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Debittering of Protein Hydrolysates Using *Aeromonas caviae* Aminopeptidase

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The bitter-tasting peptide solutions prepared from the protease hydrolysate of milk casein and soy protein were treated with aminopeptidase produced by *Aeromonas caviae* T-64. The bitterness of these solutions were significantly reduced with an increase in the amount of released free amino acids. Hydrophobic amino acids having Δf values more than 1500 cal/mol, such as valine, isoleucine, leucine, tyrosine, and phenylalanine, accounted for more than 76% of the free amino acids released by the aminopeptidase. The results suggest that the enzyme hydrolyzed bitter peptides containing hydrophobic amino acids in the N-terminal region and the bitterness of the peptides were reduced by removal of these amino acids.

Keywords: *Debittering; protein hydrolysate; Aeromonas aminopeptidase; bitter peptide*

INTRODUCTION

Enzymatic hydrolysates of various proteins frequently exhibit bitter taste caused by bitter peptides (Matoba et al., 1970; Clegg and Lim, 1974; Bumberger and Belitz, 1993). This limits their utilization in the food industry. Because this bitterness closely correlates with the hydrophobicity of the peptides (Clegg and Lim, 1974), there have been several attempts to reduce bitterness by hydrolyzing the bitter peptides with exoproteases (Umetsu et al., 1983, 1988; Minagawa et al., 1989). We have purified and characterized the aminopeptidase from *Aeromonas caviae* T-64 (Izawa et al., 1996) and revealed that this enzyme hydrolyses the peptides containing hydrophobic amino acid residues at the N-terminal and/or adjacent position with high efficiency (K_{cat}/K_m). In this report, we describe the bitterness-reducing ability of this aminopeptidase when used in milk casein and soy protein hydrolysates.

MATERIALS AND METHODS

Preparation of Protein Hydrolysates. Milk casein (Alacid720, Japan Protein Co., Tokyo, Japan) and soy protein (Type SE, Protein Technologies International Co., St. Louis, MO) were suspended in distilled water (5% w/v), and the pH's of the solutions were adjusted to 8.0 with 2 N NaOH and 2.0 with 2 N HCl, respectively. The casein solution (1000 mL) and soy protein solution (1000 mL) were incubated with 20 mg of trypsin (Type IX, Sigma Chemical Co., St. Louis, MO) and 500 mg of pepsin (1:1000, Sigma Chemical Co.), respectively, at 37 °C for 2 h. After hydrolysis, the pH's of the reaction mixtures were adjusted to 4.0 with 2 N NaOH or 2 N HCl, and then the enzymic reactions were terminated by heating at 85 °C for 15 min. The protein hydrolysate were centrifuged at 3000*g* for 20 min, and the supernatants were used in further experiments.

Preparation of Bitter-Tasting Solutions. On the basis of sensory evaluation, the supernatants obtained from the hydrolysates were diluted with Tris HCl (5 mM, pH 8.0) to exhibit equivalent bitterness corresponding to that of a 2% glycylphenylalanine solution and designated as BTmc and BTsp, respectively. The peptide concentrations of BTs (BTmc and BTsp) were determined by the Kjeldahl method.

Treatment of BTs with *Aeromonas* Aminopeptidase. *Aeromonas* aminopeptidase purified to homogeneity and having a specific activity of 55 units/mg was used throughout this experiment. BTs were hydrolyzed with the aminopeptidase at 30 °C at pH 8.0 for 1, 3, 6, and 21 h. After the scheduled periods of time, reaction was terminated by heating at 85 °C for 15 min.

Sensory Evaluation. The remaining bitterness of the aminopeptidase-treated BTs was assayed by five trained

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Table 1. Free Amino Acid Compositions (nmol/mL) Released by the Aminopeptidase Treatments in BTs after Incubation for 1, 6, and 21 h

	BTmc									BTsp		
	E/S = 1/16000			E/S = 1/8000			E/S = 1/4000			E/S = 1/8000		
amino acid	1 h	6 h	21 h	1 h	6 h	21 h	1 h	6 h	21 h	1 h	6 h	21 h
Asp	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Thr	<5	<5	12	<5	5	62	6	70	265	<5	17	30
Ser	<5	23	29	12	32	26	22	28	44	<5	11	8
Glu	<5	<5	<5	<5	<5	<5	<5	<5	13	<5	<5	<5
Gly	<5	<5	<5	<5	<5	<5	<5	<5	16	<5	<5	31
Ala	<5	<5	5	<5	6	26	7	31	137	<5	10	10
Cys	<5	<5	<5	<5	<5	6	<5	6	26	<5	<5	<5
Val	25	68	148	53	148	397	170	414	715	44	149	263
Met	<5	12	25	7	21	91	20	94	179	18	37	62
Ile	22	54	103	38	98	253	92	268	572	68	200	303
Leu	66	149	314	103	288	701	245	734	1126	154	405	612
Tyr	<5	<5	<5	<5	<5	60	32	70	192	6	58	163
Phe	76	130	177	111	176	370	165	330	449	60	200	367
Lys	<5	<5	14	<5	<5	31	<5	26	204	<5	6	22
His	<5	<5	<5	<5	<5	19	<5	10	153	<5	<5	<5
Arg	<5	<5	<5	<5	<5	102	<5	99	167	<5	<5	10
Pro	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Total	189	436	828	324	773	2145	758	2180	4259	349	1094	1881

panels by comparing with a series of untreated BTs solutions (0, 20, 40, 60, 80, and 100%). The bitter scores of these solutions were graded to be 0, 2, 4, 6, 8, and 10, respectively.

Amino Acid Analysis. The amount of amino acids released by the aminopeptidase treatment was monitored by the ninhydrin method. The free amino acid compositions in the enzyme-treated hydrolysate were determined by an amino acid analyzer (Model L-8500, Hitachi Co., Tokyo, Japan) after removal of protein and large peptides with sulfosalicylate (1%) precipitation.

RESULTS AND DISCUSSION

The supernatant of casein hydrolysate had less bitterness than that of soy protein. Therefore, the supernatants of casein hydrolysate and soy protein were diluted 2.7 and 4.4 times, respectively to obtain BTmc and BTsp (which exhibited equivalent bitterness corresponding to that of a 2% glycylphenylalanine solution). The peptide concentrations of BTmc and BTsp were 0.84 and 0.42%, respectively. The relationship between the bitterness of the aminopeptidase-treated BTs and the amount of released free amino acids is shown in Figure 1. As incubation time progressed, the bitterness in the enzyme reaction mixtures significantly decreased ($P < 0.005$) with an increase in free amino acids. After incubation for 21 h, the bitterness of BTsp was less than score 1, which corresponds to threshold concentration for bitter taste (Figure 1A). As shown in Figure 1B−D, incubation of BTmc with a series of enzyme/substrate ratios (1/16000, 1/8000, and 1/4000 (w/w, respectively)) resulted in a release of free amino acids. For the enzyme/substrate ratio of 1/8000 and 1/4000, the bitter score decreased during the first 6 h of incubation; however, a further 15 h of incubation did not result in any further reduction in bitter score. In contrast to this, the amount of free amino acids continued to increase after the initial 6 h of incubation.

The free amino acid compositions released by the aminopeptidase treatment in each BT (1, 6, and 21 h) are shown in Table 1. Amino acids having Δf values above 1500 cal/mol (Tanford, 1962), such as valine, isoleucine, leucine, tyrosine, phenylalanine, and lysine, accounted for more than 76% of total free amino acids in any stage of both enzyme treated BTs. This result reflects the substrate specificity of *Aeromonas* aminopeptidase, which hydrolyzes peptides containing hydrophobic residues in N-terminal and/or adjacent position with high hydrolysis efficiency (values of K_{cat}/K_m for Leu-Phe, Ile-Phe, Phe-Phe, Val-Phe, and Tyr-Phe are 820, 113, 75, 19, and 1.6 mM s^{-1}, respectively, while those for Ala-Phe, Ser-Phe, and Gly-Phe are 0.09, 0.02, and less than 0.001 mM s^{-1}, respectively). According to Ney (1971), the most important factor in determining the bitterness of a peptide is its degree of hydrophobicity; peptides with a Q value (calculated from Δf) above 1400 exhibit a bitter taste. In addition, Ishibashi (1987a,b) reported that the bitter taste of peptides is more intense when the content of leucine, phenylalanine, or tyrosine is high. Therefore, *Aeromonas* aminopeptidase can be considered to eliminate the bitter taste by selective hydrolysis of bitter peptides.

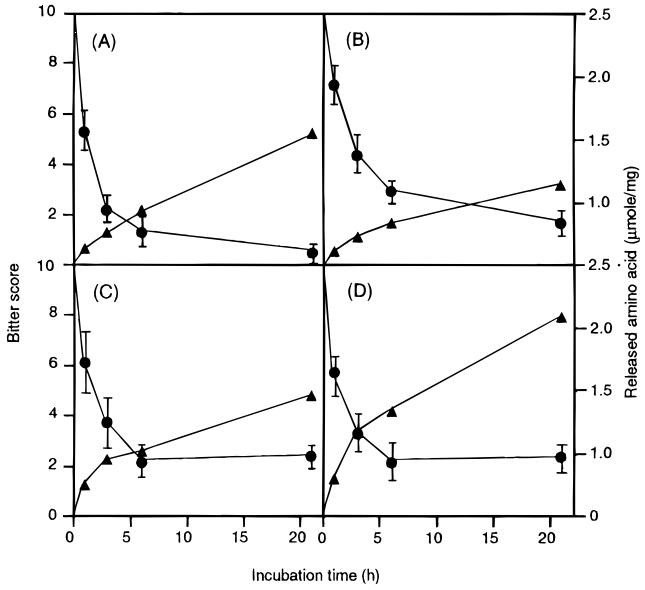

Figure 1. Reduction of the bitter taste of BTs by aminopeptidase treatment: ●, bitter score; ▲, amount of released amino acids. Untreated BTs of 100 and 10% were used as standard solutions for bitter tasting. The bitter scores of these solutions were 10 and 1, respectively: (A) Treatment of BTsp with enzyme/substrate ratio (w/w) of 1/8000. (B−D) Treatment of BTmc with enzyme/substrate ratio (w/w) of 1/16000, 1/8000, and 1/4000, respectively. Amount of released amino acids is expressed in moles per milligram of peptides in untreated BTs.

The debittering of BTmc by the enzyme was less effective than that of BTsp, as shown by the fact that the bitter score in BTmc did not decrease below 2.0 even at a high enzyme/substrate ratio (Figure 1B−D). One possible explanation of this phenomenon is that BTmc contained proline-rich bitter peptides derived from β-casein as reported by Shinoda (1986). Because *Aeromonas* aminopeptidase does not release acidic amino acids or amino acids adjacent to proline, proline-rich bitter peptides will not be susceptible to this aminopeptidase. Isibashi (1987a,b) also reported that stronger bitterness is found when leucine, phenylalanine, or tyrosine is located at the C-terminal of peptides. More effective elimination of bitterness in BTmc may be achieved by treatment with amino- and carboxypeptidase: removal of hydrophobic amino acids from both the N- and C-terminal ends of the peptides.

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