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Purification and identification of proteolytic enzymes from *Aspergillus oryzae* capable of producing the antihypertensive peptide Ile-Pro-Pro

Takanobu Gotou, Tadashi Shinoda, Seiichi Mizuno, and Naoyuki Yamamoto*

Functional Food and Drink Development Laboratory, Calpis Co., Ltd., 11-10, 5-Chome, Fuchinobe, Sagamihara-shi, Kanagawa 229-0006, Japan

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Two proteolytic enzymes capable of releasing the angiotensin I-converting enzyme (ACE) inhibitor Ile-Pro-Pro from casein were identified by purification of an *Aspergillus oryzae* extract by three-step column chromatography. First, proteins capable of producing Ile-Pro-Pro from β-casein were eluted using a DEAE-sepharose FF column with a linear sodium chloride gradient. An endopeptidase capable of releasing Pro-Ile-Pro-Gln-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro from Pro-Ile-Pro-Gln-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln and an aminopeptidase producing Ile-Pro-Pro from Gln-Asn-Ile-Pro-Pro were separated from the resultant fraction using a hydroxyapatite column. Each active enzyme was then loaded onto a Develosil 300Diol gel filtration column for high performance liquid chromatography (HPLC) and purified to homogeneity.

The endopeptidase had a molecular mass of approximately 46,000 Da and exhibited an N-terminal amino acid sequence identical to that of neutral protease I (NP I) of *A. oryzae*. Meanwhile, the aminopeptidase had a molecular mass of 36,000 Da and an N-terminal amino acid sequence similar to that of Leucine aminopeptidase (LAP), as reported in *Aspergillus sojae* and *A. oryzae*. The eluted endopeptidase and aminopeptidase were thus identified as NP I and LAP, respectively.

Analysis of peptide production using synthetic proteins containing an Ile-Pro-Pro sequence showed that NP I processed the C-terminal end and LAP processed the N terminus to produce Ile-Pro-Pro. While Ile-Pro-Pro was successfully produced from casein by the addition of these two purified enzymes, it was not generated with the addition of only a single enzyme. Based on our experimental findings, we suggest that NP I and LAP are key proteolytic enzymes in the release of Ile-Pro-Pro from casein in *A. oryzae*.

[**Key words:** Antihypertensive peptides; *Aspergillus oryzae*; Ile-Pro-Pro; Neutral protease I; Leucine aminopeptidase]

The many roles of bioactive peptides, which include specific amino acid sequences possessing *in vivo* functions, were reviewed recently (1–5). Among various physiologically functional peptides, such as opioid, immunostimulating, mineral-soluble, antimicrobial, and antihypertensive peptides, the latter have been studied most extensively.

Hypertension is a major risk factor in cardiovascular diseases such as heart disease and stroke. Potential treatment for hypertension includes angiotensin I-converting enzyme (kinase II; EC 3.4.15.1) (ACE) inhibitors, which catalyze the production of the vasoconstrictor angiotensin II and inactivate the vasodilator bradykinin (6). Various antihypertensive peptides isolated from food proteins have been proven to significantly lower blood pressure in clinical studies (7–10). In a previous study, we isolated the ACE inhibitors Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) from *Lactobacillus helveticus*-fermented milk (11). These two peptides exhibited strong, dose-dependent antihypertensive effects in spontaneously hypertensive rats (12). In addition, a fermented milk product containing both VPP and IPP significantly reduced hypertension in Japanese volunteers (7).

Recently, a new method of preparing VPP and IPP from casein was developed using a commercially available *Aspergillus oryzae* protease (13). *A. oryzae* is widely implemented in the manufacture of traditional fermented food products in Japan due to the organism's high proteolytic activity and sugar utility. To release VPP and IPP from casein, the *A. oryzae* protease must be capable of cleaving the C-terminal end of each peptide; otherwise, carboxyl peptidases would stop the reaction at Val-Pro-Pro-Phe and Ile-Pro-Pro-Leu. However, current information on *A. oryzae* proteolytic enzymes is limited, and the key enzymes required for releasing VPP and IPP from milk casein have not yet been elucidated.

The objective of the present study was to identify and characterize the key enzymes that release peptides from casein, focusing on IPP since its ACE inhibitory activity is higher and thus more clinically relevant than that of VPP. We report the purification and characterization of proteases from *A. oryzae* that may play important roles in IPP production.

MATERIALS AND METHODS

Materials Sumizyme FP was purchased from the Shin-Nihon Chemical Company (Anjyo, Japan). An anion exchange resin, DEAE sepharose FF, was purchased from Amersham Biosciences (Tokyo, Japan). Hydroxyapatite was procured from WAKO Pure Chemicals (Osaka, Japan) and Develosil 300Diol was obtained from the Nomura Chemical Company (Seto, Japan). All peptides used as substrates in peptidase activity measurements were purchased from TORAY (Tokyo, Japan).

Purification of proteolytic enzymes Three chromatographic steps were used to detect and purify the proteolytic enzymes required for IPP production in *A. oryzae*

* Corresponding author. Tel.: +81 42 769 7861; fax: +81 42 7810.
E-mail address: naoyuki.yamamoto@calpis.co.jp (N. Yamamoto).

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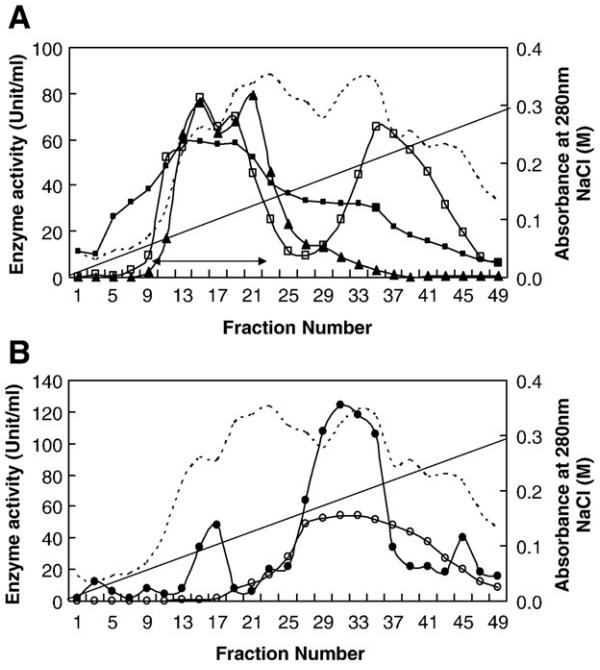

FIG. 1. Elution profiles of proteolytic enzymes derived from DEAE-sepharose FF chromatography. Proteins bound to the column were eluted using a linear gradient from 0 to 300 mM NaCl in PB, as described in Materials and methods. All units in the figure are also described in that section. (A) Dotted line, absorbance at 280 nm; closed squares, proteinase activity measured by proteolysis of FITC-casein; open squares, aminopeptidase activity; closed triangles, productivity of IPP from casein. The arrow indicates the active fraction. (B) Dotted line, absorbance at 280 nm; closed circles, XPDAP activity; open circles, carboxypeptidase activity.

extracts. The enzyme source, powdered Sumizyme FP, was dissolved in 20 mM phosphate buffer (PB; pH 7.0) to generate a 20% ($w \cdot w^{-1}$) crude enzyme solution. Next, 5 ml of the enzyme solution were loaded onto a DEAE-Sepharose FF column (1.6 cm $\Phi \times 2.5$ cm) previously equilibrated with PB. The column was washed with 30 ml of PB before protein elution with a linear gradient of 0 to 300 mM sodium chloride (NaCl) in PB at 4 °C. Fractions of 1.5 ml each were then collected for measurement of proteolytic activity and for protein characterization.

Fractions with the highest production of Ile-Pro-Pro (IPP) from casein were collected and dialyzed against 1 L of 5 mM PB (pH 7.0) at 4 °C. This dialyzed sample was loaded onto a hydroxyapatite column (1.46 cm $\Phi \times 3.0$ cm) previously equilibrated with 5 mM PB. The column was washed with 15 ml of 5 mM PB and the proteins were eluted with 15 ml of 10 mM sodium PB (pH 7.0). Fractions evidencing endopeptidase or aminopeptidase activity were collected and concentrated to a 0.5 ml volume using a Centricon (Nihon Millipore, Tokyo, Japan). This concentrated 100-µl sample was loaded onto a Develosil 300 Diol gel filtration column (0.8 cm $\Phi \times 50$ cm) using the high performance liquid chromatography (HPLC) system D-7000 (Hitachi High Technologies, Tokyo, Japan). Proteins were eluted with 20 mM PB (pH 7.0) containing 200 mM NaCl at a flow rate of 1.0 ml min^{-1}. The proteolytically active fractions were combined, concentrated using a Centricon, and stored at −20 °C.

Proteinase activity measurement Enzyme activity was determined according to a method using fluorescein isothiocyanate (FITC)-labeled casein as substrate (14) with some modification (15). The reaction mixture, containing 20 µl of 0.4% FITC (Sigma-Aldrich Japan, Tokyo, Japan) and 10 µl of the enzyme solution, was incubated at 50 °C for 5 min. Soluble peptides were prepared by the addition of 120 µl of trichloroacetic acid (TCA) to the reaction mixture and centrifugation at 15,000 ×*g* for 5 min. Twenty microliters of the supernatant (soluble in TCA solution) were then neutralized by the addition of 3 ml of 500 mM Tris–HCl buffer (pH 8.5). Fluorescence in TCA solution was measured using a Fluorescence Spectrophotometer 204 (Hitachi) at an excitation wavelength of 490 nm and an emission wavelength of 525 nm. One unit of proteolytic activity was defined as the amount of enzyme yielding 1% of total initial casein fluorescence as fluorescence soluble in TCA solution after 10 min of hydrolysis.

Specificity of proteinase towards peptide substrates After proteinase purification, its substrate specificity was measured using Pro-Ile-Pro-Gln-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln (PIPNSLPQNIPPLTQ) and Ile-Pro-Pro-Leu-Thr (IPPLT) as substrates. An aliquot of 100 µl of enzyme solution containing the purified enzyme and 1 µg of PIPNSLPQNIPPLTQ in PB buffer (pH 7.0) was incubated at 50 °C for 30 min. The reaction was stopped by heating at 95 °C for 5 min, followed by injection of the reaction mixture into a Liquid Chromatography Ion Trap Time of Flight Mass Spectrometer (LCMS-IT-TOF) (Shimadzu, Kyoto, Japan). Peptides generated from the IPP-containing substrates listed above were characterized by tandem mass spectrometry (MS/MS) spectra.

Peptidase activity measurement Proteolytic activity toward peptides (peptidase activity) were measured by using of synthetic substrates as described below differed from proteinase activity that was detectable with protein. Generally, aminopeptidase and X-prolyl–dipeptidyl aminopeptidase activity (XPDAP) have broad amino acid specificity toward X-*p*NA and X–Y-*p*NA. Thus, Ala-*p*NA (Alanine *p*-nitroanilide) and Gly-Pro-*p*NA (Gly-Pro-*p*-nitroanilide) (PEPTIDE INSTITUTE, Inc., Mino, Japan) were randomly selected and used as colorimetric substrates for analyzing aminopeptidase and XPDAP activity. Both substrates were dissolved in 20 mM sodium PB (pH 7.0). Forty-five microliters of the substrate and 5 µl of the enzyme solution were then mixed for 10 min at 50 °C. To stop the reaction, 150 µl of 5% trifluoroacetic acid (TFA) was added to the reaction mixture. Peptidase activity was measured by monitoring changes in the reaction mixture's absorbance at 405 nm according to the previously reported method (16, 17). One unit of enzyme activity was defined as the activity required to cause an 0.01 increase in absorbance at 405 nm under the above reaction conditions. To detect specific aminopeptidase activity capable of producing IPP from casein, the small synthetic peptide Gln-Asn-Ile-Pro-Pro (QNIPP) was used as a substrate throughout the purification process. Ile-Pro-Pro-Leu-Thr (IPPLT), a substrate of carboxyl peptidase, was also synthesized. During testing, 2 µg of QNIPP or IPPLT were mixed with enzyme solution to a final volume of 50 µl and incubated at 50 °C for 30 min. The enzyme mixture was then heated to 95 °C for 5 min to stop the reaction. The concentration of IPP processed from QNIPP and IPPLT by aminopeptidase and carboxyl peptidase, respectively, was analyzed using a liquid chromatography mass spectrometry (LCMS) method. One unit of peptidase activity was defined as enzymatic activity releasing 0.01 µg of IPP under the above reaction conditions. The specificity of the purified aminopeptidase was analyzed using synthetic peptides Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro (SLPQNIPP) and Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln (SLPQNIPPLTQ) as substrates, while the purified proteinase's specificity was analyzed using the substrate Pro-Ile-Pro-Gln-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln (PIPNSLPQNIPPLTQ).

Protein assay The amount of protein purified was determined using a Protein Assay Kit (Bio-Rad Laboratories, Carlsbad, CA, USA) using bovine serum albumin (BSA) as a standard.

SDS-PAGE analysis Sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE was performed by the Laemmli method (18) using a 12% polyacrylamide gel. Proteins were stained using a Silver Stain kit (WAKO). Low molecular weight proteins (Amersham Biosciences) were used as standard markers.

RESULTS

Purification of proteolytic enzyme Two kinds of proteolytic enzymes involved in releasing IPP from casein were purified from an *A. oryzae* protease extract (see Materials and methods). Initially, the extract's enzymes were separated using a DEAE-sepharose FF column with a linear NaCl gradient from 0 to 300 mM in PB. Most proteins were eluted in the 100 to 300 mM NaCl fractions (Fig. 1), while proteolytic enzymes capable of producing IPP from casein were eluted in the 80 to 150 mM NaCl fractions (Active fraction, Fig. 1A). Proteinase

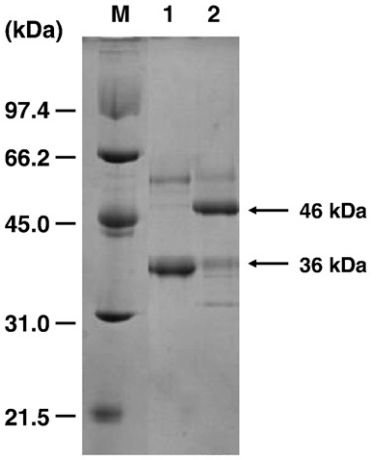

FIG. 2. SDS-12%PAGE analysis of the fraction separated by hydroxyapatite column chromatography. Lane M, molecular weight marker proteins; lane 1, 2 mM PB fraction (passed through); lane 2, 10 mM PB fraction. The following proteins were used as molecular markers: phosphorylase *b* (97.4 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21.5 kDa).