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Debittering Mechanism of Bitter Peptides from Milk Casein by Wheat Carboxypeptidase

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The bitter peptide fraction obtained from the peptic hydrolysate of casein was treated with crystalline wheat carboxypeptidase. The bitterness of the bitter fraction lessened with an increase in free amino acids. The enzymatic hydrolysate obtained from the digest of the bitter peptide fraction by wheat carboxypeptidase was chromatographed on Sephadex G-15, and the eluate was subjected to amino acid analysis. When the release percentage of total free amino acids was approximately 30%, those of hydrophobic amino acids with a Δf value (cal/mol) >1600 were 32–76%, and amino acids with a Δf value <1600 were scarcely released except for alanine and threonine. The wheat carboxypeptidase seems to have an ability to eliminate bitter taste in enzymatic protein hydrolysate by releasing hydrophobic amino acids from bitter peptides.

Enzymatic hydrolysates of various proteins have a bitter taste which may be one of the main hindrances to their utilization in food. Though unutilized protein from soybean (Fujimaki et al., 1968), fish (Fujimaki et al., 1973; Hevia et al., 1976), etc. have been modified by the treatment with proteolytic enzymes for the purpose of improving their solubility, heat stability, and resistance to precipitation in acidic environments, the use of their enzymatic hydrolysates has been limited owing to the presence of bitter flavor components. Some kinds of cheese such as Cheddar cheese rarely have a bitter taste in matured or finished products (Nelson, 1975; Hamilton et al., 1974).

Many bitter peptides have been identified and their structures estimated in enzymatic hydrolysates of soybean protein (Fujimaki et al., 1970b) and casein (Matoba et al., 1970; Clegg et al., 1974). The bitterness of peptides appears to be closely related to the contents and sequence of hydrophobic amino acids (Clegg et al., 1974).

Arai et al. (1970) showed that the bitterness of peptides from soybean protein hydrolysates was decreased by treatment of *Aspergillius* acid carboxypeptidase. It was reported that an endopeptidase-catalyzed reverse reaction, usually called the plastein reaction, was effective for debittering (Fujimaki et al., 1970a). Two debittering methods based on the removal of hydrophobic peptides from enzymatic protein hydrolysates have been proposed. Studies by Roland et al. (1978) showed that a nonbitter peptide fraction was selectively prepared from soy and casein protein hydrolysates by hydrophobic chromatography.

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Lalasidis and Sjöberg (1978) reported that bitter compounds of enzymatic protein hydrolysates were removed by the extraction of azeotropic secondary butyl alcohol.

We have isolated and crystallized carboxypeptidase from wheat and characterized its enzymatic properties (Umetsu et al., 1981). The enzyme liberated a wide range of amino acid residues containing proline from the carboxyl terminus of various peptides and proteins. Contaminants such as micotoxins may make a microbial enzyme less suitable for food application than an enzyme of plant origin. This paper describes the debittering mechanism of bitter peptides from peptic casein hydrolysates by wheat carboxypeptidase.

MATERIALS AND METHODS

Preparations of Bitter Peptide Fraction. A bitter peptide fraction from milk casein was prepared according to the method of fractionation of bitter peptides from the peptic hydrolysate of soybean protein (Fujimaki et al., 1970b) with a slight modification. Casein (Merck, according to Hammarsten; 25 g) in distilled water (1000 mL) was adjusted to pH 1.6 with HCl and incubated with pepsin (Sigma Chemicals Co., 2× crystallized; 100 mg) at 37 °C for 12 h. The enzymatic reaction was stopped by neutralization with NaOH. The peptic hydrolysate was ultrafiltrated with hollow fiber (Asahikasei Co., Ltd., Tokyo), and the ultrafiltrate was evaporated under reduced pressure. The evaporated fraction was applied to the column of Sephadex G-15 (Pharmacia Fine Chemicals; 2 × 80 cm), and elution was carried out with distilled water at 4 °C. The bitter fraction was collected and freeze-dried. The bitterness of 2% aqueous solution of the bitter peptides fraction corresponded to that of 5×10^{-3}–2.5×10^{-3}% phenylthiourea solution.

Preparation of Carboxypeptidase from Wheat. Crystalline carboxypeptidase from wheat was prepared according to a previously published method (Umetsu et al., 1981). The enzyme used here was homogeneous in disc electrophoresis at pH 4.0 and analytical ultracentrifugation.

Gel Chromatography of the Digest of the Bitter Peptide Fraction by Carboxypeptidase. The bitter peptide hydrolysate obtained by treatment of wheat carboxypeptidase was applied to the column of Sephadex G-15 (2 × 80 cm). Elution was carried out with distilled water at 4 °C. The peptide distribution in the eluate was determined by measuring the absorbance at 280 nm and carrying out the ninhydrin reaction (Cocking and Yemm, 1954) and the biuret reaction (Goa, 1953). Bovine serum albumin, Gly-Gly-Gly, and glycine were used as standards of known molecular weight.

Amino Acid Analysis. Amino acid compositions were determined by hydrolysis in 6 N HCl under vacuum at 105 °C for 22 h, followed by application to an automatic amino acid analyzer, Hitachi Model 835-30, according to the method of Moore and Stein (1963). The free amino acids were determined in the same manner but without HCl treatment. The mean numbers of amino acid residues of peptide fraction were obtained by dividing the total amino acid by the carboxyl-terminal amino acid. The total amino acid or carboxyl-terminal amino acid was determined by ninhydrin color with or without 6 N HCl treatment. The quantities of amino acid on each fraction of the eluate on Sephadex G-15 chromatography were caluculated by multiplying the quantity of amino acid at each peak of the fraction by the area of each fraction which was determined by ninhydrin color.

Sensory Test on Bitterness. The peptide solution (0.25 mL) was compared with 0.25 mL of a standard aqueous phenylthiourea solution (10^{-1}–10^{-6}%) for bitterness. The degree of bitterness is expressed in percent concentration of phenylthiourea.

Table I. Amino Acid Composition of Bitter Peptide Fraction and Its Enzymatic Hydrolysate Fractions Obtained from the Chromatography on a Column of Sephadex G-15

amino acid	amino acid from bitter peptide fraction, μmol,[a] fraction I[b] (16)[c]	amino acid recovered in the enzymatic hydrolysates obtained from the bitter peptide fraction, μmol[a] fraction II (15)	fraction III (2)	fraction IV (1)	fraction V (1)	fraction VI (1)
Asp	37.8	27.0	5.4	tr		
Thr	29.7	15.3	0.3	14.7 (49)[d]		
Ser	47.4	27.6	2.4	0.3		
Glu	82.2	62.1	20.1	tr		
Pro	66.8	34.5	4.5	26.4 (10)		
Gly	13.5	8.1	1.5	tr		
Ala	22.5	10.2	1.2	13.2 (59)		
Cys	1.5					
Val	38.7	17.1	3.6	15.3 (40)		
Met	19.2	5.4				
Ile	36.6	21.3	1.8	11.7 (32)		
Leu	57.3	12.0	1.5	43.3 (76)		
Tyr	12.0	3.0	0.9			8.0 (68)
Phe	16.2	4.5	1.2	0.3	10.1 (63)	
Lys	21.7	18.3	2.4	0.3		
His	6.3	5.3	0.6			
Arg	6.5	5.1	0.9			

[a] The quantity of amino acid is expressed as μmol/100 mg of bitter peptide. [b] Fractions I–VI correspond to the eluate fractions of the chromatography on Sephadex G-15 in Figure 2B. [c] The value in parentheses denotes the mean number of residues of given amino acid in the fraction. [d] The values in parentheses denote the percentage of amino acids released from the bitter peptide fraction (fraction I) by wheat carboxypeptidase.

RESULTS AND DISCUSSION

As shown in Figure 1, the bitterness of the peptide fraction lessened with the release of free amino acids during incubation with wheat carboxypeptidase. Though the bitterness of the peptide fraction was equivalent to that of 5×10^{-3}–2.5×10^{-3}% aqueous solution, it decreased significantly after incubation for 1 h with wheat carboxypeptidase. After incubation for 4 h this bitterness was no more than that of 10^{-5}–5×10^{-6}% aqueous solution.

Figure 2 illustrates the Sephadex G-15 elution profile of the bitter peptide fraction and the enzymatic hydrolysate obtained from the digest of the bitter peptide fraction by wheat carboxypeptidease. The bitter peptide fraction showed a single peak (fraction I), and after the digestion of the bitter peptide fraction (fraction I) the enzymatic hydrolysate was divided into five fractions (fractions II, III, IV, V, and VI).

The amino acid composition of each fraction obtained from the Sephadex G-15 elution is shown in Table I. The sum of the quantities of amino acids of fractions II–VI agreed approximately with that of the bitter peptide fraction (fraction I). The number of amino acid residues of fraction I was 16, and those of fractions II and III were 15 and 2, respectively. Fractions IV–VI were identified to be free amino acids. This result indicates that the bitter peptide fraction (fraction I) consisted of two groups: one

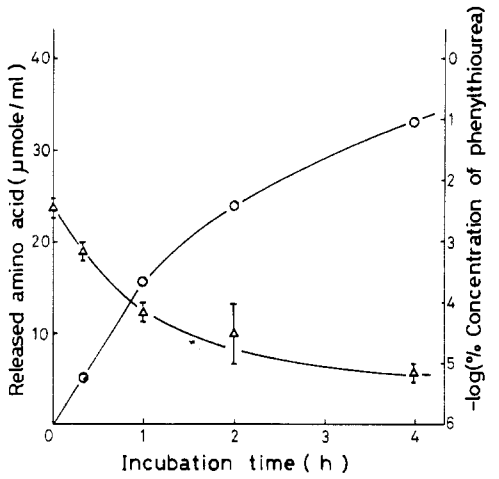

Figure 1. Correlation between released amino acids (O) and the bitterness (Δ) during the hydrolysis of the bitter peptide fraction by wheat carboxypeptidase. The substrate was hydrolyzed with the enzyme under the following conditions: substrate concentration, 2%; enzyme/substrate ratio, 1/50 (w/w); pH 5.1; temperature, 30 °C; incubation time, 0.5 1, 2, and 4 h. After the scheduled periods of time, the reaction was stopped to measure free amino acids and bitterness.

Table II. Correlation between the Δf Value and Percentages of Amino Acids Released from the Bitter Peptide Fraction by Wheat Carboxypeptidase at pH 5.1 and 30 °C

amino acid	Δf,[a] cal/mol	amino acid released by carboxypeptidase, %
Trp	3000	
Ile	2970	32
Tyr	2870	68
Phe	2650	63
Pro	2620	40
Leu	2420	76
Val	1690	40
Lys	1500	0.5
Met	1300	0
Ala	730	59
Arg	730	0
Glu	550	tr
Asp	540	tr
His	500	0
Thr	440	49
Ser	40	0.6
Gly	0	tr

[a] Δf = hydrophobicities of the side chain of amino acid residues [data from Tanford (1962)].

was easily hydrolyzed by wheat carboxypeptidase and passed into dipeptide fraction (fraction III) and amino acids; another was scarecely attacked by the enzyme followed by decreasing the number of amino acid residue from 16 to 15. The average release percentage of total free

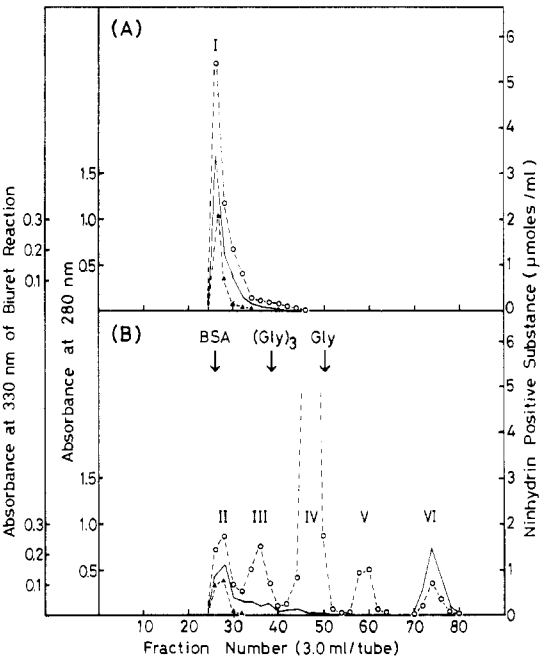

Figure 2. (A) Chromatogram of the bitter peptide fraction on a column of Sephadex G-15. Two milliliters of 5% bitter peptide solution was applied to the column. (▲) Absorbance at 330 nm of the biuret reaction; (—) absorbance at 280 nm; (O) ninhydrin-positive substance. (B) Chromatogram of the bitter peptide hydrolysates by wheat carboxypeptidase on a column of Sephadex G-15. The bitter peptide fraction was hydrolyzed with the enzyme under the following conditions: substrate concentration, 5%; enzyme/substrate ratio, 1/700 (w/w); pH 5.1; temperature, 30 °C; incubation time, 60 h. Two milliliters of the reaction mixture was applied to the column. BSA and $(Gly)_3$ denote bovine serum albumin and Gly-Gly-Gly, respectively. Other symbols are the same as in (A). Eluate fractions are designated as I, II, III, IV, V, and VI.

amino acids from the bitter peptide fraction by the enzyme was approximately 30%. On individual amino acids, the release contents of threonine, proline, alanine, valine, isoleucine, leucine, tyrosine, and phenylalanine were high, whereas aspartic acid, serine, glutamic acid, glycine, lysine, histidine, and arginine were scarcely released.

Table II shows the correlation between the Δf value and the percentage of various amino acids released from the bitter peptide fraction by wheat carboxypeptidase. The release percentage of amino acids with a Δf value >1600 cal/mol were 32–76%, while amino acids with a Δf value <1600 cal/mol except alanine and threonine were hardly released. This result indicates that the hydrophobic amino acids with a Δf value >1600 cal/mol is mainly released from the bitter peptide fraction by the enzyme with a decrease in bitterness.

The bitterness and Q value of bitter peptide fraction and its enzymatic hydrolysate fraction by wheat carboxy-

Table III. Bitterness and Q Value of the Bitter Peptide Fraction and Its Enzymatic Hydrolysate Fractions Obtained from the Chromatography on a Column of Sephadex G-15

	bitter peptide, fraction I[a]	enzymatic hydrolysates				
		fraction II	fraction II	fraction IV	fraction V	fraction VI
fraction no.[b]	26	28	36	46	60	74
NAR[c]	16	15	2	1	1	1
bitterness[d]	5×10^{-4}–10^{-4}	10^{-5}–10^{-6}	10^{-5}–10^{-6}	10^{-6}	10^{-6}	10^{-6}
Q value[e]	1400	1100	1010			

[a] Fractions I-VI denote the eluate fractions on the Sephadex G-15 chromatography in Figure 2B. [b] Fraction number is expressed as the peak of each fraction on Sephadex G-15 chromatography. [c] NAR = numbers of amino acid residues. [d] The degrees of bitterness is expressed in percent concentration of phenylthiourea. [e] Q value = $\Sigma \Delta f$/numbers of amino acid residues [data from Ney (1971)].

peptidase obtained from the Sephadex G-15 elution are shown in Table III. Ney (1971) reported that peptides with a Q value <1300 were not bitter and peptides with a Q value >1400 gave a bitter taste. The bitterness and Q value of fractions II and III apparently decreased in comparison with those of the bitter peptide fraction (fraction I). This result coincides with the prediction of bitterness of peptide described by Ney (1971). The more bitter peptides with a Q value >1400 in the bitter peptide fraction may be easily hydrolyzed by the enzyme and passed into dipeptide fraction (fraction III) and free amino acid fractions. The dipeptide fraction (fraction III), in which glutamic acid was enriched, is expected to have a masking effect for bitter taste as reported by Noguchi et al. (1972). As result of the enzymatic reaction, the bitterness of the bitter peptide fraction, on the whole, lessened.

It is concluded that wheat carboxypeptidase acts on the bitter peptides and releases hydrophobic amino acids from their carboxyl termini followed by a decrease in bitterness. Wheat carboxypeptidase may be useful for debittering protein hydrolysates in food application.

Registry No. Carboxypeptidase, 9031-98-5.

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Redistribution of Oxamyl from Treated Seeds to Peach Seedlings and Soil As Determined by High-Performance Liquid Chromatography

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Movement and degradation of oxamyl in peach seeds, seedlings, soil, and pots were studied. The determination of oxamyl and oxime was made by reverse-phase high-performance liquid chromatography using a UV detector; for analysis of seeds, soil, and pots no cleanup was required, but for roots and leaves cleanup procedures were essential. Peach seeds with endocarps removed were coated with oxamyl and planted individually in sterilized soil in clay pots. After 1 week, 5.7 and 1.4 ppm of oxamyl were found in the soil and clay pots, respectively. The corresponding oxime, methyl N-hydroxy-N',N'-dimethyl-1-thiooxamimidate, was also found, but intact oxamyl constituted 97% and 52% of the total residue in the soil and pots, respectively. There was no evidence of oxamyl degradation on the treated seeds 3 weeks after planting. Residues of oxamyl in leaves 2 and 3 weeks after sowing were 4.8 and 2.7 ppm, respectively; similarly, oxime residues were 4.3 and 4.8 ppm.

Oxamyl, methyl N',N'-dimethyl-N-[(methylcarbamoyl)oxy]-1-thiooxamimidate, is known to control a wide range of insect and nematode pests (Bromilow, 1976). Although it is ambimobile (Peterson et al., 1978), it would be more practical to rely upon its downward movement for protection of a crop from nematodes because spray application to the foliage is easier than soil application of the pesticide.

However, application of a pesticide to seed would be more efficient and more practical than soil treatment. Seeds of cereals, vegetables, soybeans, cotton, and alfalfa have been treated with oxamyl (Prasad and Rao, 1976; Rodriquez-Kabana et al., 1977; Truelove et al., 1977; Hoveland et al., 1977; Townshend and Potter, 1979) to provide protection against nematodes during and after germination. So that the fate of oxamyl applied to individual seeds can be studied, a larger seed than those mentioned above is desirable; consequently, peach seeds with their endocarps removed were chosen for study.

If it is assumed that peach seeds were planted with endocarps removed, seed treatment with oxamyl should protect the seed and roots from nematode attack at the very beginning of root formation when roots are most susceptible to nematodes (Koch, 1955). Furthermore, the active ingredient taken up by the plant should work as an insecticide to protect the leaves.

In the past, several analytical methods for oxamyl residues, a GC method (Holt & Pease, 1976), a spectropho-

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