the nomenclature proposed by ref *36*. In the MS/MS spectrum of F2-2-5 (*m*/*z* 355, **Figure 7D**) *m*/*z* 147 is the major product ion corresponding

to a C-terminal glutamine, and again, albeit weak, a signal at *m*/*z* 209 is present, suggesting the same N-terminal sequence pGlu-Pro- as the other subfractions. The fragment ion *m*/*z* 130 probably results from the loss of ammonia, which is common for glutamine (*34*). The peptide was tentatively identified as pGlu-Pro-Gln. The tentatively identified pyroglutamyl peptides are likely to have been formed during the pasteurization of the hydrolysates from the corresponding N-terminal glutamine residues, which are known to readily cyclize to pyroglutamic acid (*32*, *33*). Sato et al. (*37*) also found pyroglutamyl peptides in an enzymatic wheat gluten hydrolysate composed of proline, glycine, and Glx, that is, glutamine, pyroglutamic acid, or glutamic acid. A database search of the respective N-terminal glutamine peptide sequences Gln-Pro, Gln-Pro-Ser, Gln-Pro-Gln, and Gln-Pro-Glu, in wheat gliadin and glutenin sequences, obtained as a subset of protein sequences from the SWISS-PROT database (*38*), revealed numerous occurrences for the sequence motifs -Gln-Pro-, -Gln-Pro-Ser-, and -Gln-Pro-Gln-, in wheat gliadin and glutenin, for example, in wheat gliadins B-II and B-III. The motif -Gln-Pro-Glu- was also found several times, for example, in the wheat glutenin high molecular weight subunit 12, DY10, and PW212 precursors. The sequence element -Gln-Pro-Glu-, however, could have been also formed during acidic deamidation of the corresponding glutaminyl residue of the sequence motif -Gln-Pro-Gln-.

The bitterness of fraction F2-2 might be due to the free L-leucine, which is known to produce a bitter taste (*28*, *30*). Koibuchi et al. (*39*) described pyroglutamyl peptides consisting of two to five amino acid residues to reduce and to mask bitterness of foods and beverages and to impart an acid taste. A bitter-tasting hydrophobic peptide containing N-terminal glutamine was isolated from Cheddar cheese (*40*). The bitter taste disappeared upon cyclization of the glutaminyl to a pyroglutamic acid residue. Peptides composed of fewer than 10 amino acid residues that contain L-glutamine were also reported to remove bitter taste from foods (*41*). On the basis of these findings it is concluded that the tentatively identified pyroglutamyl peptides contribute to the taste of fraction F2-2 and that they partly reduce the bitterness elicited by the bitter-tasting free leucine.

Analysis of other fractions is currently under investigation.

ABBREVIATIONS USED

ESI-MS, electrospray ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FPLC, fast protein liquid chromatography; GPC, gel permeation chromatography; GTU, glutamate transformation unit; LAPU, leucine aminopeptidase unit; TAV, taste activity value; WGH, wheat gluten hydrolysate.

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