Appl Microbiol Biotechnol (2000) 53: 390–395

ORIGINAL PAPER

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Soybean-milk-coagulating activity of *Bacillus pumilus* derives from a serine proteinase

Received: 9 July 1999 / Received last revision: 22 October 1999 / Accepted: 5 November 1999

Abstract A proteolytic enzyme from *Bacillus pumilus* strain TYO-67, which was able to coagulate the protein in soybean milk, was characterized enzymologically. The optimum pH and temperature for its activities were 9.0 and 50 °C, respectively. The enzyme was strongly believed to be a serine proteinase because it was completely inhibited by the addition of diisopropyl fluorophosphate or phenylmethanesulfonyl fluoride. Hammerstein milk casein, cytochrome *c* and soybean protein were good substrates for the enzyme. Seven cleavages were detected using the oxidized insulin B-chain as peptide substrate for the proteolytic specificity test of the serine proteinase from *B. pumilus*. The bonds most susceptible to the action of the serine proteinase from *B. pumilus* were Leu-15–Tyr-16. The mode of action on soybean milk protein by the enzyme from *B. pumilus* was also investigated. The acidic subunit in glycinin and the α′-, α- and β-subunits in β-conglycinin were degraded during the enzyme reaction. However, the basic subunit in glycinin could not be degraded by the enzyme. The formation of coagula in soybean milk caused by the serine proteinase from *B. pumilus* was mainly due to the hydrophobic interaction.

Introduction

It is well known that proteolytic enzymes are able to coagulate milk and that chymosin and fungal proteases are used for cheese production. Some reports have shown that plant proteases such as bromelain and ficin are capable of coagulating soybean milk. Fuke and Matsuoka (1984) reported that soybean milk was coagulated by bromelain to form soybean curd and they proceeded to prepare soybean cheese. Kamata et al. (1991) also reported the preparation of a cheese analogue (from soybean curd) using bromelain. The curd obtained by using the enzyme that has soybean-milk-coagulating activity can serve not only as a material for soybean cheese production but may also be widely applicable to food processing.

We reported the preparation of tofuyo (a traditional soybean cheese in Okinawa, Japan) from tofu (soybean curd) (Yasuda and Hokama 1984; Yasuda et al. 1992, 1993, 1994, 1996). Microbial soybean-milk-coagulating enzyme (SMCE) is essential for making tofuyo. However, reports on this microbial enzyme are very limited. Park et al. (1987) reported the characterization of SMCE in the genus *Bacillus*. Yasuda et al. (1999) reported the purification and characterization of SMCE from *B. pumilus* TYO-67 isolated from tofu.

Proteinases are classified into four groups, i.e. serine proteinases, cysteine proteinases, metal proteinases and aspartic proteinases. As bromelain is a cysteine proteinase, it is necessary to establish the proteinase category to which SMCE from *B. pumilus* TYO-67 belongs.

We now report the identity and characterization of the *B. pumilus* TYO-67 proteinase that has SMCE activity, and its mode of action on the protein in soybean milk is described.

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Materials and methods

The microorganism, its cultivation and purification of the enzyme

The microorganism used throughout this study was *B. pumilus* TYO-67, which was isolated in our laboratory from the tofu used for the manufacture of tofuyo. The culture of the microorganism

and the purification of the enzyme used in this work were carried out as previously reported (Yasuda et al. 1999).

Preparation of soybean milk

Soybean milk was prepared according to the method reported previously (Yasuda et al. 1999).

Chemicals

Soybean protein isolate was obtained from the Fuji Oil Co. (Japan). Oxidized insulin B-chain, subtilisin Carlsberg and stem blomelain were obtained from Sigma Chemicals (USA). The other chemicals were of analytical grade.

Enzyme assay and protein determination

Proteinase activity was assayed at pH 9.0, as described previously (Yasuda and Sakaguchi 1998). SMCE activity was assayed at 65 °C, as described previously (Yasuda et al. 1999). Protein concentrations were determined using the BCA Protein Assay Reagent Kit (PIERCE CO., Ltd.) with desalted bovine serum albumin as the standard.

Digestion of the oxidized insulin B-chain and separation of the digested peptides

First, 100 μg oxidized insulin B-chain was dissolved in 20 mM $H_3BO_3 \cdot$KCl-NaOH buffer (pH 9.0) and it was incubated with the enzyme (0.5 U for soybean protein) at 50 °C for an appropriate reaction time. At intervals of 5, 10, 30 and 60 min, duplicate 0.1-ml aliquots were removed and placed in an ice bath. The digested peptides (50 μl) were then separated by reversed-phase high-performance liquid chromatography (HPLC) at 40 °C on a COSMOSIL 5C18-AR-300 column (4.6-mm internal diameter × 150 mm, nacalai tesque CO., Ltd.), with a linear gradient of 0–60% acetonitrile solution containing 0.05% trifluoroacetic acid. Each fraction of the peptide peaks was collected and analysed using an amino acid analyser and an amino acid sequencer to examine cleavage sites on the oxidized insulin B-chain.

Amino acid analysis and amino-terminal sequence of the peptides from oxidized insulin B-chain

Amino acid analysis was performed according to the method of Simpson et al. (1976) on a Shimadzu HPLC LC6A automatic amino acid analyser. Peptides were hydrolysed with 6-N HCl at 110 °C for 24, 48, and 72 h in evacuated sealed tubes.

Amino-terminal sequence analysis was subjected to automated Edman degradation with an ABI 437 gas liquid-phase protein sequencer by the method of Edman and Begg (1967), as previously reported (Yasuda et al. 1999).

Slab-SDS-PAGE

The samples for gel electrophoresis were prepared as follows. The reaction mixture, containing 50 μl soybean milk (pH 6.1) and 5 μl enzyme solution (SMCE activity, 3.6 U/ml), was incubated at 65 °C (the optimum temperature for soybean-milk-coagulating activities; Yasuda et al. 1999) for an appropriate reaction time. After the enzyme reaction, the activity was stopped by the addition of 15 μl 4% sodium dodecyl sulphate (SDS) in 50 mM Tris buffer (pH 6.8) containing 4% 2-mercaptoethanol and the mixture was boiled at 100 °C for 2 min. These samples were used for slabSDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% polyacrylamide gel (10 × 14 × 0.2 cm) was performed according to the method of Laemmli with a pH 8.3 buffer system (Laemmli 1970). After the run, the protein was stained with 0.25% Coomassie brilliant blue in a methanol/acetic acid solution (methanol:acetic acid:water, 50:10:43, v/v/v) at room temperature.

Analysis of the sulfhydryl group

We incubated 1.8 ml soybean milk (pH 6.1) with 0.2 ml of the enzyme solution (SMCE activity, 3.6 U/ml) at 65 °C for an appropriate reaction time. At intervals of 0.5, 1, 3, 5, 7, 10 and 20 min, each sample was placed in a cool bath (7 °C) and the sulfhydryl group in the reaction mixture was immediately measured using the method of Obata et al. (1989) with 2,2′-dithiobis-(5-nitropyridine).

Results

Effects of pH and temperature on activity of the proteolytic enzyme

The effect of pH on the activity of the purified proteolytic enzyme is shown in Fig. 1. The optimum pH for enzyme activity was 9.0. The enzyme was considered to be an alkaline proteinase.

The enzyme reaction was carried out at various temperatures at pH 9.0 for 60 min. The apparent optimum temperature of the proteolytic enzyme under these experimental conditions was 50 °C (Fig. 2).

Inhibitory studies

The effects of proteinase inhibitors on enzyme activity were investigated. As shown in Table 1, the enzyme was completely inhibited by 1 mM diisopropyl fluorophosphate, by 0.1 mM chymostatin and by 1 mM phenylmethanesulfonyl fluoride (PMSF). The enzyme was inhibited in 51% by 1 mM antipain. However, the enzyme was not inhibited by monoiodoacetic acid, $AgNO_3$, *p*-chloromercuribenzoic acid, ethylenediaminetetraacetic acid, pepstatin A or soybean trypsin inhibi-

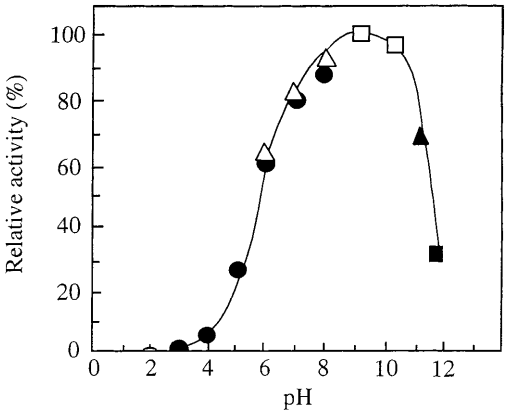

Fig. 1 Effect of pH on proteinase activity of the enzyme from *Bacillus pumilus*. The enzyme activity was measured at 50 °C in each buffer: ○ HCl-KCl buffer, △ phosphate buffer, ● McIlvaine buffer, □ $H_3BO_3 \cdot$KCl-NaOH buffer, ▲ Atkins–Pantin buffer, ■ Na_2HPO_4-NaOH buffer

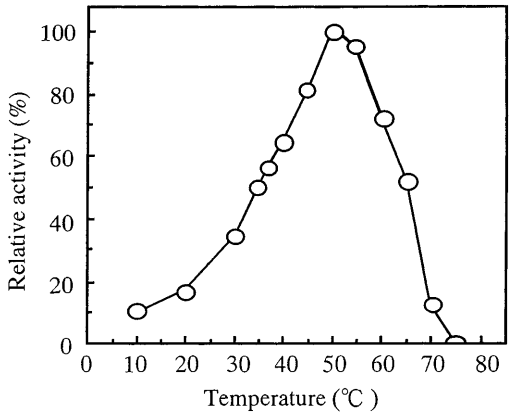

Fig. 2 Effect of temperature on proteinase activity of the enzyme from *B. pumilus*. The enzyme activity was measured at pH 9.0

tor. These results strongly suggested that the enzyme was a serine proteinase.

Substrate specificity

The substrate specificity of the proteinase was investigated. As shown in Table 2, the enzyme catalyses the hydrolysis of various proteins, of which cytochrome *c* and Hammerstein milk casein were better substrates than soybean protein. Human haemoglobin, and bovine albumin were moderately hydrolysed. The enzyme could not hydrolyse egg albumin, bovine β-lactoglobulin or gelatin.

Some esters such as N^{α}-L-arginine methyl ester, *N*-acetyl-L-phenylalanine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester were good substrates for the enzyme. However, the enzyme could not hydrolyse benzoyl-L-arginine ethyl ester, peptides such as benzyloxycarbonyl (Cbz)-L-alanyl-phenylalanine, Cbz-phenylalanyl-alanine, Cbz-alanyl-glutamic acid, Cbz-glutamyl-tyrosine or leucyl-glycyl-glycine and could not hydrolyse amides such as L-leucyl 2-naphtylamide and L-phenyl-2-naphthylamide (Table 3).

Table 1 Effect of inhibitors on the enzyme activity of *Bacillus pumilus*

Compound[a]	Concentration (mM)	Relative activity (%)
None	–	100
DFP	1.0	0
	0.1	71
Chymostatin	0.1	0
	1.0	0
PMSF	0.1	8
	0.02	62
TPCK	1.0	76
TLCK	1.0	92
Antipain	1.0	51
	0.1	83
Leupeptin	1.0	88
NEM	1.0	104
PCMB	1.0	98
MIA	1.0	96
Cysteine	1.0	104
L-AsA	1.0	93
$AgNO_3$	1.0	91
E-64	1.0	102
EDTA	1.0	90
Phosphoramidon	1.0	109
Pepstatin A	0.1	94
SDS	1.0	98
STI	1 (mg/ml)	110

[a] Abbreviations: *DFP* Diisopropyl fluorophosphate, *PMSF* phenylmethanesulfonyl fluoride, *TPCK* *N*-tosyl-L-phenylalanyl chloromethyl ketone, *TLCK* N^{α}-tosyl-L-lysyl chloromethyl ketone; *NEM* *N*-ethylmaleimide, *PCMB* *p*-chloromercuribenzoic acid, *MIA* monoiodacetic acid, L-AsA L-ascorbic acid, *EDTA* ethylenediamine tetraacetic acid; *SDS* sodium dodecyl sulfate, *STI* soybean trypsin inhibitor

Table 2 Substrate specificity of the enzyme from *B. pumilus*

Substrate	Relative activity (%)
Soybean protein isolate	100
Cytochrome *c*	145
Hammerstein milk casein	130
Human haemoglobin	45
Albumin (bovine)	40
Gluten	13
Albumin (egg)	0
β-Lactoglobulin (bovine)	0
Gelatin	0

Analysis of hydrolysis of the oxidized insulin B-chain by the enzyme

The reversed-phase chromatographic separation of the peptides produced after 5–60 min of the enzyme reaction was carried out using HPLC. Two peptides were determined within a 5-min digestion and eight peptides were identified after a 60-min digestion. From the analysis of amino acid composition and N-terminal amino acid sequence in each peptide, it was found that the oxidized insulin B-chain was split at the peptide bond Leu-15–Tyr-16 by the enzyme during the initial reaction stage. The other detectable cleavages that occurred were of the peptide bonds Gln-4–His-5, His-5–Leu-6, Gly-8–Ser-9, Ser-9–His-10, Glu-13–Ala-14 and Tyr-26–Thr-27. Com-

Table 3 Kinetic parameters for various synthetic substrate hydrolyses by the enzyme from *B. pumilus*

	Substrate	K_m (mM)	V_{max}
Esters	Tos–Arg–OMe	25.2	4.7
	Ac–Phe–OEt	15.4	438.5
	Ac–Tyr–OEt	36.5	141.0
	Bz–Arg–OEt	–	–
Peptides	CBz–Ala–Phe	–	–
	CBz–Phe–Ala	–	–
	CBz–Ala–Glu	–	–
	CBz–Glu–Tyr	–	–
	Leu–Gly–Gly	–	–
Amides	Leu–BNA	–	–
	Phe–BNA	–	–

parison of the cleavage sites with those of the other serine proteinase and bromelain are shown in Fig. 3. Cleavage sites in the oxidized insulin B-chain used by the enzyme from *B. pumilus* TYO-67 were identical to four sites in subtilisin Carlsberg from *Bacillus licheniformis* (Markland and Smith 1971), three sites in subtilisin BPN′ from *Bacillus subtilis* (Morihara and Tsuzuki 1969), three or two sites in SMCEs I or II from *Bacillus* sp. K-295 G-7 (Park et al. 1987) and two sites in bromelain from mature pineapple (Kortt et al. 1974). Cleavage sites in the oxidized insulin B-chain used by the *B. pumilus* enzyme were markedly different from those of bromelain.

Mode of action toward protein in soybean milk by the enzyme

Slab-SDS-PAGE results are shown in Fig. 4. Coagulation of the protein in soybean milk occurred after a 3-min enzyme reaction. The polypeptide band of the acidic subunit in glycinin disappeared after the 0.5-min reaction, then a new polypeptide band (molecular mass 28 kDa) appeared and was degraded during the enzyme reaction. However, the basic subunit in glycinin still remained even after 30 min. The α′-, α- and β-subunits in β-conglycinin degraded gradually, but a small amount remained after 30 min. Polypeptide bands of 14–20 kDa and those smaller than 5.6 kDa appeared during the enzyme reaction.

The SDS-PAGE pattern of protein digestion of in soybean milk by bromelain and that for subtilisin Carlsberg are shown in Figs. 5 and 6, respectively. In the case of bromelain (Fig. 5), the acidic subunit in glycinin remained after 0.5 min of the enzyme reaction; polypeptide bands of 28 kDa and 20 kDa appeared. After a 3-min reaction, polypeptide bands of acidic and basic subunits and α′-, α-, and β-subunits in β-conglycinin completely disappeared. After a 30-min reaction, the polypeptide bands of 28 kDa and 20 kDa disappeared; however, those smaller than 6.5 kDa increased.

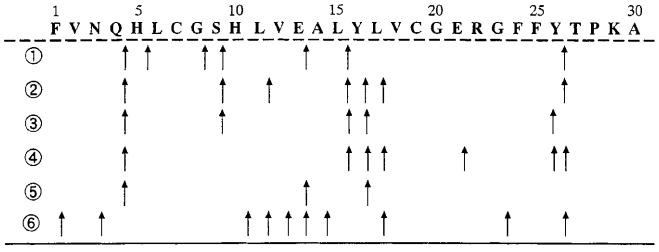

Fig. 3 Comparison of the cleavage sites of oxidized insulin B-chain treated with different proteinases. Circled numbers on the left represent the following enzymes: 1, the enzyme from *B. pumilus*; 2, subtilisin Carlsberg from *Bacillus licheniformis* (Markland and Smith 1971); 3, subtilisin BPN′ from *Bacillus subtilis* (Morihara and Tsuzuki 1969); 4, Enzyme I from *Bacillus* sp. K-295 G-7 (Park et al. 1987); 5, Enzyme II from *Bacillus* sp. K-295 G-7 (Park et al. 1987); 6, stem bromelain from mature pineapple (Kortt et al. 1974). The *arrows* indicate the cleavage sites

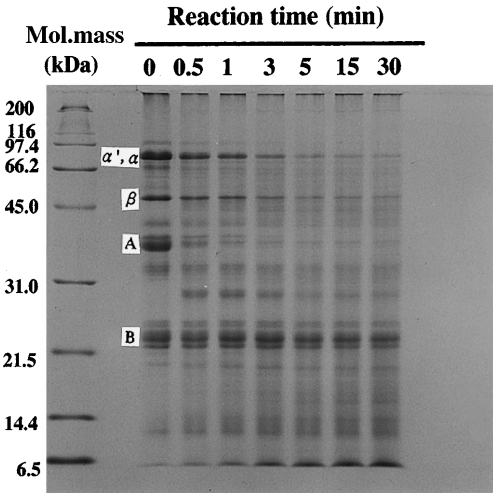

Fig. 4 Degradation of soybean milk protein with the enzyme from *B. pumilus*. The molecular masses of the protein standards (Bio-Rad) are as follows: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, 6.5 kDa. α′, α, β: α′-, α- and β-subunits in β-conglycinin. A, B: acidic and basic subunits, respectively, in glycinin

In the case of subtilisin Carlsberg (Fig. 6), the SDS-PAGE pattern was relatively similar to that of SMCE. However, each subunit of glycinin and β-conglycinin was completely degraded by subtilisin Carlsberg.

Changes in the content of the sulfhydryl group in soybean milk during the enzyme reaction

Changes in the content of the sulfhydryl group in soybean milk during the enzyme reaction are shown in Fig. 7. The amount of the detectable sulfhydryl group in soybean milk increased in accordance with the progress of soybean protein degradation (within 2 min). After

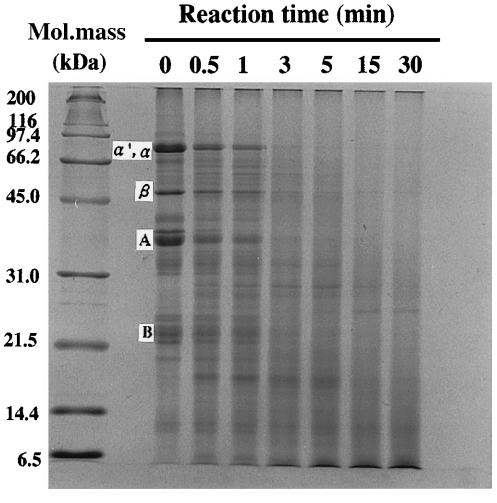

Fig. 5 Degradation of protein in soybean milk treated with bromelain

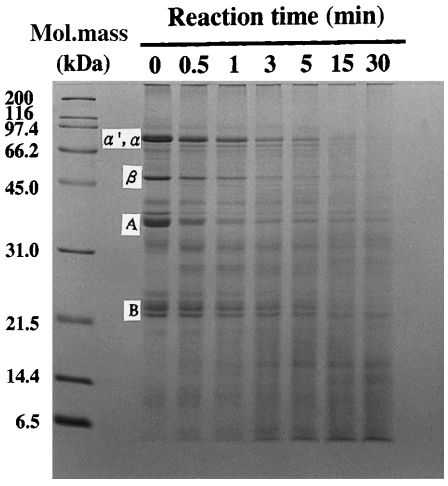

Fig. 6 Degradation of protein in soybean milk treated with subtilisin Carlsberg

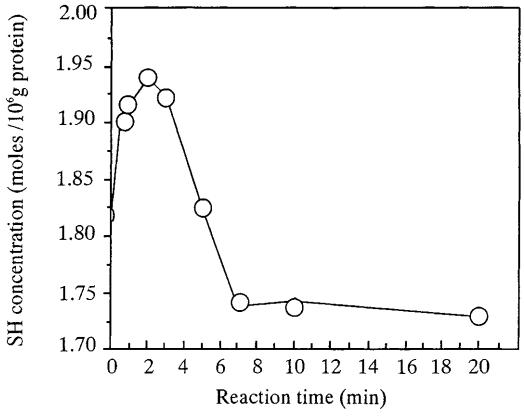

Fig. 7 Changes in sulfhydryl (SH) group content in soybean milk treated with the enzyme from *B. pumilus*

that, the amount decreased until 7 min and remained at a constant value. Coagulation of protein in soybean milk was observed after a 3-min reaction. The coagula obtained were completely dissolved in the presence of more than 0.5% SDS without 2-mercaptoethanol.

Discussion

SMCE from *B. pumilus* isolated in our laboratory was purified and characterized (Yasuda et al. 1999). In order to establish the category of proteinase to which the SMCE from *B. pumilus* belongs, further characterizations of the enzyme as proteinase were investigated. The enzyme was an alkaline serine proteinase because it was completely inhibited by 1 mM diisopropyl fluorophosphate or 1 mM PMSF and its optimum pH was 9.0.

In comparisons with the subtilisins, which are typical serine proteases from the genus *Bacillus*, a K_m value of this enzyme against *N*-acetyl-L-phenylalanine ethyl ester (K_m value 15.4 mM) was found to be similar to that (K_m value 16.6 mM) of subtilisin BPN′ (Markland and Smith 1971). The data for other subtilisins such as subtilisin Carlsberg or subtilisin Novo have not been reported.

The oxidized insulin B-chain was split in seven places by the enzyme. The specificities of bromelain from mature pineapple and other serine proteinases such as subtilisin Carlsberg from *B. licheniformis* (Markland and Smith 1971), subtilisin BPN′ from *B. subtilis* (Morihara and Tsuzuki 1969) and SMCEs I and II from *Bacillus* sp. K-295 G-7 (Park et al. 1987) on the same substrate were compared. Interestingly, the peptide bond Gln-4–His-5 was split by all the serine proteinases listed in Fig. 3 but Tyr-16–Leu-17 could not be split by the *B. pumilus* enzyme or bromelain. Apparently, the specificity of the *B. pumilus* enzyme differs more from those of bromelain (Kortt et al. 1974), SMCEs I and II from *Bacillus* sp. K-295 G-7 (Park et al. 1987) and subtilisin BPN′ from *B. subtilis* (Morihara and Tsuzuki 1969) than it does compared to that of subtilisin Carlsberg from *B. licheniformis* (Morihara 1974).

The mode of action of this enzyme on the protein in soybean milk was examined by using the SDS-PAGE method. The polypeptide bands of an acidic subunit in glycinin and the α′-, α- and β-subunits in β-conglycinin were degraded during the enzyme reaction. However, the basic subunit in glycinin remained. In the cases of bromelain and subtilisin Carlsberg, the polypeptide bands of the acidic and basic subunits in glycinin and the α′-, α- and β-subunits in β-conglycinin completely disappeared. These results indicated that the degradation of soybean protein by this enzyme was different from that produced by bromelain or subtilisin Carlsberg. It is considered that the differences in the patterns of degradation of soybean protein with these enzymes may greatly relate to the physical properties in curd formation.

Changes in the sulfhydryl (SH)-group content and in the coagulation in soybean milk obtained with this enzyme were investigated. When the coagulation of soybean milk was completed, the amount of detectable SH groups in soybean milk decreased. There are some reports that heat-induced aggregates of soybean protein occurred with the formation of S–S bonds (Kelly and Pressey 1966; Haga and Ohashi 1979). However, the coagula caused by this enzyme were dissolved in 0.5% SDS solution without 2-mercaptoethanol. This phenomenon was in good agreement with the case of bromelain (Fuke et al. 1985). The formation of coagula caused by this enzyme may be mainly due to the hydrophobic interaction but is not due to disulfide bond formation.

Although the overall structure (isoelectric point, amino acid composition, and amino-terminal sequences) and specificity to the oxidized insulin B-chain of the enzyme from *B. pumilus* TYO-67 were similar to those of subtilisin Carlsberg from *B. licheniformis* (Yasuda et al. 1999), the two enzymes may exhibit subtle differences in terms of catalytic properties such as soybean protein degradation.

Fuke and Matsuoka (1984) reported that bromelain coagulated protein in soybean milk and that the result-

ing curd had a soft and smooth texture. They prepared soybean cheese using the curd. Specificities for the oxidized insulin B-chain and soybean protein degradation by bromelain were different from those of the *B. pumilus* enzyme. These data may explain the difference in the physical properties of the soybean curd obtained enzymatically. The physical properties of the curd obtained using the *B. pumilus* enzyme and also its applications in food processing should be investigated in detail.

Acknowledgements The authors wish to thank Dr. S. Toyama for his helpful advice. We also thank Mr. M. Sakaguchi for his technical assistance.

References

Edman P, Begg G (1967) A protein sequenator. Eur J Biochem 1: 80–91

Fuke Y, Matsuoka H (1984) Preparation of fermented soybean curd using stem bromelain. J Food Sci 49: 312–313

Fuke Y, Sekiguchi M, Matsuoka H (1985) Nature of stem bromelain treatment on the aggregation and gelation of soybean proteins. J Food Sci 50: 1283–1288

Haga S, Ohashi T (1979) Effect of disulfide bond on the gel formation miosin B and/or soybean protein CIF. J Jpn Soc Food Sci Technol 26: 422–428

Kamata Y, Ohta K, Yamauchi F, Yamada M (1991) Cheese analogs from soymilk-curd by limited proteolysis. J Jpn Soc Food Sci Technol 38: 1143–1148

Kelly JJ, Pressey R (1966) Studies with soybean protein and fiber formation. Cereal Chem 43: 195–206

Kortt AA, Hinds JA, Zerner B (1974) On the specificity and pH dependence of ficin-catalyzed hydrolyses. Some comparisons with bromelain specificity. Biochemistry 13: 2029–2037

Laemmili UK (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 277: 680–685

Markland FS Jr, Smith EL (1971) Subtilisins: primary structure, chemical and physical properties. In: Boyer PD (ed) The enzymes, vol 3, 3rd edn. Academic Press, New York, pp 508–561

Morihara K (1974) Comparative specificity of microbiol proteinases. In: Meister A (ed) Advances in enzymology, vol 41. John Wiley & Sons, New York, pp 179–188

Morihara K, Tuzuki H (1969) Comparison of the specificities of various serine proteinases from microorganisms. Arch Biochem Biophys 129: 620–634

Obata A, Matsuura M, Fukushima D (1989) Spectrophotometric determination of sulfhydryl groups in soymilk using 2,2′-dithiobis-(5-nitropyridine). J Jpn Soc Food Sci Technol 36: 707–711

Park YW, Kobayashi H, Kusakabe I, Murakami K (1987) Purification and characterization of soymilk-clotting enzymes from *Bacillus* sp. K-295 G-7. Agric Biol Chem 51: 2343–2349

Simpson RJ, Neuberger MR, Liu TY (1976) Complete amino acid analysis of proteins from a single hydrolysate. J Biol Chem 251: 1936–1940

Yasuda M, Hokama I (1984) Production of soybean curd for tofuyo-manufacture. J Jpn Soc Food Sci Technol 31: 19–23

Yasuda M, Sakaguchi M (1998) Degradation of soybean protein by an acid proteinase from *Monascus anka*. Food Sci Tech Int Tokyo 4: 6–8

Yasuda M, Sakaguchi M, Otonari H, Okuhama S, Kinjyo S, Hongo F, Toyama S (1992) The role of microorganisms during dehydration of tofu cubes. J Jpn Soc Food Sci Technol 39: 870–877

Yasuda M, Matsumoto T, Sakaguchi M, Kobamoto N (1993) Changes in chemical components of tofuyo prepared by *Monascus* fungus during fermentation. J Jpn Soc Food Sci Technol 40: 331–338

Yasuda M, Matsumoto T, Sakaguchi M, Kinjyo S (1994) Changes in protein and nitrogen compounds of tofuyo prepared by *Aspergillus oryzae* during fermentation. J Jpn Soc Food Sci Technol 41: 184–190

Yasuda M, Kinjyo S, Miki E (1996) Changes in breaking characteristics, creep behavior and microstructure of tofuyo during fermentation. J Jpn Soc Food Sci Technol 43: 322–327

Yasuda M, Aoyama M, Sakaguchi M, Nakachi K, Kobamoto N (1999) Purification and characterization of a soybean-milk-coagulating enzyme from *Bacillus pumilus* TYO-67. Appl Microbiol Biotechnol 51: 474–479