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Purification and Characterization of a Proteolytic Enzyme from *Bacillus subtilis* M2-4

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The proteolytic enzymes produced by naturally occurring microorganisms during the bread-making process were investigated. We isolated one Gram-positive and aerobic endospore-forming rod bacterium designated *B. subtilis* M2-4 producing a proteolytic enzyme from traditional and naturally fermented wheat flour. An analysis of the 16S rRNA sequence of the isolated strain revealed it to be 99% identical to *Bacillus subtilis*. We purified the proteolytic enzyme to electrophoretic homogeneity from the culture supernatant by column chromatography. The calculated molecular mass of the purified enzyme determined by gel filtration was 33 kDa. The proteolytic enzyme showed optimal activity at 55 ℃and pH 11.0, and was stable below 50℃ and in the pH range of 5.0-11.0. The *N*-terminal amino acid sequence of the purified protease from *B. subtilis* M2-4 was AQSVPYGISQIKAPA, the same as other subtilisins. However, this is the first report of its isolation from traditional fermented wheat flour. The purified protease digested acid casein into fragments with hydrophilic and hydrophobic amino acids at the C-terminal, in particular Arg, Glu, Val and Ile.

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Keywords: *Bacillus subtilis* M2-4, proteolytic enzyme, naturally fermented food, traditional fermented food, wheat flour.

INTRODUCTION

For many thousand of years, people have prepared foods by traditional and natural fermentation. Naturally occurring microorganisms such as bacteria, yeast and moulds produce the proteolytic enzymes to make foods. Multi-proteolytic enzymes[1] which produced from fungal (*Aspergillus orizae*) and bacterial (*Lactobacillus* sp.[2] and *Bacillus* sp.) are used during the bread and biscuits processing. Similarities have been found among the microorganisms of the traditional fermented wheat flour foods and its dough collected from many places around the world.[3)-5)] The bacteria were isolated from traditional wheat flour dough and identified as *Bacillus* sp., *Lactobacillus* sp., *Leuconostoc* sp. and *Lactococcus* sp.[5] The gliadin of wheat flour digested by proteolytic enzyme from *Bacillus* sp. was more pronounced than that of yeast.[4] Proteolytic enzymes from these microorganisms not only improve the taste, texture, quality and other properties of the foods, but also degrade the protein allergens of the wheat flour to hypoallergens.[4)5)] As the numbers of wheat allergen patients are growing increasingly, hypoallergenic wheat flour and their products are necessary for these patients. It has been reported that hypoallergenic wheat flour might be processed with proteases from microorganisms.[6)7)] The mantou processed by *Bacillus* sp. and yeast was inhibited about 1/20-1/30 RAST inhibition rates than that by yeast only.[5]

This study purposes to isolate and identify a microorganism from traditional fermented wheat flour foods with high proteolytic activity and hydrophobic digestion activity. Furthermore, we also describe the purification and characterization of the proteolytic enzyme secreted from the microorganism.

MATERIALS AND METHODS

Microorganism

In order to screen the microorganism which produces protease with the ability to hydrolyze hydrophobic protein, strain M2-4, isolated from the traditional fermented wheat flour food named mantou from the Mongolian People's Republic, was used in this study.

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Medium

Proteolytic enzyme-producing microorganisms were screened on an insoluble collagen-medium (0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% citrate$\cdot 2H_2O$, 0.1% yeast extract, 0.3% insoluble collagen (type I, Sigma Co., USA) and 1.5% agar, pH 7.4). Except for the insoluble collagen, the medium was sterilized at 121℃ for 15 min. Collagen was dissolved with sterilized distilled water, then added into the sterilized pre-medium.

The medium for producing enzyme contained 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% citrate$\cdot 2H_2O$, 0.1% yeast extract, 0.3% gelatin, 0.01% $CaCl_2 \cdot 2H_2O$, 1% glucose and 1% polypeptone at pH 7.4. The medium was sterilized at 121℃ for 15 min.

Screening procedure

The microorganism was inoculated aerobically in a screening medium for 2–3 days at 35℃,[8] then the diluted culture was spread on an insoluble collagen-agar-medium and incubated at 35℃ for 48 h. Colonies forming large halos were regarded as collagen degradation microorganisms and collected. After assaying the proteolytic activity, the separated microorganism with the highest activity was selected.

Identification of bacterium

The bacterium was identified by the API 50 CH kit[9)10)] (Bio Mérieux Co., Ltd., Marcy-l'Etoile, France). The biological profile obtained for the strain after the final reading can be identified using the identification software with the database (Version 3.0) according to Bergey's Manual[11)] and information of Bio Mérieux Co., Ltd.

DNA sequencing

Nucleotide sequences of 16S ribosome RNA gene (16S rRNA) was analyzed by the dideoxynucleotide chain termination method using an automatic DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, USA). The extraction of amplified genomic DNA from the isolated microorganism was performed by the Microseq 16S rRNA gene kit (Applied Biosystems).

Collagen degradation assay

To assay collagen degradation activity, 400 μl of 50 mM Tris-HCl containing 4 mM $CaCl_2$ (pH 7.5) was pre-incubated with 10 mg insoluble collagen for 10 min at 37℃. Then 100 μl enzyme was added, the reaction was carried out at 37℃ for 30 min with shaking and then was stopped by adding 1 ml of 0.1 M acetic acid. The reaction mixture was centrifuged at 10,000×g for 10 min at 4℃. The amount of soluble degraded protein released from insoluble collagen was measured by the ninhydrin method.[12)] One unit of collagenolytic activity was defined as the amount of enzyme releasing 1 μmol of leucine equivalent per minute from insoluble collagen.

α_s-casein degradation assay

One milliliter of 0.6% α_s-casein (pH 7.5) was pre-incubated for 10 min at 37℃, then 100 μl enzyme was added and incubated for 10 min at 37℃ with shaking. The reaction was stopped by adding 500 μl of 10% trichloroacetic acid (TCA), and was then centrifuged at 10,000×g for 10 min at 4℃. The absorbance of supernatant was measured at 273 nm. One unit of caseinolytic activity was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per minute from α_s-casein.

Enzyme purification

All purification steps were carried out at 4℃. One liter of culture supernatant of *B. subtilis* M2-4 was concentrated to 49 ml by ultrafiltration using membrane 3000 CUT (Millipore Co., USA). The concentrated enzyme was loaded onto the first DEAE Sepharose CL-6B column (2.5×40 cm, Pharmacia Biotech Co., USA) pre-equilibrated with 10 mM Tris-HCl (containing 4 mM $CaCl_2$, pH 7.5). The column was eluted using a linear gradient from 0 to 2.0 M NaCl in 10 mM Tris-HCl (containing 4 mM $CaCl_2$, pH 7.5) at a flow rate of 100 drops/min. The active fractions were pooled and dialyzed against 10 mM Tris-HCl (containing 4 mM $CaCl_2$, pH 7.5) overnight. The dialyzed enzyme was loaded onto a CM-cellulose column (1.5×13 cm; Pharmacia Biotech Co.) pre-equilibrated with 10 mM Tris-HCl (containing 4 mM $CaCl_2$, pH 6.0). The column was eluted using a linear gradient from 0 to 1.0 M NaCl in 10 mM Tris-HCl (containing 4 mM $CaCl_2$, pH 6.0) at a flow rate of 100 drops/min. The active fractions were pooled and applied onto a pre-equilibrated Sephadex G-75 column (2.5×40 cm; Pharmacia Biotech Co.). The column was eluted with 10 mM Tris-HCl (containing 4 mM $CaCl_2$, pH 7.5) at a flow rate of 100 drops/min. The active fractions were pooled and used as a purified enzyme.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method developed by Laemmli[13)] with 12.5% polyacrylamide gel. Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14 kDa) were used as molecular mass standard.

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Enzyme properties

The effect of pH on the enzyme activity was assessed by incubating the purified enzyme with 10 mg insoluble collagen in 50 mM sodium acetic acid buffer (pH 3.0-6.0), 50 mM phosphate buffer (pH 6.0-8.0), 50 mM sodium carbonate-boric acid buffer (pH 8.0-10.0) and 50 mM di-sodium hydrogenphosphate sodium hydroxide buffer (pH 11.0-12.0) at 37℃ for 10 min. The pH stability of the enzyme was monitored by incubating the enzyme at various pHs for 30 min, and then residual activity was measured under standard assay conditions. The effect of temperature on the activity was assessed by incubating the purified enzyme with 0.5% gelatin in 50 mM Tris-HCl buffer (containing 4 mM $CaCl_2$, pH 11.0) at different temperatures in the range of 25℃ to 70℃ for 30 min. The thermostability of the purified enzyme was monitored by incubating the enzyme at each temperature for 30 min, and then the residual activity was measured under standard assay conditions, and insoluble collagen was used as the substrate. To study the effect of various additives on the activity, each additive (final concentration of 1 mM) was added to the standard assay mixture.

Molecular mass

The estimated molecular mass of the purified enzyme was analyzed with gel filtration on Sephadex G-75 (2.5×40 cm; Pharmacia Biotech Co.). To estimate the molecular mass, γ-Bovine globulin (150,000 Da), Bovine albumin (66,267 Da), Ovalbumin (42,700 Da), α-Chymotrypsinogen (25,000 Da) and Cytochome C (12,327 Da) were used as the standard.

The *N*-terminal amino acid sequence

The purified enzyme was elect blotted from SDS-PAGE onto PVDF membrane. The protease band on PVDF membrane was stained with Coomassie Brilliant blue G250 and washed with 50% methanol. The desired band was cut and subjected for *N*-terminal amino acid sequence by an ABI 471 peptide sequencer (Applied Biosystems).

Protein assay

Protein content was determined by the Lowry method[14] with alkaline cooper sulfate solution and phenol solution, using bovine albumin as the standard.

Analysis of peptide digestion site by protease from *B. subtilis* M2-4

Analysis of peptide digestion site was in the same method as described previously.[15][16] The 5% acid casein in 5 ml of solution (20 mM potassium phosphate buffer/pH7) was incubated at 37℃ for 15 min with 0.5 ml of protease of various protease concentrations (0.1-2.5 units) from *B. subtilis* M2-4 (from the step of DEAE sepharose CL 6B), Proleather FG-F (*Bacillus* sp.), Protease N (*B. subtilis*) and Protease A (*Aspergillus oryzae*) (Amano Enzyme Inc.). The peptide fragments were digested further at 37℃ for 60 min by carboxypeptidase Y. The released free amino acids were analyzed to assumed C-terminal amino acids of digested peptide fragment.

RESULTS AND DISCUSSION

Isolation and identification of proteolytic enzyme bacterium

There was one colony with the largest halo on an insoluble collagen medium isolated from strain M2-4. It was a Gram-positive bacterium with endospore. The bacterium was selected for further study because it showed the highest proteoytic activity. *Bacillus* spp. was identified to species level according to growth in 7% NaCl, at pH 5.7 and at 50℃, Voges-Proskauer reaction, hydrolysis of starch and utilization propionate.[11] Assay of the 16S rRNA sequence of the isolated strain revealed it to be 99% identical to *Bacillus subtilis* (Accession no. Z99104),[17] *Bacillus subtilis* No. 66 (Accession no. AB 110598),[18] *B. subtilis* FS-2[15] and *Bacillus subtilis* CN2 (Accession no. AB 383135).[16] According phylogenetic analysis on 16S rRNA gene using NJ analyses (Fig. 1), *Bacillus subtilis* M2-4 was distinguish from other *B. subtilis* strains.[17][18] The identified *Bacillus* was confirmed by testing their ability to fermented 49 different carbohydrates using the API 50 CH galleries and API 50 CHB medium (Table 1). The identified *Bacillus subtilis* M2-4 was reacted with salicin, D-lactose and xylitol. To our knowledge, *B. subtilis* M2-4 (Accession no. AB 379850) from phenotypic and genotypic identification is the first report of *B. subtilis* isolated from traditional fermented wheat flour food producing a proteolytic enzyme.

Purification of proteolytic enzyme

A summary of the purification of the proteolytic enzyme from *B. subtilis* M2-4 is shown in Table 2. The purified proteolytic enzyme moved as a single band on SDS-PAGE (Fig. 2). The total yield from the culture supernatant was 4.1%. About 3.8 mg of purified enzyme was obtained from 1,000 ml of culture broth. The total collaganase activity of 3.8 from *B. subtilis* M2-4 was 2 fold higher than that from *B. subtilis* FS-2.[15] The specific activity of the purified enzyme was 0.95 unit/mg for collagen reduction, slight higher than that of 0.71 unit/mg protein from *B. subtilis* FS-2.[15] It was free from caseinolytic activity, and showed specific only for insoluble collagen which was same as

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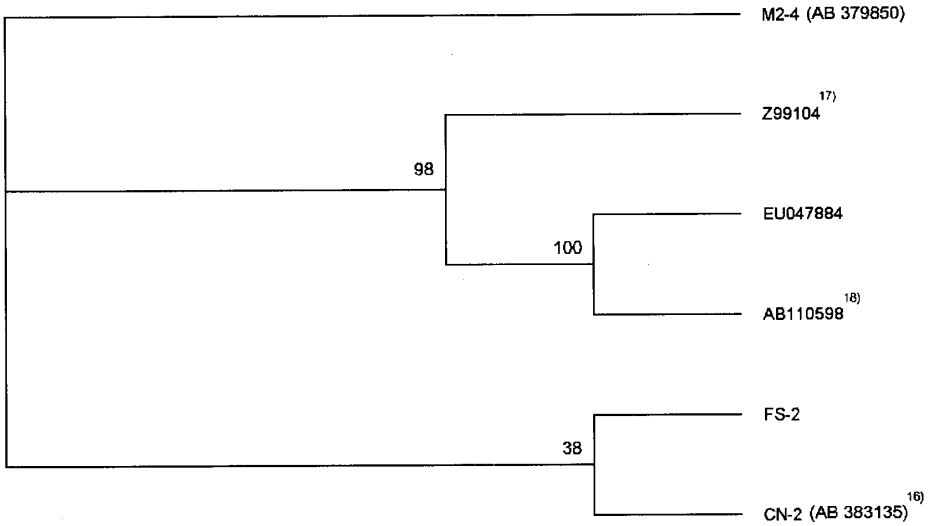

Fig. 1. Neighbor-joining phylogenetic trees base on 16S rRNA gene Sequence of isolated *B. subtilis* strains

GenBank accession numbers are provided for each strain except *B. subtilis* FS-2.

Table 1. Microbiological characterizations of *B. subtilis* M2-4, FS-2[15] and CN-2[16]

Test	Strain			Test	Strain		
	M2-4	FS-2	CN-2		M2-4	FS-2	CN-2
Shape	Rod	Rod	Rod				
Gram stain	+	+	+	Arbutin	±	±	−
Aerobic growth	+	+	+	Esculin ferric citrate	+	+	+
Sporulation	+	+	+	Salicin	+	−	±
Glycerol	+	+	+	D-Cellobiose	+	+	+
Erythritol	−	−	−	D-Maltose	+	+	+
D-Arabinose	−	−	−	D-Lactose	+	−	−
L-Arabinose	+	+	+	D-Melibiose	+	+	+
D-Ribose	+	+	+	D-Saccharose	+	+	+
D-Xylose	+	+	+	D-Trehalose	+	+	+
L-Xylose	−	−	−	Inuline	+	+	±
D-Adonitol	−	−	−	D-Melezitose	−	−	−
Methyl-βD-xylopyranoside	−	−	−	D-Raffinose	+	+	+
D-Galactose	−	−	−	Amidon (starch)	+	+	±
D-Glucose	+	+	+	Glycogene	+	+	±
D-Fructose	+	+	+	Xylitol	+	+	−
D-Mannose	+	+	+	β-Gentiobiose	−	−	−
L-Sorbose	−	−	−	D-Turanose	+	+	+
L-Rhamnose	−	−	−	D-Lyxose	−	−	−
Dulcitol	−	−	−	D-Tagatose	−	−	−
Inositol	+	+	+	D-Fucose	−	−	−
D-Mannitol	+	+	+	L-Fucose	−	−	−
D-Sorbitol	+	+	+	D-Arabitol	−	−	−
α-Methyl-D-mannoside	−	−	−	L-Arabitol	−	−	−
α-Methyl-D-glucoside	±	+	−	Potassium gluconate	−	−	−
N-Acetyl-glucosamine	−	−	−	Potassium 2-ketogluconate	−	−	−
Amygdalin	+	+	+	Potassium 5-ketogluconate	−	−	−

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Table 2. Purification of proteolytic enzyme from *B. subtilis* M2-4

Step	Volume (ml)	Total protein (mg)	Collagenolytic			Caseinolytic	
			Total activity (U)	Specific activity (U/mg Pr)	Yield (%)	Total (U)	Specific activity (U/mg Pr)
Culture supernatant	1,000	450.0	88.0	0.2 (1.0)	100.0	65.8	0.15
Ultra filtration	49	112.5	32.5	0.3 (1.5)	36.9	22.3	0.2
DEAE Sepharose-CL6B	50	42.2	19.0	0.5 (2.5)	21.6	13.5	0.3
CM-cellulose	21	8.4	6.5	0.8 (4.0)	7.4	—	—
Sephadex G-75	29	3.8	3.6	0.95 (4.8)	4.1	—	—

those purified proteolytic enzyme secreted from *B. subtilis* FS-2.[15]

Molecular mass

The relative molecular mass of the purified proteolytic enzyme by SDS-PAGE was estimated to be 30 kDa (Fig. 2). Furthermore, the calculated molecular mass of the native enzyme by Sephadex G-75 gel filtration was determined to be 33 kDa. This suggests that the purified enzyme from *B. subtilis* M2-4 was a low-molecular mass proteolytic enzyme without subunit. It was almost the same as that of the proteolytic enzyme from *B. lichenniformis* N22[19] and *B. subtilis* CN2[20] which were 29 kDa and 27 kDa, respectively. The molecular mass was lower as compared to those proteolytic enzymes from *Bacillus* Soc 67[21] (350 kDa), *Cytophaga* sp. L43-1[22] (120 kDa) and *Pseudomonas* sp.[8] (52 kDa). It was confirmed that the proteolytic enzyme from *Pseudomonas* sp. was composed from two subunits with molecular masses of 33 kDa and 19.8 kDa, respectively. The molecular mass of the purified proteolytic enzyme from *B. subtilis* M2-4 was much lower than that of collagenase from *B. subtilis* FS-2[15] (125 kDa) isolated from traditional fermented fish sauce. The molecular mass of *Bacillus subtilis* CN2 protease was determined to 31 kDa by SDS-PAGE and 27,636±3 Da by Electrospay Interface-Mass Spectrromery (ESI-MS).

The *N*-terminal amino acid sequence of *B. subtilis* M2-4 protease was AQSVPYGISQIKAPA, which was same as those of subtilisin from *B. amyloliquefaciens*,[23] protease from *Bacillus subtilis* CN2,[20] subtilisin E from *Bacillus subtilis*[24] and one Nattokinase.[25] Protelytic enzyme, especially subtilisins, were extensively studied and transformed long time ago. Moreover, these microorganisms were isolated from different origins and subtilisin E was a genetic protease encoded by *spr*E gene of *Bacillus subtilis*.[24] The protease from *B. subtilis* M2-4 was the first report that

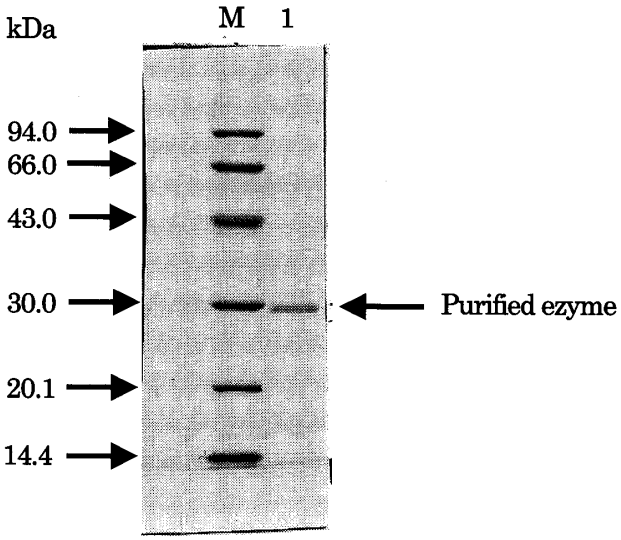

Fig. 2. SDS-PAGE of purified proteolytic enzyme from *B. subtilis* M2-4

Lane 1 indicates the final purified proteolytic enzyme. It was analyzed by SDS-PAGE using 12.5% polyacrylamide gels, followed by Commassie Brilliant Blue R-250 staining. Lane M indicates molecular mass marker.

indicated the presence of proteolytic enzyme on traditional wheat flour fermented foods.

Enzymatic properties of purified proteolytic enzyme

Some properties of the purified proteolytic enzyme from *B. subtilis* M2-4 were investigated with insoluble collagen as the substrate except at optimum temperature. The purified enzyme showed its highest activity at pH 11.0 (Fig. 3A) and was stable between pH 5.0-11.0 (Fig. 3B). Its optimal pH was higher than with the pH 7.4-9.0 reported for *B. subtilis* FS-2,[15] *Pseudomonas* sp.,[8] *Cytophaga* sp. L43-1,[22] *B. licheniformis* N22[19] and *B. amyloliquefaciens*.[23] It performed proteolytic activity at a strong alkaline condition and was suggested

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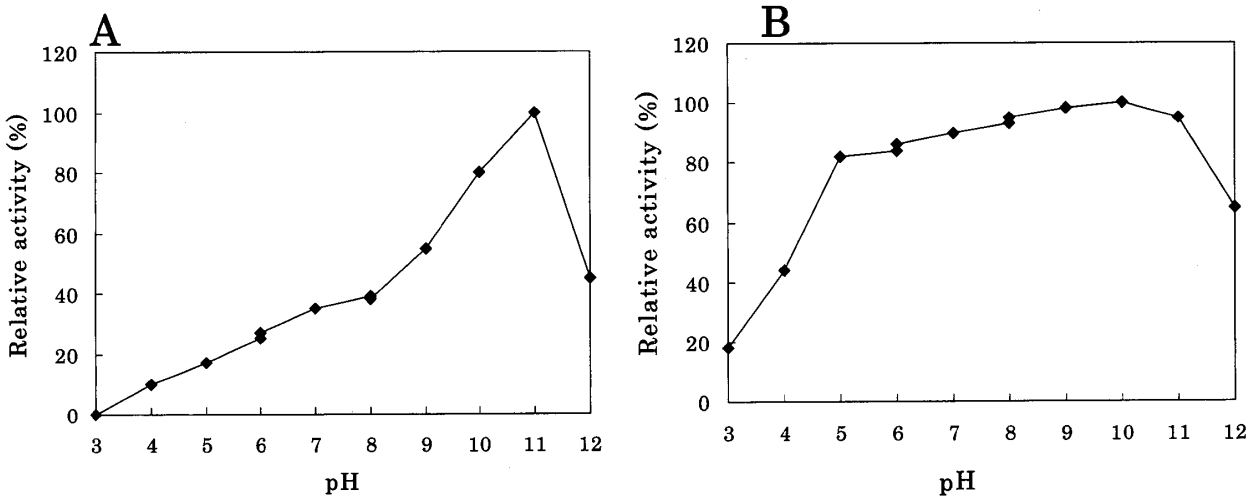

Fig. 3. Effect of pH on proteolytic enzyme from *B. subtilis* M2-4

(A) Effect of pH on collagen-degraded activity. 100 μl of purified enzyme was incubated with 10 mg insoluble collagen for 10 min at 37℃ in 400 μl of various buffer. (B) Effect of pH on stability. The enzyme was incubated for 30 min at 37℃ in various buffers, then the residual collagenolytic activity was measured. 50 mM sodium acetic acid buffer (pH 3.0-6.0), 50 mM phosphate buffer (pH 6.0-8.0), 50 mM sodium carbonate-boric acid buffer (pH 8.0-10.0) and 50 mM di-sodium hydrogenphosphate sodium hydroxide buffer (pH 11.0-12.0) were used.

to be an alkaline protease. It was stable at wider pH than that from *Cytophaga* sp. L43-1[22] (pH 6.0-8.0). However, there was a minor difference from *B. subtilis* FS-2[15] (pH 5.0-10.0).

The optimal temperature of the enzyme was 55℃ (Fig. 4A) utilizing gelatin as substrate, and it was only slighter higher than the 50℃ from *B. subtilis* FS-2[15] and the 48℃ from *B. amyloliquefaciens.*[23] However, it was higher than the 30℃ from *Cytophaga* sp. L43-1.[22] The enzyme was stable up to 50℃ (Fig. 4B), and 80% of its proteolytic activity remained after treatment at 60℃ for 30 min. It was higher than that (40-45℃) from *Cytophaga* sp. L43-1,[22] *B. licheniformis* N22[19] and *Pseudomonas* sp.[8] The enzyme was revealed to be a thermostable proteolytic enzyme.

The effect of metal ions and other chemical reagents on the proteolytic activity is shown in Table 3. *N*-ethylmaleimide, L-cystein, 2-mercaptoethanol or soybean trypsin-inhibitor at 1 mM or 0.1 mg/ml did not influence the proteolytic activity.

The proteolytic activity was inhibited by 1mM PMSF (33%), which are well-known inhibitors of serine proteases. EDTA (74%) slightly influenced its activity. The sensitivity of *B. subtilis* M2-4 enzyme to EDTA was similar to those from *B. subtilis* FS-2,[15] *Pseudomonas* sp.[8] and *B. licheniformis* N 22,[19] but quite different from those of *Cytophaga* sp. L43-1[22] and *C. histolyticum.*[26] This was in accord with the report in which the alkaline protease was inhibited very little by EDTA.[27] In addition, the enzyme activity was strongly inhabited by the presence of Zn^{2+}(47%), Mn^{2+}(54%), Co^{2+} (59%), or Cu^{2+}(25%), but was not significantly affected by Mg^{2+}, Ca^{2+}, Fe^{2+}.

Digestion of the acid casein by protease

Digestion site of acid casein is very important for functional peptide. The C-terminal amino acid of the peptide fragments that digested by *B. subtilis* M2-4 protease (after DEAE Sepharose-CL6B, in Table 2) were confirmed by a digestion with carboxypeptidase Y and then free amino acids of the acid casein were analyzed by an amino analyzing system (Table 4). The hydrophobic digestion site number of purified protease from *B. subtilis* M2-4 on acid casein is higher than from commercial enzyme of *B. subtilis* (Prolleather FG-F), *Bacillus* sp. (Protease N) and *Aspergillus oryzae* (Protease A). The protease from *B. subtilis* M2-4 digested acid casein into fragments with hydrophilic and hydrophobic amino acids at C-terminal, in particular Arg, Glu, Val and Ile. Especially, the Glu was an unique fragment comparing with another digesting fragments. There was no report of acid casein digestion of those four protease mentioned above. The protease from *B. subtilis* M2-4 was suggested to be a novel proteolytic enzyme. It was demonstrated that such proteolytic enzyme could delete gliadin of wheat flour protein[5] and degrade allergens of bread (mantou) to hypoallergens.[5][28] It shows the possibility to apply in food

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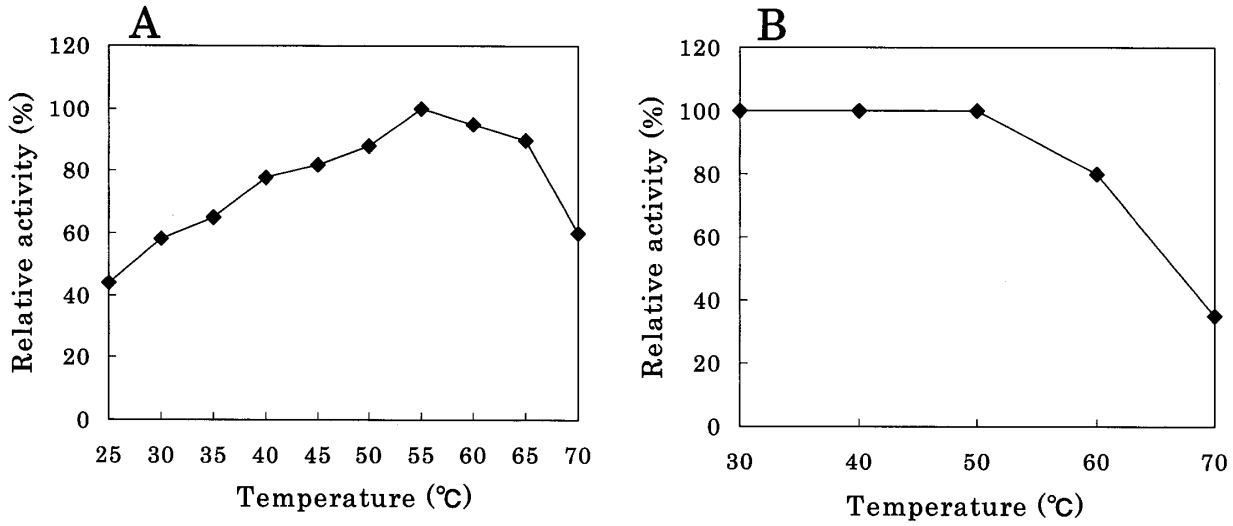

Fig. 4. Effect of temperature on proteolytic activity from *B. subtilis* M2-4

(A) Effect of temperature on collagen degraded activity. 100 μl of purified enzyme was incubated with 400 μl of 0.5% gelatin in 50 mM Tris-HCl buffer (containing 4 mM $CaCl_2$, pH 11.0) at different temperatures. (B) Effect of temperature on stability. Pre-incubation was carried out at indicated temperature for 30 min in the same buffer, then residual activities were measured under standard assay conditions.

Table 3. Effects of additives on proteolytic activity

Additives	Concentration (mM)	Relative activity (%)
None	—	100
$ZnCl_2$	1	47
$MgSO_4$	1	86
$MnCl_2$	1	54
$CoCl_2$	1	59
$CaCl_2$	1	125
$CuSO_4$	1	25
$FeSO_4$	1	107
N-Ethylmaleimide	1	94
L-Cystein	1	100
EDTA	1	74
Idoacetamide	1	81
2-Mercaptoethanol	1	99
Soybean trypsin-inhibitor	0.1 (mg/ml)	86
PMSF	1	35

industry.

As conclusion, we isolated a Gram-positive rod and endo-spore producing bacterium, designated as *B. subtilis* M2-4 which produced an effective alkaline protease. The purified enzyme showed substrate specific only for insoluble collagen and was thermostable. It was a novel protease and may thus be useful for food technology in general and in hypoallergenic and functionally foods in particular. Because the properties of protease from *B. subtilis* M2-4 was same as those from *Bacillus* sp. and *Aspergillus* sp., which produced an effective same enzyme as *B. subtilis* FS-2.[1] We suggest to clone the protease genes from *B. subtilis* M2-4 for further study of enzyme specificities, amino acid sequences and secretion mechanism.

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Table 4. Specificity of peptide digestion sites of acid casein by enzyme from *B. subtilis* M2-4

	His	Arg	Asn	Gln	Ser	Asp	Gly	Glu	Thr	Ala	Pro	Met	Val	Cys	Lys	Phe	Ile	Leu
B. subtilis M2-4		↑		↑	↑			↑	↑	↑			↑		↑	↑	↑	↑
B. subtilis FS-2		↑	↑	↑	↑		↑		↑	↑			↑		↑	↑	↑	↑
Proleather FG-F				↑	↑					↑					↑	↑		↑
Protease N									↑							↑		↑
Protease A		↑		↑	↑				↑	↑					↑	↑		↑

The arrows indicate digestion sites of protease from *B. subtilis* M2-4, *B. subtilis* FS-2, Proleather FG-F (*B. subtilis*), Protease N (*Bacillus* sp.), Protease A (*Aspergillus oryzae*). ↑: the C-terminal amino acids of digested fragment.

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Bacillus subtilis M2-4 由来タンパク分解酵素の精製とその性質

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パンに代表される伝統的な自然発酵食品は，古くから世界中で食され，自然発酵微生物の生産するタンパク質分解酵素はパン作りに利用されてきた．今回は，小麦粉発酵食品よりタンパク分解酵素を産生するグラム陽性桿菌*B. subtilis* M2-4を分離した．分離菌株は，16S rRNAによる遺伝子解析の結果，*B. subtilis*と99%一致した．*B. subtilis* M2-4の産生するタンパク分解粗酵素は，カラムクロマトグラフィーを用い，単一バンドまで精製した．ゲルろ過により精製酵素の分子量は，33 kDaと推定された．至適温度は55℃で，至適pHは11.0であり，その酵素安定性は，50℃まで，ならびにpH 5.0-11.0の範囲で安定であった．*N*-末端アミノ酸配列はAQSVPYGISQIKAPAであり，サブチリシンと同じであった．しかし，小麦粉発酵食品が分離源であることは初めてであった．酸性カゼインに対する分解フラッグメントのC末端は，特に親水性アミノ酸アルギニン，グルタミン，疎水性アミノ酸バリン，イソロイシン等のアミノ酸であった．

キーワード：*Bacillus subtilis* M2-4，タンパク分解酵素，自然発酵食品，伝統発酵食品，小麦粉．

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