Purification and Debittering Effect of Aminopeptidase II from *Penicillium caseicolum*

Hiroatsu Matsuoka,*,† Yoko Fuke,† Shuichi Kaminogawa,‡ and Kunio Yamauchi§

Department of Food and Nutrition, Tachikawa College of Tokyo, 3-6-33 Azumacho, Akishima-shi, Tokyo 196, Japan, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan, and Department of Food Technology, College of Agriculture and Veterinary Medicine, Nihon University, Tokyo, Japan

An extracellular aminopeptidase II was purified by chromatographic techniques from *Penicillium caseicolum*. The enzyme was most active at pH 8.0 and was stable over a wide pH range. The activity was inhibited by metal chelating agents and by 2-mercaptoethanol. The enzyme was remarkably activated by the addition of Co^{2+}. The molecular weight of the enzyme was estimated to be 20 000 by Sephadex G-100, and the enzyme was the monomer when determined by SDS–PAGE. The enzyme has a wide specificity for various substrates, and it was capable of cleaving amino-terminal leucine and phenylalanine residues of dipeptides and oligopeptides. The effect of debittering for bitter peptide fraction from peptic casein was clearly found by APase II treatment for 3 h.

INTRODUCTION

Penicillium caseicolum has been used as a mold starter for manufacturing Camembert and Brie cheese (Kosikowski, 1979). Many studies (Furtado et al., 1984; Noomen, 1983; Rousseau, 1984) on ripening of surface-ripened white mold cheeses suggest that extracellular proteases from the mold contribute to the breakdown of protein into amino acids and peptides. The purification and properties of extracellular endopeptidases from *P. caseicolum* have been reported (Lenoir et al., 1979; Matsuoka et al., 1963; Trieu-Cuot et al., 1982). However, few papers exist on extracellular exopeptidases from *P. caseicolum*. The purification and characterization have been described for aminopeptidase I from *P. caseicolum* (Fuke et al., 1988). The possibility that aminopeptidase I might produce free amino acids and contribute to the formation of the flavor of cheeselike products prepared from soy milk with *P. caseicolum* has been discussed (Fuke et al., 1987, 1988).

Some kinds of hard-type cheeses such as Cheddar cheese rarely have a bitter taste during ripening or as finished products (Nelson et al., 1975). Mechanisms by which bitter peptides from milk casein were debittered by wheat carboxypeptidase (Umetsu et al., 1983) and by aminopeptidase T from *Thermus aquaticus* YT-1 (Minagawa et al., 1989) have been reported.

The objective of the present study was to purify and characterize aminopeptidase II (APase II) and to clarify its effect on bitter peptide fraction.

MATERIALS AND METHODS

Culture. A strain of *P. caseicolum* isolated from Camembert cheese (Tsugo et al., 1959) was used. Lyophilized spores of the mold were inoculated onto a wheat bran medium and cultured for 7 days at 20 °C.

Reagents. DEAE-Sephacel, Sephadex G-100, PBE-94 gel, and Poly buffer were obtained from Pharmacia Fine Chem. Peptides were purchased from the Protein Research Foundation, from Nakarai Chemicals, from Tokyo-Kasei, and from Sigma Chemical Co. Bestatin and amastatin were obtained from Sigma, and other inhibitors of the enzyme were from Tokyo-Kasei.

Enzyme Assay. The activity of APase II was measured by using amino acid 2-naphthylamides (AA-2-NA) (Sigma), amino acid 4-nitroanilides (AA-4-NA) (Sigma), and various synthetic peptides as the substrates. The activity was assayed according to a previous paper (Fuke et al., 1988).

Molecular Weight Determination. The apparent molecular weight of the enzyme was determined by gel filtration using Sephadex G-100. Bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A were used as the standard proteins (Pharmacia).

Enzyme Kinetics. Leucine-2-NA (pH 7.5) was used as a substrate to measure the enzyme activity at 40 °C for 15 min.

Electrophoresis. Disc PAGE was performed on a 7.5% gel according to the method of Davis (1964). A current was applied at 2 mA per tube at 5 °C. Gels were then stained with 0.25% Coomassie brilliant blue R-250.

Amino Acid Analysis. The amino acid compositions were estimated by using a SC-71 column (4.6 × 15 cm, 60 °C) in a ATTO MLC-20 amino acid analyzer. The total amino acids were analyzed after the samples were hydrolyzed for 24 h in 6 N HCl at 110 °C. Tryptophan and cysteine were not determined because little of the purified enzyme remained for further analysis. The free amino acids were determined according to the same method but without the HCl treatment.

Preparation and Purification of APase II. An extracellular enzyme was extracted from the medium and prepared according to a previous paper (Fuke et al., 1988). The precipitate between 30% and 80% ammonium sulfate saturation was collected and then dissolved in about 100 mL of a 0.02 M phosphate buffer (PB), pH 6.5, and finally dialyzed against PB at 4 °C. The crude enzyme solution was applied to a column (2 × 45 cm) of DEAE-Sephacel equilibrated with PB. After the column was washed with PB, the enzyme activity was eluted with a linear gradient of 0–0.3 M KCl in PB. The flow rate was 30 mL/h, and each fraction of 5 mL was collected. The fractions with high enzyme activity were pooled and dialyzed against the same buffer. The concentrated solution was applied to a column (1.5 × 40 cm) of DEAE-Sephacel equilibrated with PB. Elution was performed with a stepwise concentration gradient of 0.13 and 0.14 M KCl. After enzyme fractions were pooled, they were concentrated by collodion bag and dialyzed as before. The enzyme preparation was subjected to chromatofocusing on a PBE-94 gel column (1.0 × 15 cm) using a Poly buffer (pH 6.0 to 4.0). The enzyme eluted from the column was dialyzed against PB. A total of 58–65 fractions were pooled, dialyzed against PB, and concentrated. Further purification was performed by using disc electrophoresis. After the samples were applied to the gels and electrophoresis was carried out, the gels were sliced into pieces

* Address correspondence to this author.
† Tachikawa College of Tokyo.
‡ University of Tokyo.
§ Nihon University.

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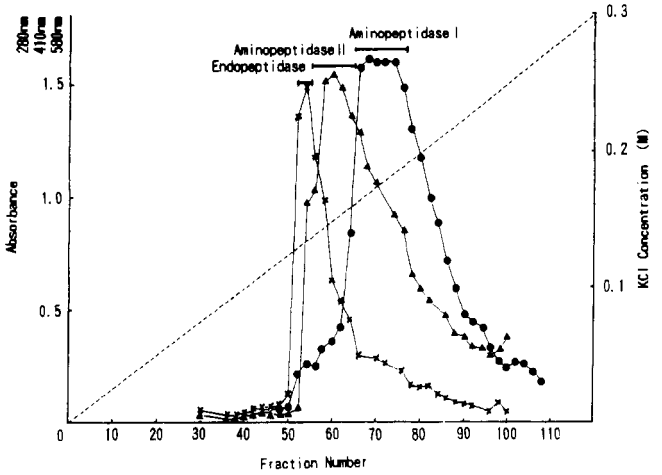

Figure 1. Elution profile of ammonium sulfate precipitated fraction from *P. caseicolum* on DEAE-Sephacel chromatography: (×) endopeptidase activity; (●) APase I activity; (▲) APase II activity; (- - -) KCl concentration.

measuring about 2 mm, and the enzyme was extracted from sliced gel with PB at 4 °C overnight. The activity of each extraction was determined, and the extracts containing the high enzyme activity were pooled. For all purification steps, the aminopeptidase activity was assayed against Leu-4-NA.

Preparation of Bitter Peptide Fractions. Casein (Merck) was dissolved to 2% in deionized water and adjusted to pH 2.0 with 1 N HCl. The hydrolysis of whole casein with pepsin (Sigma) (2× crystallized) was carried out at 30 °C for 20 h with an enzyme/substrate ratio (w/w) of 1:250. The reaction was stopped by neutralization with NaOH. The peptic hydrolysate was centrifuged at 6000 rpm for 20 min. The supernatant, adjusted to pH 7.0, was applied to the Sephadex G-15 column (Pharmacia) (2 × 60 cm), and elution was carried out with deionized water at 4 °C. The bitter peptide fractions were collected and freeze-dried. A solution of bitter peptide dissolved in 0.02 M phosphate buffer (pH 8.0) was hydrolyzed with APase II at an enzyme/substrate ratio of 1:500 (w/w) at 40 °C for 3 h. Sensory evaluation was performed at each setting time. For analysis of amino acids, the reaction was stopped by using a 0.4 M trichloroacetic acid solution.

Sensory Evaluation of Bitterness. The bitterness was evaluated with the enzymatic hydrolysates, compared with a standard quinine sulfate solution. The degree of bitterness is expressed in mole concentration of quinine sulfate solution. Sensory evaluation was performed by four panelists trained in fundamental sensory test at the cooking science laboratory at Tachikawa College of Tokyo; the panelists evaluated the bitter taste in our laboratory. Before tasting, the panelist's mouth was thoroughly rinsed with deionized water. A peptide solution of between 0.1 and 0.2 mL was used for the test. The test for bitterness was carried out in duplicate for each sample.

The degree of bitterness was evaluated as follows: 10^{-8} M, not bitter; 10^{-7} M, slightly bitter; 10^{-6} M, distinctly bitter; 10^{-5} M, very bitter; 10^{-4} M, extremely bitter.

RESULTS AND DISCUSSION

Enzyme Purification. Figure 1 shows the elution pattern on DEAE-Sephacel chromatography. The activity toward Leu-4-NA was eluted with a linear gradient at around 0.13 M KCl concentration, and the other activity for Arg-2-NA was obtained as the peak delayed. The former aminopeptidase was designated APase II and the latter APase I. Detailed properties of purified APase I have been reported (Fuke et al., 1988).

APase II was further purified by DEAE-Sephacel chromatography, chromatofocusing, and disc electrophoresis. APase II eluted with 0.13 M KCl on DEAE-Sephacel chromatography was applied on a PBE-94 gel with a Poly buffer at pH 4.5. The elution profile is shown in Figure 2. The enzyme fraction obtained on chromatofocusing was applied

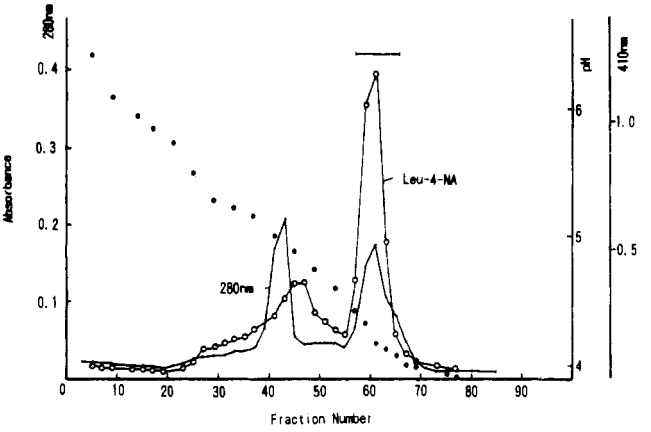

Figure 2. Elution profile of APase II on chromatofocusing through PBE-94 gel (column, 1 × 15 cm; eluent, Poly buffer pH 6.0 to 4.0; flow rate, 20 mL/h): (—) absorbance at 280 nm; (○) APase II activity; (●) pH of buffer.

Table I. Amino Acid (AA) Composition of APase II from *P. caseicolum*

AA	nmol/mL	AA	nmol/mL
Asp	16.1	Met	1.1
Thr	17.4	Ile	9.9
Ser	62.6	Leu	12.0
Glu	15.6	Tyr	5.5
Pro	6.4	Phe	7.3
Gly	46.4	Lys	8.8
Ala	21.3	His	11.0
Val	13.8	Arg	2.3

to disc electrophoresis. A main band and two slight bands appeared on the gel. The enzyme extracted from the sliced gels had a single band on disc PAGE, and the enzyme activity was detected at the same position of the gel.

The purity of the enzyme obtained was approximately 800 times greater than that of the cell-free extract.

Molecular Weight. APase II had a molecular weight of about 20 000 as estimated by Sephadex G-100 and gave only one band with the mobility of molecular weight 20 000 on SDS-PAGE. These results shows the enzyme was the monomer. The molecular weight is much smaller than those of aminopeptidases I–III from *Aspergillus oryzae* (Nakadai et al., 1973a–c): 26 500, 61 000, and 56 000, respectively.

Amino Acid Composition. The amino acid composition of the enzyme is shown in Table I. The contents of serine and acidic amino acids were relatively high, which is similar to a proline iminopeptidase from *P. caseicolum* (Fuke et al., 1991) and a prolidase from *Streptococcus cremoris* H61 (Kaminogawa et al., 1984).

General Properties. *Effect of pH.* The effect of pH on the APase II activity was examined over the pH range 3.0–10.0 by using Leu-2-NA as a substrate. The optimum pH for enzyme activity was found to be 8.0. The pH stability was also determined after the enzyme was preincubated for 2 h at various pHs at room temperature. The activity of APase II was stable over a wide pH range.

Effect of Temperature. The activity of APase II was measured at different temperatures, the maximum activity being obtained at 45 °C. The enzyme was preincubated at various temperatures for 10 min and the thermal stability estimated by determining the residual activity. APase II was stable below 55 °C, and 20% of the activity remained at 60 °C.

Effect of Various Chemical Reagents and Metal Ions. The activity of APase II was inhibited by chelating agents such as EDTA and 1,10-phenanthroline (Table II). The activity was remarkably increased by Co^{2+} addition and

Table II. Effects of Various Reagents on the Activity of APase II from *P. caseicolum*

reagent	concn, mM	rel act., %
control		100
EDTA	1.0	33
1,10-phenanthroline	1.0	0
N-ethylmaleimide	1.0	100
monoiodoacetic acid	1.0	100
2-mercaptoethanol	1.0	47
PCMB	1.0	82
cysteine	1.0	70
bestatin	0.05	100
amastatin	0.05	28

Table III. Effects of Metal Ions on the Activity of APase II from *P. caseicolum*

metal ion	concn, mM	rel act., %
control		100
$CaCl_2$	1.0	71
$MgCl_2$	1.0	65
$MnCl_2$	1.0	164
$ZnCl_2$	1.0	145
$CoCl_2$	1.0	1,025
$CuSO_4$	1.0	133
$SnCl_2$	1.0	52
$FeSO_4$	1.0	32

Table IV. Relative Activity of APase II from *P. caseicolum* for Various Synthetic Peptides

peptide	rel act.[a]	peptide	rel act.	AA-2/4-NA	rel act.
Gly-Leu	10	Tyr-Leu	11	2-NA	0
Gly-Phe	11	Tyr-Glu	2	Gly-2-NA	100
Gly-Val	1	Val-Ala	10	Leu-2-NA	66
Leu-Ala	68	Val-Gly	4	Ala-2-NA	81
Leu-Gly	79	Val-Leu	12	Arg-2-NA	1
Leu-Leu	72	Pro-Lys	1	Glu-2-NA	14
Leu-Phe	4	Pro-Ala	5	Phe-2-NA	12
Leu-Tyr	100	Pro-Gly	2	Pro-2-NA	25
Leu-Val	36	Pro-Leu	5	Ser-Tyr-2-NA	5
Ala-Ala	24	Pro-Tyr	5	Arg-Arg-2-NA	
Ala-Leu	13	Pro-Pro	0		
Ala-Met	0	Ala-Pro	0		
Ala-Phe	7	Leu-Pro	0	4-NA	
Ala-Ser	0	Phe-Pro	0		
Ala-Val	0	Gly-Gly-Gly	7	Leu-4-NA	100
Arg-Asp	6	Gly-Gly-His	4	Lys-4-NA	73
Asp-Gly	8	Gly-Phe-Ala	21	Ala-4-NA	19
Glu-Ala	7	Leu-Gly-Gly	85	Ala-Ala-4-NA	20
Glu-Glu	2	Leu-Gly-Phe	44		
Glu-Val	1	Ala-Gly-Gly	16		
Glu-Phe	4	Glu-Glu-Phe	3	Cbz-Glu-Phe	0
His-Ala	17	Glu-Val-Phe	17	Cbz-Gly-Gly	0
His-Gly	11	Phe-Gly-Gly	47	Cbz-Tyr-Glu	0
His-Leu	53	Val-Gly-Gly	41		
Lys-Gly	55	Ala-Ala-Ala-His	31		
Phe-Ala	25	Gly-Gly-Gly-Gly	15		
Phe-Leu	86	Leu-Leu-Leu-Leu	10		
Tyr-Ala	30	Ala-Gly-Gly-Gly-Gly	100		

[a] Hydrolyzing activity for peptides, AA-2-NA, and AA-4-NA were taken as 100 for Leu-Leu, Ala-2-NA, and Leu-4-NA, respectively.

also was activated by Mn^{2+}, Zn^{2+}, and Cu^{2+} (Table III). The Co^{2+} addition to enzyme treated with 1 mM EDTA raised the reduced activity resulting from EDTA treatment. The activity was inhibited to a considerable extent by 2-mercaptoethanol and amastatin. These results indicate that APase II is one kind of metalloenzyme.

Specificity of Various Substrates. The specificity of APase II toward various peptides is shown in Table IV. The relative activities for peptides, AA-2-NA, and AA-4-NA are indicated as percentages of the hydrolyzing activity against Leu-Tyr, Leu-2-NA, and Leu-4-NA, respectively. Such peptide bonds as Leu–X, Phe–X, Lys–X, His–X, and Tyr–X were readily cleaved. The enzyme also hydrolyzed Pro–X weakly but not X–Pro. APase II had a high capability for hydrolyzing hydrophobic N-terminal amino acid residues, like APase I. The enzyme hydrolyzed Gly–X peptides; however, APase I (Fuke et al., 1988) was not capable of hydrolyzing. It had a high activity for AA-2-NA such as Leu-2-NA, Arg-2-NA, and Ala-2-NA. For AA-4-NA, the enzyme had high hydrolyzing activity. APase II preferentially hydrolyzed the oligopeptides containing Leu as the N-terminal amino acid, indicating that the enzyme is a kind of leucine aminopeptidase such as aminopeptidases from *A. oryzae* (Nakadai et al., 1973a–c).

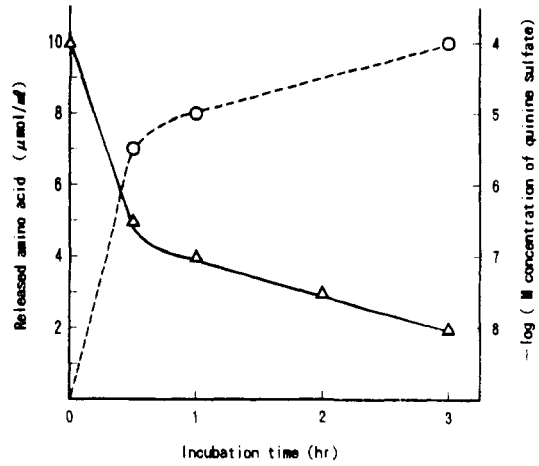

Figure 3. Correlation between released amino acids and the bitterness during the hydrolysis of bitter peptides fraction: (○) released amino acid; (△) bitterness.

These results suggest that the enzyme will be useful for specifically removing the amino acid residues at the N terminus.

Kinetic Properties of APase II. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of the enzyme were determined for the hydrolysis of Leu-2-NA at pH 7.0 at 37 °C. The V_{max} value was estimated to be 567 units mL^{-1} min^{-1} and K_m was 8×10^{-5} M.

Debittering Effect. The bitterness of the bitter peptide fractions decreased with the release of free amino acids during incubation with APase II (Figure 3). After incubation for 1 h, the bitterness of the peptide fraction decreased significantly and disappeared completely after 3 h. The total amino acid composition and the amounts of free amino acids released by hydrolyzing bitter peptide fractions with APase II are shown in Table V. Amino acids such as Thr, Lys, Ser, Leu, Phe, and Met were mainly released from the peptide fractions treated with APase II for 3 h. However, Cys, Pro, Asp, and Glu were scarcely released.

It was proved that APase II was able to decrease the bitterness of the peptide fractions from whole casein. Minagawa et al. (1989) reported that APase T from *T. aquaticus* YT-1 was able to remove the bitterness of the bitter peptides of enzymatic hydrolysates from milk casein. Umetsu et al. (1983) isolated a carboxypeptidase from wheat bran that acted on bitter peptides to release hydrophobic amino acids from their carboxyl termini followed by a decrease in bitterness. The wide specificity of the enzyme toward various dipeptides and oligopeptides suggests that it may be related to the liberation of amino acids from the N-terminal amino acid residues of peptides resulting from protein hydrolysis with endopeptidases of the mold during ripening. Therefore, in white mold cheeses such as Camembert and cheeselike products from soy milk, APase II may be useful for improving the taste of products.

Table V. Amino Acid (AA) Composition of Bitter Peptide Fraction and Its Enzymatic Hydrolysate Fraction

		AA liberated					
		0.5 h		1 h		3 h	
AA	total AA, μM/mL	μM/mL	%	μM/mL	%	μM/mL	%
Asp	7.2	0.02	0.3	0.04	0.6	0.08	1.2
Thr	4.3	0.32	7.5	0.44	10.2	0.69	16.0
Ser	8.1	0.51	6.4	0.61	7.5	0.79	9.8
Glu	26.0	0.28	1.1	0.32	1.2	0.40	1.5
Pro	15.5	0.01	0.1	0.03	0.2	0.08	0.5
Gly	3.4	0.09	2.6	0.09	2.6	0.11	3.2
Ala	8.7	0.38	4.4	0.44	5.1	0.63	7.2
Cys	0.1	0	0	0	0	0	0
Val	12.4	0.47	3.8	0.61	4.9	0.85	6.9
Met	3.5	0.18	5.1	0.21	6.0	0.26	7.4
Ile	8.2	0.35	4.3	0.47	5.7	0.60	7.3
Leu	12.4	0.97	7.8	1.01	8.1	1.15	9.3
Tyr	4.9	0.32	6.5	0.32	6.5	0.36	7.3
Phe	8.2	0.75	9.1	0.73	8.9	0.73	8.9
Lys	17.5	1.20	6.9	1.61	9.2	2.34	1.34
His	4.1						
Arg	5.4	0.13	2.4	0.20	3.7	0.31	5.7

The enzyme may be effective for debittering protein hydrolysates in food applications because it has a wide specificity for peptides.

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