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The Production of Recombinant APRP, an Alkaline Protease Derived from *Bacillus pumilus* TYO-67, by *In Vitro* Refolding of Pro-enzyme Fixed on a Solid Surface

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***Bacillus pumilus* TYO-67 has been isolated from *tofuyo*, a traditional fermented food made from soybean milk in Okinawa, Japan. This bacterium secretes a soybean-milk-coagulating enzyme (SMCE), which can be applied for the production of processed foods from soybean milk. Thus, an easy method of producing the recombinant enzyme was developed in this study. SMCE is an alkaline serine protease belonging to the subtilisin family; its candidate gene, *aprP*, which encodes a prepro-enzyme, was isolated in a previous study. Recombinant APRP was then produced by *in vitro* refolding of pro-APRP-His, *i.e.*, N-terminally His-tagged pro-APRP. A large amount of pro-APRP-His was produced in *Escherichia coli* BL21(DE3) (*ca.* 8 mg from a 20-ml culture), collected as insoluble protein, dissolved in 6 M guanidine-HCl (pH 8.0), bound to Ni-NTA, and refolded on the resin at pH 10.0 to become mature APRP by autocleavage. Then, 0.16 mg of purified mature APRP was obtained through single-step chromatography from the refolded sample using 10 mg of pro-APRP-His. The N-terminal sequence and the enzymatic properties of refolded APRP were identical to those of SMCE. In addition, the pro-sequence was found to be essential for the production of mature APRP, suggesting that it could function as an intramolecular chaperone.**

Key words: autocleavage, intramolecular chaperone, *in vitro* refolding, pro-sequence, soybean-milk-coagulating enzyme, subtilisin.

Abbreviations: AAPX; *N*-succinyl-L-Ala-L-Ala-L-Pro-L-X-*p*-nitroanilide; BPN′, subtilisin BPN′; CBB, Coomassie Brilliant Blue R-250; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani; M9CA, M9 medium containing casamino acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NaPB, sodium phosphate buffer; SBE, subtilisin E; SMCE, soybean-milk-coagulating enzyme; TCA, trichloroacetic acid.

Soybeans are widely used as food materials throughout the world and have excellent nutritional properties, *e.g.*, they are rich in proteins and high-quality oils. Moreover, many investigations have demonstrated improvement of hypercholesterolemia by ingestion of soybeans and soy protein-containing foods (*1*, *2*). *Bacillus pumilus* TYO-67 was isolated as the best producer of a soybean-milk-coagulating enzyme (SMCE) from the manufacturing process of *tofuyo*, a traditional fermented food made from soybean milk in Okinawa, Japan (*3*). SMCE is an alkaline serine protease and efficiently coagulates soybean milk by digesting soybean proteins (*4*). Thus, SMCE can be applied for the production of various soybean-milk-derived foods (*3*). However, because its purification requires multi-step chromatography, easy methods of recombinant enzyme production and purification are desirable. SMCE belongs to the subtilisin family based on the homology of the N-terminal amino acid sequence of the purified enzyme (*3*). A candidate gene of SMCE, *aprP*, has been isolated from the chromosomal DNA of *B. pumilus* TYO-67 by PCR based on its amino acid sequence (*5*). The gene encodes a prepro-enzyme belonging to the subtilisin family, and the N-terminal sequence of the deduced mature protease is identical to that of SMCE. However, no further evidence has been reported showing a relationship between the *aprP* gene and SMCE because of the absence of enzymatic investigations using the recombinant protein (APRP) and the difficulty of disrupting the specific gene of *B. pumilus* TYO-67.

We have studied subtilisin E (SBE), a member of the 'true' subtilisin family derived from the *Bacillus subtilis* I168 strain, to elucidate the mechanism of mature enzyme production and introduce functional improvements by protein engineering (*6*–*14*). SBE is synthesized in the bacterium as a prepro-enzyme with a signal peptide and a pro-sequence attached to the N-terminal side of the mature enzyme sequence. After the signal peptide is cleaved off upon secretion across the plasma membrane, the resultant pro-SBE folds into the appropriate tertiary structure. In this phase, the pro-sequence plays an essential role as an intramolecular chaperone for the correct folding of the mature enzyme sequence (*15*). Then, the pro-sequence is autocatalytically cleaved off and degraded to form active mature SBE (*13*, *16*).

Production systems of active recombinant SBE have been established by using not only the direct expression

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of prepro-SBE in bacteria, *e.g.*, *B. subtilis* (*9*, *12*), but also the *in vitro* refolding of pro-SBE (*11*, *17*). In this study, we produced active recombinant APRP using the *in vitro* refolding method to investigate the relationships between the *aprP* gene and SMCE from enzymatic and biochemical properties. In particular, we designed an *in vitro* refolding system with pro-APRP bound to a solid surface by an additional N-terminal tag sequence. Then, we compared the proteolytic activities of purified recombinant APRP with those of SMCE purified from *B. pumilus* TYO-67. Moreover, the necessity of the prosequence for mature APRP production was investigated in both the *in vitro* refolding and the *B. subtilis* expression systems.

MATERIALS AND METHODS

Bacterial Cultures and Chemicals—Escherichia coli strain DH5α [*deo*R *end*A1 *gyr*A96 *hsd*R17($r_k^-m_k^+$) *rec*A1 *rel*A1 *pho*A *sup*E44 *thi*-1 Δ(*lac*ZYA-*arg*F)U169 ϕ80d *lac*ZΔM15 F^- λ^-] was used as a host for the construction of the plasmids. *E. coli* strain BL21(DE3) [F^- *dcm omp*T *hsd*S_B ($r_B^-m_B^+$) *gal*(λ*cl*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7*gene*1) λ(DE3)] was used for the production of recombinant protein as a host for expressing recombinant pET11d plasmids (Novagen, Madison, WI). *E. coli* strain C600 (*rec*A^+ F^- *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *ton*A21 *sup*E44 λ^-) was used for the amplification of expression plasmids for *B. subtilis*. *B. subtilis* DB403 (*trp*C2 *apr*E^- *epr*E^- *npr*E^-), a three-protease-deficient strain, was a gift from Dr. R. H. Doi. The bacteria were cultured in Luria-Bertani (LB) medium or M9 medium containing 2% casamino acid (M9CA) (*18*). Synthetic peptide substrates, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-X-*p*-nitroanilide [AAPX; X = Ala (A), Leu (L), Lys (K), Met (M), Phe (F) and Val (V)], were purchased from Bachem AG (Bubendorf, Switzerland) and Sigma (St. Louis, MO). Bovine milk casein (Casein nach Hammarsten) was from Merck (Helsingborg, Sweden). Subtilisin BPN′ (BPN′), 2-(*N*-morpholino)ethanesulfonic acid (MES), and Folin & Ciocalteu's Phenol Reagent were purchased from Sigma. Coomassie Brilliant Blue R-250 (CBB) and other general chemicals were obtained from Nacalai Tesque (Kyoto, Japan).

Plasmid Constructions—To construct pNK, a plasmid expressing the *aprP* gene in *B. subtilis*, the full-length *aprP* was amplified from the genomic DNA of *B. pumilus* TYO-67 by PCR with aprp(+) (5′-CGGGATCCCCAAGCGACTTAATTCCC-3′) and aprp(–) (5′-GCTCTAGAGCTTTCCCAAGTCAATCC-3′) primers and TAKARA Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Japan). The amplified fragment (1.5 kb) containing both the deduced transcription promoter and the terminator was digested with *Bam*HI and *Xba*I, and then the resulting fragment was subcloned into pHY300PLK (Takara Bio Inc.), a shuttle vector between *E. coli* and *B. subtilis*.

To construct pET-proAPRP-His, a DNA fragment encoding pro-APRP with a 6× His tag sequence at the N-terminus was amplified from pNK by PCR with APRP-His(+) (5′-CATGCCATGGCACATCACCATCACCATCACAATGCCGAGACTGCCT-3′) and APRP-*Bam*HI(–) (5′-CGGGATCCTTAGTTAGAAGCTGCTTGAAC-3′) primers. The amplified fragment (1.1 kb) was digested with *Nco*I and *Bam*HI, and then subcloned into a pET11d vector.

pHY-APRP(Δpro) and pET-ΔproAPRP-His plasmids were constructed on pHY300PLK and pET11d vectors, respectively. These plasmids carry prepro-APRP- and pro-APRP-coding sequences, respectively, but they lack most of the pro-sequence. First, the full-length *aprP* fragment was excised from pNK with *Bam*HI and *Xba*I, and then the resulting fragment was subcloned into pBS(ΔSma-Xho), a modified pBluescript SK^- vector lacking cloning sites between *Sma*I and *Xho*I, resulting in pBS-APRP. Then, a *Pst*I site was introduced into the prosequence-coding region at the site corresponding to Tyr (–68) of APRP by site-directed mutagenesis to produce pBS-APRP(+Pst). Briefly, two sets of the first PCR were performed separately with pBS-APRP and Ex Taq polymerase. One set was carried out with M13-40 (5′-GTTTTCCCAGTCACGAC-3′) and APRP(-68)PstI(–) (5′-CGATATAGCCTGCAGCACTTTCTG-3′) primers, while the other set was carried out with APRP(–68)PstI(+) (5′-CAGAAAGTGCTGCAGGCTATATCG-3′) and APRP115(–) (5′-CATCCATGTTATTGGCAAC-3′). The amplified fragments (360 and 560 bp, respectively) were purified, mixed and further subjected to the second PCR with M13–40 and APRP115(–). The amplified fragment in the second PCR (900 bp) was digested with *Bam*HI and *Hin*dIII to produce a 300-bp fragment. This 300-bp fragment was ligated with a 4.2-kb fragment prepared from pBS-APRP with *Bam*HI/*Hin*dIII digestion, resulting in pBS-APRP(+Pst). Then, the 400-bp fragment was amplified from pBS-APRP with APRP(-8)Pst(+) (5′-GACTGCAGAAGACCACAAAGCAGA-3′) and APRP 115(–) (5′-CATCCATGTTATTGGCAAC-3′) primers, and digested with *Pst*I and *Nhe*I to produce an 180-bp fragment encoding the autocleavage region of pro-APRP. This 180-bp fragment was ligated with the 4.3-kb fragment prepared from pBS-APRP(+Pst) with *Pst*I and *Nhe*I to produce pBS-APRP(Δpro). To construct pHY-APRP(Δpro), the whole insert (1.5 kb) of pNK was exchanged with the full-length insert (1.3 kb) of the pBS-APRP(Δpro) excised by *Bam*HI/*Xba*I digestion. The 5′-terminal region of Δpro-APRP-His (600 bp) was then amplified by PCR from pBS-APRP(Δpro) with APRP-His(+) and APRP190(–) (5′-AATTCAGGACCTGCGCT-3′) primers, and the product was digested with *Nco*I and *Nhe*I to produce a 200-bp fragment. To construct pET-ΔproAPRP-His, the *Nco*I/*Nhe*I fragment (400 bp) excised from pET-proAPRP-His was exchanged with this 200-bp fragment.

The nucleotide sequences were confirmed with an ABI PRISM™377 DNA sequencing system (Applied Biosystems Japan Ltd., Tokyo, Japan) using a ThermoSequenase™ dye terminator cycle sequencing kit (Amersham-Pharmacia Biotech, Buckinghamshire, UK).

Expression of pNK and pHY-APRP(Δpro) Plasmids in B. subtilis DB403—The pNK and pHY-APRP(Δpro) plasmids were amplified once in *E. coli* C600 to become concatemers, and then they were introduced into the *B. subtilis* DB403 strain by electroporation (*19*). Transformed cells were cultured at 37°C for 24 h in LB medium containing 20 μg/ml tetracycline in order to investigate the proteolytic activities of the culture supernatants.

Production of pro-APRP-His and Δpro-APRP-His—E. coli strain BL21(DE3) was transformed with pET-proAPRP-His and pET-ΔproAPRP-His. Individual transformants were cultured at 37°C in M9CA medium con-

taining 50 μg/ml ampicillin, and the production of pro-APRP-His and Δpro-APRP-His was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacteria were collected by centrifugation at 3,000 rpm for 10 min at 4°C, suspended in 10 mM NaPB (pH 6.2), then disrupted by sonication with a Bioruptor UCD-200T (CosmoBio, Japan). The inclusion body fractions were collected by centrifugation at 15,000 rpm for 10 min at 4°C, then solubilized in denaturing buffer (pH 8.0) containing 6 M guanidine-HCl, 10 mM Tris and 0.1% Tween 20.

Production of Recombinant APRP by In Vitro Refolding—*In vitro* refolding of pro-APRP-His was performed based on the rapid dilution method (*11*, *17*), except that the protein was in a bound state on a solid surface. First, 10 mg of pro-APRP-His protein solubilized in denaturing buffer (pH 8.0) was bound to 2.5 ml of Ni-NTA agarose resin (Qiagen). After washes with suspension buffer (pH 6.3) containing 6 M guanidine-HCl, 10 mM NaPB and 0.1% Tween 20, the resin was suspended in the same buffer, resulting in a total volume of 5 ml. Binding to the resin was confirmed by elution with 6 M guanidine-HCl (pH 4.5) from a small part of the resin. Then, the resin suspension was slowly (*i.e.*, over *ca.* 5 min) dropped into 100 volumes of a refolding buffer containing 700 mM $(NH_4)_2SO_4$, 50 mM buffer [Tris-HCl (pH 8.5, 9.0 or 9.5) or Gly-NaOH (pH 10.0 or 10.5)], 1 mM $CaCl_2$ and 0.1% Tween 20, at 4°C with gentle stirring. After overnight incubation at 4°C, the Ni-NTA resin was removed by centrifugation. Then, the refolded sample was subjected to ultrafiltration in order to concentrate mature APRP by removing molecules smaller than 10 kDa, and then dialyzed with 10 mM NaPB (pH 6.2), 1 mM $CaCl_2$. The active enzyme was purified from the dialyzed sample using an ÄKTA FPLC system (Pharmacia) with a MonoS HR 5/5 column (Pharmacia), which was equilibrated with 50 mM NaPB (pH 6.2) and 1 mM $CaCl_2$. The enzyme was then eluted with a 0–0.2 M KCl linear gradient, and the active fractions were identified by their AAPF-hydrolyzing activity. The purified enzyme was dialyzed against 10 mM NaPB (pH 6.0) containing 1 mM $CaCl_2$. The N-terminal amino acid sequence of purified APRP was determined by the Edman degradation method. The purified enzyme was divided into small aliquots, frozen with liquid nitrogen, and stored at –80°C until use.

Preparation of SMCE—As described in the previous report, SMCE was purified from the culture supernatant of *B. pumilus* TYO-67 by ammonium sulfate precipitation and multi-step column chromatography, *e.g.*, hydrophobic and ion-exchange chromatography (*3*).

Hydrolysis of Synthetic Peptide Substrates—The hydrolytic activities for synthetic peptide substrates (*i.e.*, AAPA, AAPF, AAPK, AAPL, AAPM and AAPV) were investigated as described previously (*20*). Enzyme reactions were performed in assay buffer containing 50 mM Tris-HCl (pH 9.0) and 1 mM $CaCl_2$ at 45°C unless otherwise stated. Recombinant wild-type SBE was prepared as described previously and used as a control at pH 8.5 and 37°C (*12*). The amount of *p*-nitroaniline release was estimated by measuring the absorbance at 410 nm using a Beckman Spectrophotometer DU640 (Beckman Instruments, Inc., Fullerton, CA). The specific activities of the refolded samples and the purified enzymes are shown as units (U) per mg protein. One unit was defined as the amount of an enzyme that could release 1 nmol of *p*-nitroaniline per min. The kinetic values of the hydrolysis reactions were determined from the initial rates of the reactions. To investigate the pH profiles of enzymatic activity, 50 mM acetate (pH 4.0–5.5), MES (pH 5.5–7.0), Tris-HCl (pH 7.0–9.5) or Gly-NaOH (pH 9.5–11.0) buffer containing 1 mM $CaCl_2$ was used.

Caseinolytic and Soybean-Milk-Coagulating Activities—The hydrolysis of bovine milk casein was investigated using a modified version of the method of Hagihara *et al.* (*21*). Reaction mixtures containing 0.6% casein, 50 mM Tris (pH 9.0), 1 mM $CaCl_2$ and a protease sample were incubated at 37°C. The reactions were stopped by the addition of a trichloroacetic acid (TCA)-containing reagent. The precipitate was then removed by centrifugation, the supernatant containing the digested peptides was mixed with Folin's phenol reagent, and absorbance was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to increase the absorbance by the equivalent of 1 μg of tyrosine per min. Soybean milk-coagulating activity was determined at pH 6.1 and 65°C by a modified version of the method of Arima *et al.*; in brief, the time was measured until the soybean milk had coagulated (*3*, *22*). One unit of activity was defined as the amount of enzyme required to coagulate 1 ml of soybean milk per min. Soybean milk was prepared as described previously (*23*).

Protein Concentration and SDS-PAGE—Protein concentration was measured with a Protein Assay kit (Nippon Bio-Rad Laboratories, Tokyo) on the basis of Bradford's method or by using Micro BCA Protein Assay Reagent (Pierce, Helsingborg, Sweden). BPN′ was used as a standard protein in both assays.

To investigate various recombinant APRP samples, *e.g.*, inclusion body fractions and the purified enzyme, on SDS-PAGE, the proteins were denatured in 6 M guanidine-HCl (pH 4.8 or 8.0) and precipitated with a final concentration of 0.6 M TCA to avoid self-hydrolysis. The precipitates were washed with ethanol at –20°C, solubilized in the SDS-PAGE sample buffer, boiled, and applied to SDS-PAGE according to the method of Laemmli *et al.* (*24*). SDS-PAGE gels were stained with CBB.

RESULTS

In vitro refolding of pro-APRP-His Produced by E. coli BL21(DE3)—To investigate whether the *aprP* gene product is an active protease, the gene was first expressed in the *B. subtilis* DB403 strain. When the strain was transformed with the pNK plasmid carrying the full-length *aprP*, the transformants showed significant proteolytic activity (data not shown), suggesting that *aprP* encoded an active protease. However, because the active enzyme could not be purified completely, another system for recombinant protein production became necessary for accurate investigations. Thus, the development of an *in vitro* refolding system was examined.

APRP is a member of the subtilisin family and must be synthesized as a prepro-enzyme in *B. pumilus* TYO-67 (Fig. 1A). The pre-sequence could be a signal peptide for secretion, while the pro-sequence could be essential for the correct folding of the mature enzyme sequence, acting

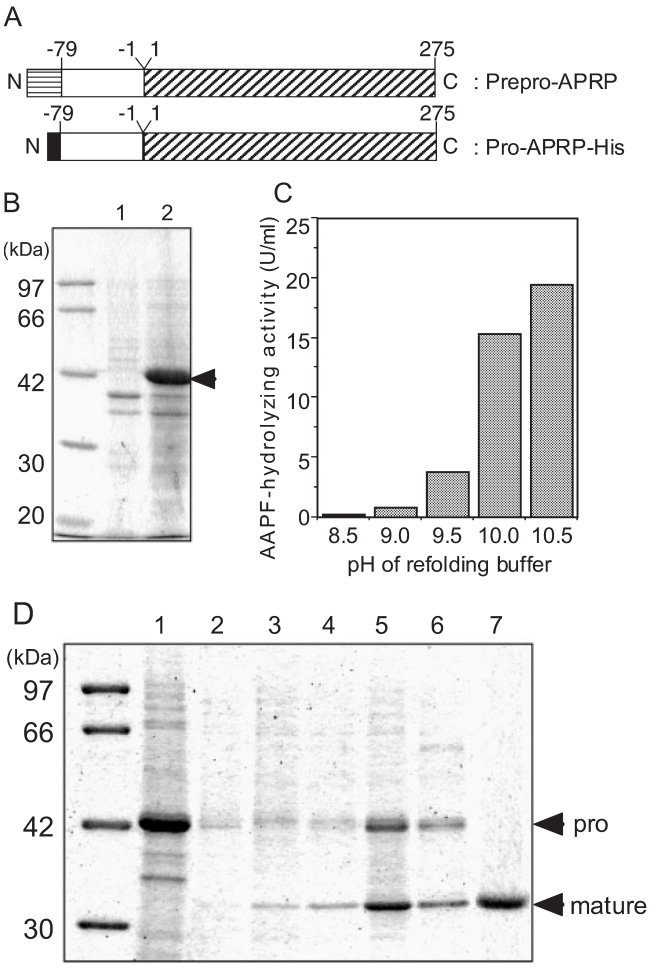

Fig. 1. ***In vitro* refolding of recombinant pro-APRP-His on Ni-NTA resin.** (A) Schematic drawings of prepro-APRP (383 amino acid residues) and pro-APRP-His (362 residues). Horizontally striped, open, hatched and filled boxes indicate the signal peptide (29 residues), the pro-sequence (79 residues), the mature enzyme sequence (275 residues) and the 6× His tag, respectively. (B) Production of pro-APRP-His in *E. coli* BL21(DE3). The precipitate fraction prepared from the lysate of BL21(DE3) transformed with pET11d (lane 1) or pET-proAPRP-His (lane 2) was analyzed on SDS-PAGE. An arrowhead indicates pro-APRP-His. (C, D) Investigation of the optimal pH of the refolding buffer. A 0.2-ml suspension of Ni-NTA resin bound with *ca.* 0.4 mg of pro-APRP-His was dropped into 20 ml of refolding buffer at pH 8.5–10.5, and was incubated with gentle stirring overnight at 4°C. The AAPF-hydrolyzing activity of the refolded sample was measured at pH 9.0 and 45°C in (C). Proteins in 1 ml of the individual refolded samples were precipitated with TCA reagent and analyzed by SDS-PAGE to investigate mature enzyme formation. Lane 1, *ca.* 10 μg of crude pro-APRP-His produced by BL21(DE3); lanes 2–6, refolded sample at pH 8.5, 9.0, 9.5, 10.0 and 10.5, respectively; lane 7, *ca.* 3 μg of purified SMCE. Arrowheads labeled with 'pro' and 'mature' denote pro-APRP-His and mature APRP, respectively.

as an intramolecular chaperone, as do other subtilisin family members (*15*, *16*). As regards SBE, a member of the subtilisin family, the *in vitro* refolding method using the pro-enzyme was established to produce active mature SBE (*11*, *17*). However, in preliminary experiments, the production of active mature APRP by the *in vitro* refolding of pro-APRP failed under the same conditions as

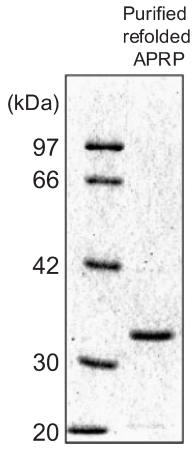

Fig. 2. **SDS-PAGE of mature APRP purified from the sample refolded *in vitro*.** Approximately 3 μg of protein was applied to the gel.

those for the SBE production. We then modified the system by using pro-APRP-His, *i.e.*, N-terminally 6× His-tagged pro-APRP (Fig. 1A), in order to design a pro-enzyme that could bind to a solid surface during the refolding procedure *via* the His tag, because such binding might mimic the attachment to the bacterial surface at the time of secretion.

A large amount of pro-APRP-His was produced in *E. coli* BL21(DE3) transformed with pET-proAPRP-His (Fig. 1B). Approximately 8 mg of crude pro-APRP-His was obtained in the insoluble protein fraction prepared from a 20-ml bacterial culture. The optimal constitution of the refolding buffer was determined by varying constituents one by one, based on that required for pro-SBE. At the final step, the pH of the refolding buffer was investigated between pH 8.5–10.5. As shown in Fig. 1C, the AAPF-hydrolyzing activity of the refolded sample increased remarkably at pH 10.0–10.5. Moreover, a remarkable increase in mature APRP was detected on SDS-PAGE in the samples refolded at pH 10.0–10.5, and the Mw of mature APRP seemed identical to that of SMCE purified from *B. pumilus* TYO-67 (Fig. 1D). Thus, the optimal pH for the refolding of APRP was determined to be 10.0.

Purification of Refolded mature APRP—A large-scale *in vitro* refolding experiment was performed with 10 mg pro-APRP-His under the conditions described above. As described in Table 1, 0.16 mg of active mature APRP was purified from 500 ml of refolded sample through ultrafiltration, dialysis and cation-exchange chromatography. The AAPF-hydrolyzing activity of purified APRP was 160,000 U/mg protein at pH 9.0 and 45°C, and the enzyme showed a single band on SDS-PAGE (Fig. 2). Because the N-terminal amino acid sequence was identical to that of SMCE, *i.e.*, Ala-Gln-Thr-Val-Pro-, the autocleavage of pro-APRP occurred at the correct site.

pH and Temperature Profiles—The AAPF-hydrolyzing activity of the refolded mature APRP and SMCE was investigated at various pHs and at a fixed temperature of 45°C (Fig. 3A). Both enzymes showed precisely the same pH profile, and the optimal pH was 9.0. Then, the temperature profiles of both enzymes for AAPF-hydrolyzing

Table 1. **Purification of recombinant APRP produced by *in vitro* refolding of pro-APRP-His.**

Step	Volume (ml)	Total protein (mg)	Total activity(U)	Specific activity (U/mg)	Purification (fold)
Refolded sample	500	10	7,300	730	1
Ultrafiltration and dialysis	45	1.1	32,000	29,000	39
Mono S HR 5/5	3.2	0.16	26,000	160,000	220

AAPF-hydrolyzing activity was measured at pH 9.0 and 45°C. One unit was defined as the amount of enzyme that could release 1 nmol of *p*-nitroaniline per min.

Table 2. **Kinetic parameters of APRP, SMCE and SBE in synthetic peptide hydrolysis.**

Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	*n*
AAPF	APRP	0.71 ± 0.13	370 ± 65	520 ± 31	3
	SMCE	0.70 ± 0.037	220 ± 19	310 ± 10	3
	SBE	1.1 ± 0.17	11 ± 1.5	10 ± 0.26	6
AAPL	APRP	0.50 ± 0.074	95 ± 9.5	190 ± 9.4	3
	SMCE	0.29 ± 0.051	50 ± 15	170 ± 24	3
AAPM	APRP	0.42 ± 0.060	78 ± 1.6	190 ± 27	3
	SMCE	0.52 ± 0.16	58 ± 12	120 ± 35	3

Data are shown as the mean ± SD. '*n*' denotes the number of experiments.

activity were investigated at pH 9.0 (Fig. 3B). There were no significant differences in the temperature profiles, and the optimal temperature was 50–55°C. However, the maximum activity of APRP (160,000 U/mg) was larger than that of SMCE (100,000 U/mg). SMCE might have been partially denatured during purification from the *B. pumilus* culture supernatant, because the purification process requires multi-step column choromatography (*3*).

Substrate Specificity with Synthetic Peptides—The specificity for the P1-residue, namely, the amino acid residue at the N-terminal side of the scissile peptide bond, of refolded mature APRP and SMCE was investigated with various synthetic peptide substrates at pH 9.0 and 45°C (Fig. 4). The order of preference of APRP for the substrates, *i.e.*, AAPF > AAPL > AAPM, was the same as that of SMCE (Fig. 4). It was also clearly different from that of SBE, *i.e.*, AAPM > AAPF > AAPL > AAPK (Fig. 4). Moreover, the specific activity of APRP and SMCE in AAPF hydrolysis (160,000 and 100,000 U/mg, respectively) was much larger than that of SBE (2,400 U/mg).

Kinetic Analyses of Synthetic Peptide Hydrolyses—The kinetics of AAPF-, AAPL- and AAPM-hydrolyzing reactions with refolded mature APRP and SMCE were investigated. As shown in Table 2, there seemed to be no significant differences in K_m values between APRP and SMCE for individual substrates, while k_{cat} and k_{cat}/K_m values of APRP were each about 1.5 times larger than those of SMCE. The differences may be due to the partial denaturation of SMCE during the multi-step purification (*3*). Moreover, the order of the k_{cat}/K_m values of each enzyme was almost the same as that of the specific activities shown in Fig. 4, *i.e.*, AAPF > AAPL > AAPM. This result seemed to be primarily due to the k_{cat} differences. In addition, the k_{cat}/K_m values of APRP and SMCE for AAPF were 30–50 times larger than that of SBE.

Caseinolytic and Soybean-Milk-Coagulating Activities—Because SMCE is a strong alkaline protease and induces soybean-milk-coagulation by the digestion of soy proteins (*4*), the caseinolytic and soybean-milk-coagulating activities of refolded APRP were investigated in comparison with those of SMCE. The caseinolytic activities of refolded APRP and SMCE were 4,400 ± 530 and 4,300 ± 560 U/mg (mean ± SD, n = 3), respectively, while their soybean-milk-coagulating activities were 120 ± 3.6 and 97 ± 13 U/mg (mean ± SD, n = 3), respectively. Thus, both enzymes had similar activities.

Necessity of Pro-sequence for Maturation—APRP, like SBE and BPN′, is a member of the subtilisin family. The

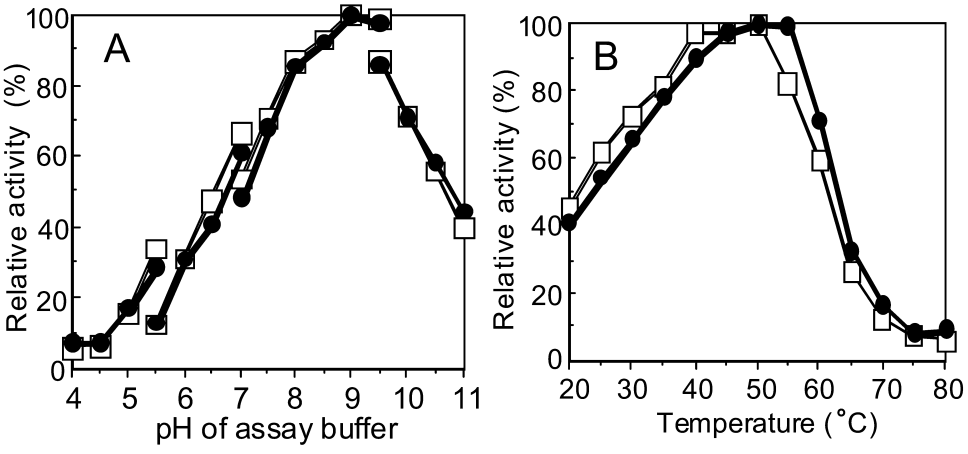

Fig. 3. **Comparison of pH and temperature profiles of AAPF-hydrolyzing activities of refolded mature APRP and SMCE.** The activities were measured by varying either the pH of the assay buffer at 45°C (A) or the assay temperature at pH 9.0 (B). The relative activities of APRP (closed circles) and SMCE (open boxes) are shown as percentages of the highest values (A, 160,000 and 100,000 U/mg at pH 9.0 for APRP and SMCE, respectively; B, 170,000 U/mg at 55°C for APRP and 110,000 U/mg at 50°C for SMCE).

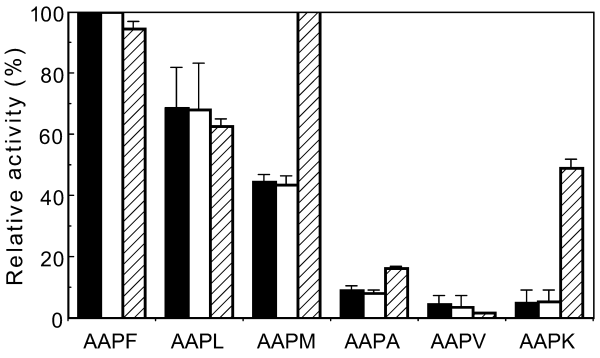

Fig. 4. **P1-specificity of refolded mature APRP, SMCE and SBE for synthetic peptides.** The activities of APRP (closed bars) and SMCE (open bars) were measured at pH 9.0 and 45°C, while those of SBE were measured at pH 8.5 and 37°C (hatched bars). Relative activities are shown as a percentage of the highest value (APRP, 160,000 U/mg for AAPF; SMCE, 100,000 U/mg for AAPF; SBE, 2,600 U/mg for AAPM). Each bar shows the mean ± SD (n = 3). The order of preference for substrates of APRP was the same as that of SMCE, but clearly differed from that of SBE.

pro-sequence of SBE has been investigated in detail, and it is known to play an essential role as an intramolecular chaperone for the correct folding of the mature enzyme sequence (*13*). Because the pro-sequence of APRP has about 48% amino acid residue identity with those of SBE and BPN′, the pro-sequence of APRP is also likely to function as an intramolecular chaperone (Fig. 5A). Therefore, pET-ΔproAPRP-His and pHY-APRP(Δpro) plasmids were constructed in order to investigate the importance of the pro-sequence of APRP to produce the active enzyme by both *in vitro* refolding and *in vivo* expression systems, respectively. The Δpro-APRP-His protein lacked most of the middle portion of the pro-sequence, which was assumed to be important for intramolecular chaperone activity (Fig. 5A). The pro-APRP-His and Δpro-APRP-His proteins produced in BL21(DE3) were bound to Ni-NTA resin and refolded overnight at 4°C, then the AAPF-hydrolyzing activity of the refolded samples was investigated. The refolded sample of pro-APRP-His showed AAPF-hydrolyzing activity of 15.9 ± 3.14 U/ml (mean ± SD, n = 5), whereas no activity (< 0.05 U/ml) was detected in that of Δpro-APRP-His. In agreement with this result, mature APRP was detected in the refolded sample of pro-APRP-His by SDS-PAGE, whereas it was not detected in that of Δpro-APRP-His (Fig. 5B). Moreover, AAPF-hydrolyzing activity was not detected in the culture supernatant of *B. subtilis* DB403 transformed with pHY-APRP(Δpro), whereas remarkable activity was detected in that of the transformant expressing the full-length prepro-APRP (Fig. 5C).

DISCUSSION

There have been many reports concerning the improvement of hypercholesterolemia with diets of soybeans and soy proteins (*1*, *2*). SMCE purified from *B. pumilus* TYO-67 shows excellent soybean-milk coagulation by digesting soy proteins (*4*), and can be applied in the production of various processed foods from soybean milk. However, because the purification of SMCE requires multi-step chromatography, it is desirable to develop an easier method of producing the purified recombinant enzyme. In this study, this aim was achieved by *in vitro* refolding of the pro-enzyme.

When large amounts of a recombinant protein are produced in *E. coli*, they are frequently included in the insol-

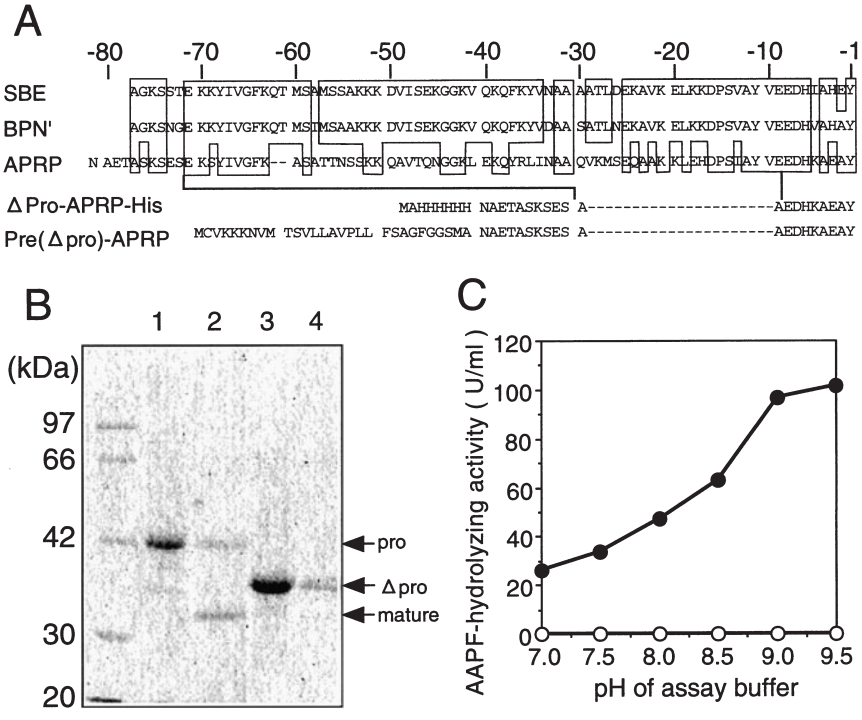

Fig. 5. **Necessity of the pro-sequence of APRP for active enzyme production.** (A) Comparison of pro-sequences of SBE, BPN′ and APRP with the N-terminal sequences of Δpro-APRP-His and pre(Δpro)-APRP. The identical amino acids among SBE, BPN′ and APRP are enclosed in boxes. About eight to ten amino acid residues in the N- and C-termini of the pro-sequence of APRP were left in Δpro-APRP-His and pre(Δpro)-APRP in order to avoid the failures in signal peptide cleavage and autocatalytic processing to produce mature APRP. (B) SDS-PAGE analysis of mature APRP formation from pro- and Δpro-APRP-His by *in vitro* refolding. Lanes 1 and 3, *ca.* 10 μg crude pro- and Δpro-APRP-His produced in *E. coli* BL21(DE3), respectively. Lanes 2 and 4, proteins in 1 ml of refolded samples of pro- and Δpro-APRP-His, respectively. Arrows labeled with pro, Δpro, and mature indicate pro-APRP-His, Δpro-APRP-His, and mature APRP, respectively. Mature APRP was detected only in lane 2. The bands of pro- and Δpro-APRP-His detected in lanes 2 and 4 were due to release from the Ni-NTA resin during overnight incubation. (C) The pro-sequence was necessary for active APRP production in the *B. subtilis* expression system. The AAPF-hydrolyzing activities of the culture supernatant of *B. subtilis* DB403 expressing prepro-APRP (closed circles) or pre(Δpro)-APRP (open circles) were investigated by varying the pH of the assay buffer at 45°C.

uble protein fraction. In such cases, a method of *in vitro* refolding of a denatured insoluble protein is widely employed (*25*). To improve refolding efficiency, various reagents including cyclodextrins and cycloamylose are sometimes added to the refolding buffer as "artificial chaperones" (*26–29*). The candidate gene of SMCE, *aprP*, encodes a prepro-protease belonging to the subtilisin family. Because the pro-sequence of SBE is an intramolecular chaperone, the refolding of denatured pro-SBE needs no additional chaperones. Denatured pro-SBE can efficiently refold itself into the correct tertiary structure *in vitro* and becomes mature SBE through the autocatalytic cleavage of the pro-sequence (*11*, *17*, *30*). Thus, we attempted to modify the *in vitro* refolding system for pro-SBE to produce recombinant APRP. Moreover, we designed the refolding process such that it occurred on a solid surface, since such a system might mimic the state of pro-APRP attached to the surface of a bacterium after secretion. First, a large amount of pro-APRP-His was produced in *E. coli* BL21(DE3) (*ca.* 8 mg pro-enzyme from 20 ml of bacterial culture). Then, pro-APRP-His was denatured, bound to Ni-NTA resin, and refolded on the resin by the rapid dilution method. After cation-exchange chromatography, 0.16 mg of purified recombinant APRP was obtained from 10 mg of denatured inclusion body protein. The enzymatic properties of recombinant APRP with the synthetic peptide substrates, *i.e.*, the pH and temperature profiles and the P1-substrate specificity, were identical to those of SMCE (Figs. 3 and 4). However, the specific activities of APRP seemed to be somewhat greater than those of SMCE, possibly because our system needed only simple purification (Table 2). In addition, the apparent Mw and N-terminal sequences of both enzymes were also identical (Fig. 1D). Thus, based on these enzymatic and biochemical properties, recombinant APRP is identical to SMCE. In contrast, APRP/SMCE was shown to have greater activity than SBE, and also to have different P1-substrate specificity from SBE. In order to explain these results in terms of molecular structure, the X-ray crystalographic structure around the substrate-binding pocket and the catalytic center of APRP/SMCE must be investigated and compared with those of SBE. Nevertheless, the greater activity of APRP/SMCE could provide the desired effect in terms of soymilk coagulation.

There have been reports concerned with the aggregation of soybean milk and soy proteins induced by proteases such as bromelain and subtilisin Carlsberg (*31–33*). Such protease-induced coagulation is an important technique in the manufacture of processed foods from soybeans. When soy proteins such as conglycinin and glycinin contained in soybean milk are partially digested with APRP/SMCE, hydrophobic aggregation starts and soy protein curd will form. A traditional food, *tofuyo*, is made by the fermentation of this soy protein curd (*23*). Therefore, it is very important that both recombinant APRP and SMCE show almost equal activity levels, not only with respect to synthetic substrate hydrolysis, but also caseinolysis and soybean-milk-coagulation.

The essential region in the pro-sequence of SBE required for activity as an intramolecular chaperone has been thoroughly investigated, and several conserved residues and secondary structures in the middle portion of the pro-sequence have been shown to be important (*34*). Thus, we used *in vitro* refolding and *in vivo* expression systems to investigate whether or not the pro-sequence of APRP was necessary for the production of the active mature enzyme. No AAPF-hydrolyzing activity was detected either in the *in vitro* refolded sample of Δpro-APRP-His protein lacking most of the middle part of the pro-sequence or in the culture supernatant of *B. subtilis* DB403 transformed with pHY-APRP(Δpro) (Fig. 5C). In addition, mature APRP was not detected in the refolded sample of Δpro-APRP-His by SDS-PAGE, although it was certainly detected in the refolded sample of pro-APRP-His (Fig. 5B). Thus, the pro-sequence of APRP was suggested to be an intramolecular chaperone according to both *in vitro* refolding and *in vivo* expression systems.

In summary, a system for the production of active recombinant APRP was developed using an *in vitro* refolding method, and all of the enzymatic and biochemical properties of recombinant APRP were identical to those of SMCE purified from *B. pumilus* TYO-67. It is expected that recombinant APRP could be used in the production of soy protein curd, tofu. A specific gene disruption system using *B. pumilus* TYO-67 will be necessary to elucidate whether or not the single *aprP* gene encodes SMCE in this strain. Pro-APRP-His was refolded with the assistance of the pro-sequence on a solid surface, and the pro-sequence was correctly cleaved to release active mature APRP. This system has potential application for the recombinant production of similar autoprocessing-type proteases.

This work was supported in part by grants from the Fukui Prefectural Scientific Research Foundation. In this article, the amino acid residues of the pro-sequence are numbered from the C-terminus to the N-terminus in reverse order as follows: –1, –2, –3, *etc.*

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