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Salt-Tolerant Acid Proteases: Purification, Identification, Enzyme Characteristics, and Applications for Soybean Paste and Sauce Industry

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1. Introduction

Soybean, the most cultivated plant in the world, is rich in proteins (40–50%) and contributes many essential nutrients and health-promoting bioactive compounds. Fermented soybean products including soybean paste and soybean curd, *etc.* are consumed in considerable amounts in Asian countries such as China, Japan, and Korea for a long history. Nowadays, they are considered as healthy foods and soy sauce become quite popular all over the world. The fermentation process improves the nutritional quality of the soybeans and contributes to the elimination of trypsin inhibitors (Kim et al., 2010). They are known to be highly digestible and nutritious, and affect a number of physiological activities such as antioxidative activity (Lin et al., 2006), fibrinolytic activity, lowering of blood pressure, and prevention of osteoporosis (Kim et al., 2011). Fermentation starters are essential for the production of fermented soybean products. Microorganisms including *Bacillus*, *Aspergillus*, and *Rhizopus* species and proteases are commonly used as a starter for fermenting soybeans. Proteases produced from microorganisms are widely used for baking, photographic, brewing, fermentation, protein hydrolysates, gelatin industries, meat, leather, and detergents, *etc.* (Murakami et al., 1991). In this study, salt-tolerant acid proteases and their application in the manufacture of fermented soybean products are discussed.

2. Fermented soybean product

2.1 Soybean paste

Soybean paste was originated in China about 2,500 years ago. It is commonly known as Jiang in China, Miso in Japan, Doenjang in Korea, Tao-tjo in Indonesia and Thailand, and Tao-si in Philippines. There are series of soybean paste products in each country. Though they are made with soybean and cereals in the presence of salt, they have special tastes and flavors because of the different ratio of substrates, salt concentration, and the length of fermentation and aging (Fukushima, 1979). The salt concentration of fermented soybean paste is ranged from 4-11%, and the pH is about 5 (Shibasaki and Hesseltine, 1962). Soybean

paste is consumed in large different ways in different countries. It is used as the base for sauces served with meat, seafood, poultry, or vegetable dishes in China, while it is used as the base for soups in Japan and Korea (Fukushima, 1979).

2.2 Soy sauce

Soy sauce is recognized as Jiang-you in China, Shoyu in Japan and Ganjang in Korea. There are two types of soy sauce based on the color. One type of soy sauce is an all purpose liquid seasoning, characterized by a strong aroma, a myriad flavor, and a deep red-brownish color. The other type is in a lighter brown color used mainly for cooking when the original flavor and color of the foodstuff need to be preserved. The difference in aromas observed between Japanese and Chinese styles of soy sauce are ascribed to the different ratios of wheat and soybeans used and the existence of pasteurized process (Fukushima, 1979). The salt concentration of soy sauce is around 16-17%, and the pH is around 4-5 (Shibasaki and Hesseltine, 1962).

2.3 Fermented whole soybean products

Fermented whole soybean products are known as Dou-chi in China, Natto in Japn, and Tempeh in Indonesian (Ogawa et al., 2004), which are much different from their counterparts. These products are solid, and the shape of cooked whole soybean particles is kept as it is. Although these products from different countries share this common characteristic, they have distinctive qualities because of the different bacterial and fungal communities in these products (Kim et al., 2010).

2.4 Soybean curd

Sufu (Fu-ru, or Dou-fu-ru in Chinese) is a fermented soybean curd and a highly flavored, soft creamy product originating in China. It is a cheese-like product consumed as a side dish mainly with breakfast rice or steamed bread (Han et al., 2001). It has a long history and written records date back to the Wei Dynasty 220–265 AD in China. Several types of sufu can be distinguished, according to processing method or according to color and flavor.

3. Starters for the fermented soybean product

Fermentation of soybean is complicated and time-consuming. High amount of salt used for the manufacture and the acid environment inhibit the growth of microorganism and enzyme activity. Therefore, starter is used to shorten the natural fermentation period. Soybean is full of protein and carbohydrate, thus the starter should produce or contain various hydrolytic enzymes such as protease and amylase with strong activity in solid or submerged cultures.

3.1 Microorganisms

Microorganisms used as soybean fermentation starters include bacteria, molds and yeasts. The first procedure for soybean fermentation is the preparation of Qu (in Chinese) or called koji (in Japanese). Koji is a source of microorganisms and their enzymes for converting the carbohydrates and proteins into sugars, peptides, and amino acids, etc. contained in the materials. The nutrients which were produced through the action of these enzymes will be used by yeasts and lactic acid bacteria during further fermentation period (Abe et al., 2006).

3.2 Soybean paste

Cultures of *Aspergillus oryzae*, which known as a seed mold, are commonly used as starter for the production of soybean paste (Fukushima, 1979). Rice or barley is steamed and inoculated with *Aspergillus oryzae* to make koji. After completion of fermentation, the resulting koji is mixed with salt, cooked whole soybeans, pure cultured yeasts, lactic acid bacteria, and water, and then kept for an appropriate period for the second fermentation. The resulting aged mixture is mashed and packaged as miso. In general, miso is a paste of bright yellow to dark brown color. Whiter miso contains more rice than soybeans, whereas the darker miso contains 50 to 90% soybeans (Abe et al., 2006). The molds produce amylases and proteases which hydrolyze the cereal starch and the proteins in soybeans and cereals.

3.3 Soy sauce

Cultures of *Aspergillus oryzae* are also used as starter for the production of soy sauce. The manufacturing of soy sauce is carried out as follows (Fukushima, 1979). Soybeans, or defatted soybean flakes, are moistened and cooked under pressure until they are sufficiently soft. The cooking was done in a batch type or a continuous cooker which allows a high pressure. On the other hand, the wheat is roasted by continuous roasting and then cracked into four to five pieces. The cooked soybean and wheat are mixed and then inoculated with a pure culture of *Aspergillus oryzae* to make koji. After two or three days, the well fermented koji is mixed with yeasts, lactic acid bacteria in the presence of more than 18% salt water which excludes undesirable microorganisms in the second period fermentation. The pH drops from an initial value of 6.5-7.0 down to 4.7-4.8. After fermentation, the product is filtered, pasteurized and packaged as soy sauce. The salt concentration of soy sauce from different country ranges from 10-21% (Stute et al., 2002).

3.4 Fermented whole soybean products

Fermented whole soybean products are made of microorganisms with nindehulled soybean. *Bacillus natto* (Murooka and Yamashita, 2008) and *Bacillus subtilis* (Kim et al., 2011) are the bacteria used for Natto production in Japan and Chungkookjang in Korea, respectively. *Rhizopus oligosporus* is the principal fungus used for the Tempeh preparation in Indonesia. *Bacillus subtilis* and *Rhizopus microsporus* are used in different kinds of Dou-chi production in China (Wang et al., 2008).

3.5 Soybean curd

Actinomucor spp., i.e. *A. elegans* and *A. taiwanensis*, and *Rhizopus* spp., i.e. *Mucor sufu* and *Mucor wutungkiao* are used as starters for high quality sufu making (Han et al., 2001). After preparation of tofu from soybean, one or more types of the above microorganisms are inoculated to ferment pehtze. The pehtze is transferred to salt-saturated solution to adsorb salt until the salt content of pehtze reaches about 16%, which takes 6–12 days. After maturation with different kinds of dressing mixture, different kinds of sufu are produced.

3.6 Enzymes

Manufacturing processing of fermented soybean product requires to uniform of the quality of the product. However, it is difficult to control the natural fermentation according to the traditional method using microorganisms as starters. Because high salt concentration is needed in fermentation of soybean products, salt-tolerant hydrolytic enzymes, especially

protease with high proteolytic activity is used as starter to uniform the quality of the product, and shorten the ripening periods of fermentation. During the fermentation, the pH value decreased down to lower than 5 (Fukushima, 1979), thus the salt-tolerant acid protease is more valuable for the production of fermented soybean products.

4. Salt-tolerant acid proteases

4.1 Microorganisms for the production of salt-tolerant proteases

Salt-tolerant proteases were produced from halotolerant microorganisms; Halobacterium salinarium, Bacillus sp., Halobacterium halobium, Halomonas sp., Halobacillus sp., Virgibacillus sp., Oceanobacillus sp., Saccharomyces cerevisiae, Aspergillus sp.,etc (Barbosa et al., 2006; Jeong et al., 2001; Kim et al., 2004). However, few of them have been purified and characterized to be acid proteases. They are proteases produced by B. subtilis JM3 from anchovy sauce (W.J. Kim and S.M. Kim, 2005), B. megaterium KLP-98 from fermented squid (Fu et al., 2008), Rhizopus japonicus (Chung, 1984), and A. oryzae LK-101 from the traditional Korean soybean paste (Hwang et al., 2010).

4.2 Production of salt-tolerant acid protease

4.2.1 Production of salt-tolerant acid protease by *B. subtilis* JM-3

For the isolation of protiolytic bacteria, anchovy sauce fermented at 15 ± 3 °C for 3 years was inoculated on a brain–heart infusion (BHI) agar and incubated at 27.5 °C. The highest transparent colony was isolated and identified by the genetic mapping method to be *B. subtilis*. Five hundred milliliters of the medium in a 1-L wide-mouth culture flask was inoculated with 10 mL of *B. subtilis* JM-3 suspension prepared from BHI broth media cultivated at 37 °C for 3 days. The culture was incubated at 37 °C for 8 days in a shaking incubator at 150 rpm. The optimal protease production reached 1500 U/L (W.J. Kim and S.M. Kim, 2005).

4.2.2 Production of salt-tolerant acid protease by *B. megaterium* KLP-98

Proteolytic bacteria from the fermented squid were isolated by BHI agar. After inoculating 0.1 mL of fluid of the squid with 10% NaCl concentration fermented at room temperature for 2 months, the culture media were incubated at 37 °C for 48 h. The highest transparent colony on the culture medium was isolated, identified and inoculated in BHI broth media with 10% NaCl. 500 mL of medium in a 1 L wide-mouth culture flask was inoculated with 10 mL suspension prepared from BHI broth media cultivated at 37 °C for 72 h. The culture was incubated at 37 °C for 5 days in a shaking incubator at 150 rpm. The optimal protease production reached 520 U/L (Hwang et al., 2010).

4.2.3 Production of salt-tolerant acid protease by *A. oryzae* LK-101

Proteolytic bacterium was isolated from traditional Korean soybean paste on agar plates incubated for 2 to 4 weeks. Spores suspension was transferred to the seed culture medium (pH 3.0) containing glucose, corn steep liquor, Hyflo Super-Cel, and a defoaming agent. The bacterium was identified by rDNA sequencing method and and named as *A. oryzae* LK-101. For the production of protease, *A. oryzae* LK-101 was cultivated in a 500 mL flask containing 200 mL of 2% defatted soybean flour culture broth at 27 °C for 4 days in a shaking incubator at 150 rpm. The optimal protease production reached 973 U/L (Fu et al., 2008).

4.3 Extraction and purification of salt-tolerant acid protease

Five kinds of reported salt-tolerant acid proteases were extracellular enzymes. Thus, they were extracted and purified from the culture media of each corresponding microorganism.

4.3.1 Purification of salt-tolerant acid protease of *B. subtilis* JM-3 and *B. megaterium* KLP-98

As shown in Table 1 and 2, proteases produced by *B. subtilis* JM3 and *B. megaterium* KLP-98 were purified by a similar procedure. Proteases in the culture media were precipitated by ammonium sulfate. The precipitates were dissolved, dialyzed, and applied to a DEAE-Sephadex ion exchange column. Proteins were eluted with an increasing gradient of NaCl. Fractions containing greater than 50% of maximal peak activity were pooled, dialyzed and applied to a Sephadex G-75 gel filtration column, and eluted with sodium acetate buffer (pH 5.5). Proteases produced by *B. subtilis* JM3 and *B. megaterium* KLP-98 were purified by 35.56 folds with 5.33% of yield and 18.83 folds with 15.3% of yield, respectively.

Steps	Volume (mL)	Total Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,000	600.0	3000.0	5.0	100.0	1.0
Ammonium sulfate	10	13.1	320.5	24.5	10.68	4.9
Ultrafiltrate	16	8.5	235.2	27.7	7.84	5.5
DEAE-Sephadex	35	2.2	185.2	84.2	6.17	16.8
Sephadex G-75	50	0.9	160.0	177.8	5.33	35.56

Table 1. Purification of *Bacillus subtilis* JM-3 protease from anchovy sauce (W.J. Kim and S.M. Kim, 2005).

Steps	Volume (mL)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,000	1,234	1040	0.84	100.0	1.00
Ammonium sulfate	14	814	1000	1.23	96.2	1.46
DEAE-Sephadex	40	32	172	5.36	16.5	6.37
Sephadex G-75	40	10	159	15.86	15.3	18.83

Table 2. Purification of *Bacillus megaterium* KLP-98 protease from fermented squid (Fu et al., 2008).

4.3.2 Purification of salt-tolerant acid proteases of *Rhizopus japonicus*

As shown in Table 3, two acid proteases were produced by *R. japonicus*. They were precipitated by ammonium sulfate, and purified by twice applications of CMC column (Fig. 1). Proteases I and II were purified by 165.5 folds with 61.6% of yield and 176.5 folds with 2.9% of yield, respectively.

Purification Steps		Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme		385000	25300	15.2	100	1.0
Ammonium sulfate		225000	3150	71.4	58.4	4.7
First CMC column		26880	20.5	1311	7	86.25
Second CMC column	Protease I	6050	2.4	2521	1.6	165.5
	Protease II	11000	4.1	2683	2.9	176.5

Table 3. Purification of acid salt-tolerant proteases from *Rhizopus japonicus* (Chung, 1984).

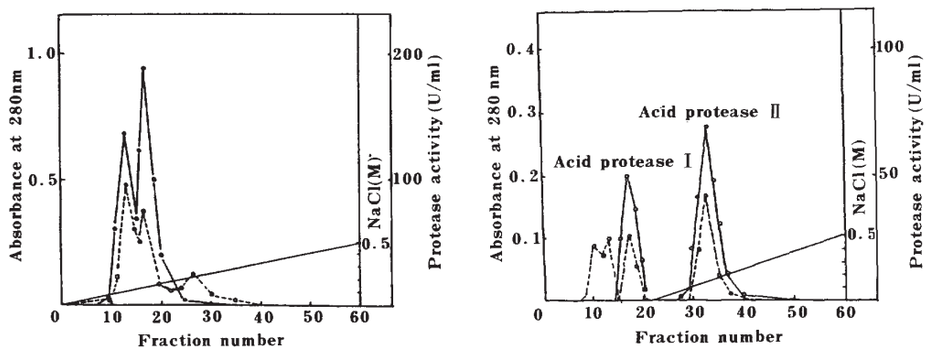


Fig. 1. First (A) and second (B) CMC column chromatography. - - - - Abs 280nm, — — protease activity (Chung, 1984).

4.3.3 Purification of salt-tolerant acid proteases of *A. oryzae LK-101*

As shown in Table 4, purification of protease produced by *A. oryzae* LK-101 was carried out as follows. Culture medium was centrifuged. Ammonium sulfate was added to precipitate the protein. The precipitate was dissolved, dialyzed, and concentrated by ultrafiltration. The resulting concentrate was purified by DEAE-Sephadex ion exchange column and Sephadex G-100 gel filtration column consequently. Protease produced by *A. oryzae* LK-101 was purified by 11.6 folds with 6.8% of yield.

Purification steps	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude extract	97,270	490.7	198.2	100.0	1.00
Ammonium sulfate	21,229	19.4	1,094.3	21.8	5.52
Ultrafiltration	13,351	10.8	1,236.2	13.7	6.23
DEAE-Sephadex	8,913	5.24	1,700.9	9.2	8.57
Sephadex G-100	6,581	2.86	2,301.0	6.8	11.59

Table 4. Purification of *A. oryzae* LK-101 from the traditional Korean soybean paste (Hwang et al., 2010).

4.4 Determination of protease activity

Protease activity was determined according to the modified method of Anson (1939). One mL of enzyme solution was added to 5 mL of 0.6% casein solution in 1/15 M phosphate buffer, pH 6.5 and reacted at 37 °C for 10 min. The reaction was stopped by adding 5 mL of 0.44 M trichloroacetic acid (TCA) and then stood for 30 min. The solution was then filtered with Whatman No. 2. Two mL of the filtrate was mixed with 5 mL of 0.55 M Na_2CO_3 solution and 1 mL of 1 N Folin reagent, and then stood for at room temperature for 30 min. The absorbance was measured at 660 nm with spectrophotometer, and then converted to the amount of tyrosine equivalent based on a standard curve. One unit (U) of protease activity was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per 10 min. Protein concentration was determined according to the method of Lowry (Lowry et al., 1951) with egg ovalbumin as the standard. During column chromatography, protein concentration in the fractions was estimated by measuring the absorbance at 280 nm.

4.5 Characterization of salt-tolerant acid protease

Characteristics of proteases purified from the culture medium of *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* LK-101 were well studied, while those of the protease from *Rhizopus japonicus* except specific activity were not reported. Thus, the characteristics of the three kinds of proteases were compared and discussed in this study.

4.5.1 Enzyme activity

As shown in Table 5, the protease from different origin varied a lot in specific activity (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Chung, 1984; Hwang et al., 2010). The specific activity of the *R. japonicus* protease II was the highest among these five proteases, and that of the *B. megaterium* KLP-98 was the lowest.

Source of protease		Specific activity (U/mg)
Bacillus subtilis JM3		177.8
Bacillus megaterium KLP-98		15.86
Rhizopus japonicus	Protease I	2521
	Protease II	2683
Aspergillus oryzae LK-101		2301

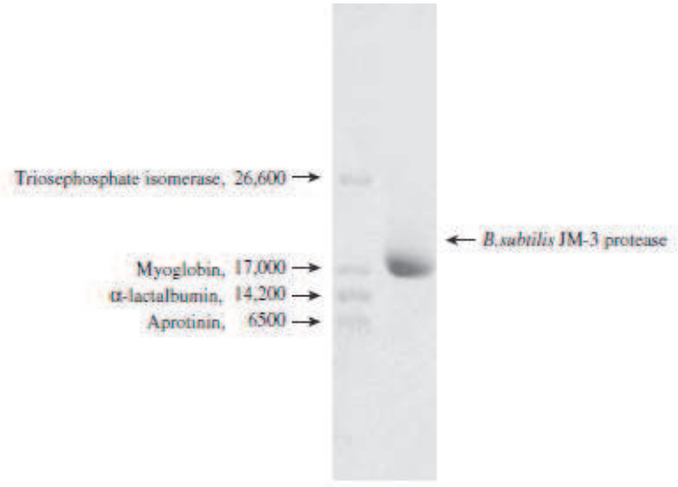
Table 5. Comparison of specific activities of purified salt-tolerant acid proteases.

4.5.2 Molecular mass

The molecular masses and purities of the protease were determined by SDS-PAGE electrophoresis (Fig. 2) (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Hwang et al., 2010).

Source of protease	Mr (kDa)
Bacillus subtilis JM3	17
Bacillus megaterium KLP-98	64
Aspergillus oryzae LK-101	25

Table 6. Comparison of molecular masses of purified salt-tolerant acid proteases.



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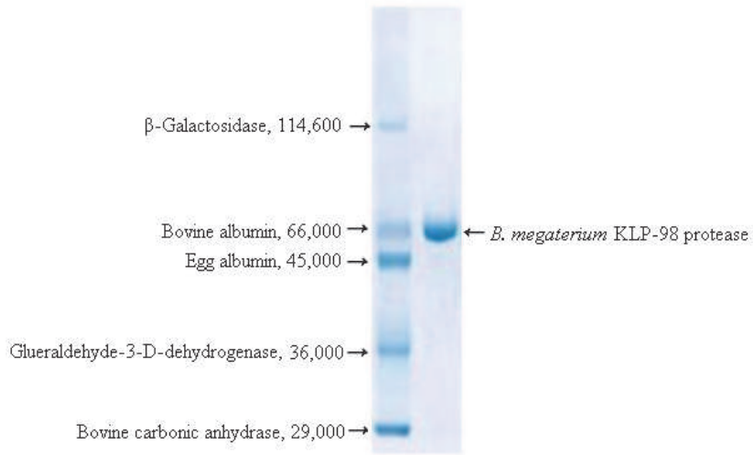

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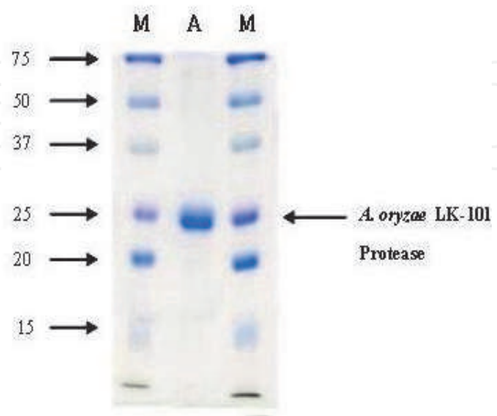

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Fig. 2. SDS-PAGE electrophoresis of proteases produced by *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* LK-101.

Each purified protease was homogeneous on SDS-PAGE. As shown in Table 6, different protease had different molecular masses ranging from 17 kDa to 64 kDa.

4.5.3 Effects of temperature on enzyme activity and stability

As shown in Table 7, the optimal temperature for the activity of protease produced by *A. oryzae* LK-101 was lower than those of the other two proteases, while the stability of proteases produced by *A.* LK-101 and *B. megaterium* KLP-98 was better than that of the protease produced by *B. subtilis* JM3 (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Hwang et al., 2010).

Source of protease	Optimal temperature for enzyme activity (°C)	Temperature range for enzyme stability (°C)
Bacillus subtilis JM3	60	≤ 30
Bacillus megaterium KLP-98	60	≤ 40
Aspergillus oryzae LK-101	50	≤ 40

Table 7. Effects of temperature on activity and stability of purified salt-tolerant acid proteases.

4.5.4 Effects of pH on enzyme activity and stability

Relative activity increased up to pH 5.5 and decreased rapidly at higher pH (Fig. 3A). The optimal pH for the hydrolysis of azocasein was 5.5. Therefore, the *B. subtilis* JM-3 protease was classified as an acid protease. *B. subtilis* JM-3 protease showed the optimal stability at pH 5.5 (Fig. 3B) of weak acidic condition, but was unstable above pH 6.0.

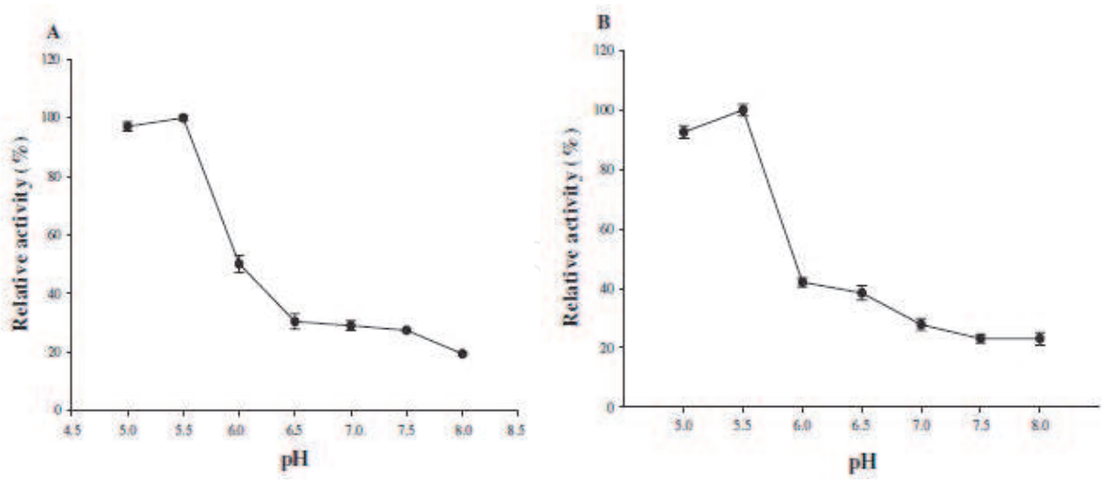


Fig. 3. Effects of pH on activity (A) and stability (B) of purified *Bacillus subtilis* JM3 protease (W.J. Kim and S.M. Kim, 2005).

Relative activity increased up to pH 5.5 and then decreased significantly (Fig. 4A). The optimal pH for the hydrolysis of azocasein was 5.5, and it had high proteolytic activity at weak acidic conditions (pH 5.0-6.0). The optimal pH of *B. megaterium* KLP-98 protease was similar to those of *B. subtilis* JM-3 protease. *B. megaterium* KLP-98 protease was stable around

pH 4.0-5.5 of weak acidic conditions. The pH stability range of *B. megaterium* KLP-98 protease and *B. subtilis* JM-3 protease was narrow.

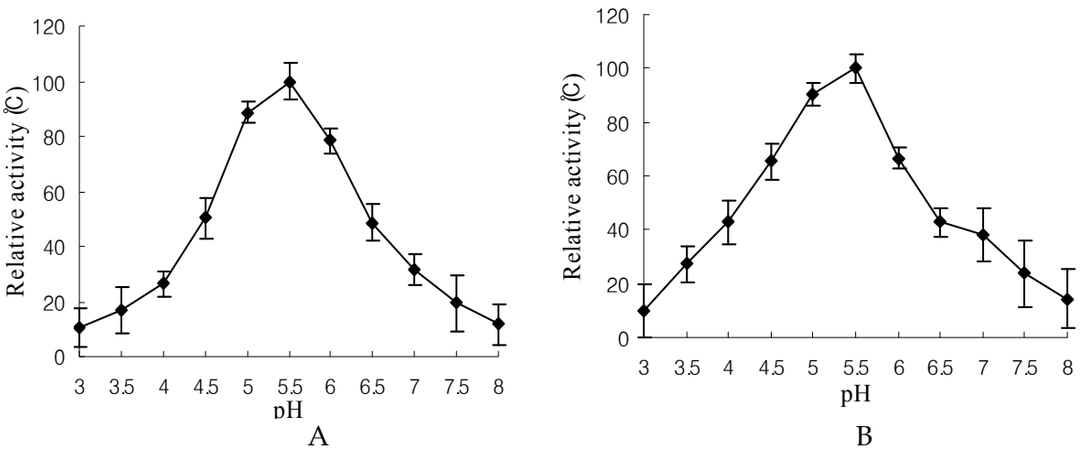


Fig. 4. Effects of pH on activity (A) and stability (B) of purified *Bacillus megaterium* KLP-98 protease (Fu et al., 2008).

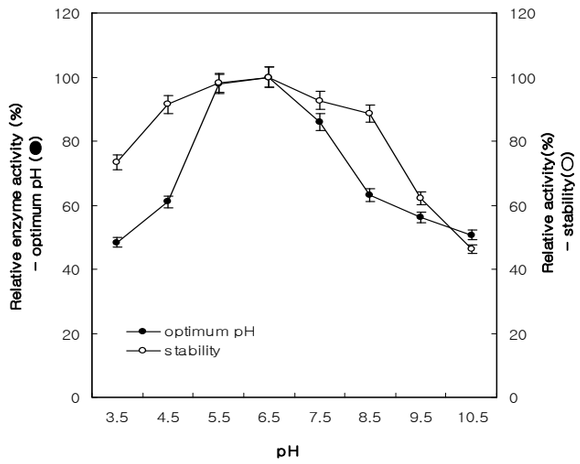


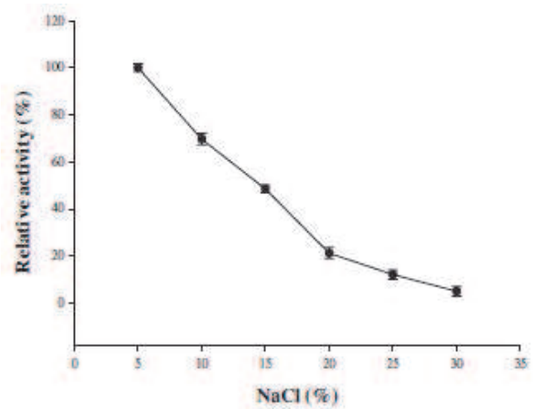
Fig. 5. Effects of pH on activity (A) and stability (B) of purified *Aspergillus oryzae* LK-101protease (Hwang et al., 2010).

The optimum pH for *A. oryzae* AOLK-101 protease activity was determined to be 6.5. It had higher proteolytic activity at weak acidic conditions than at the alkaline region. Thus, it was an acid protease. It remained more than 80% of activity in the region of pH 4.5-8.5 (Fig. 5). Thus the stability of this protease was better than those of *B. subtilis* JM-3 and *B. megaterium* KLP-98 proteases.

4.5.5 Effects of NaCl concentration on enzyme activity

The relative activities of purified proteases decreased as NaCl concentration increased. However, the protease produced by *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* LK-

101 still remained 65, 75 and 50% activity at 10% NaCl concentration and 21, 35 and 22% at 20% NaCl concentration, respectively (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008). Thus, these proteases were salt-tolerant proteases which can be used in soybean paste and soy sauce productions.



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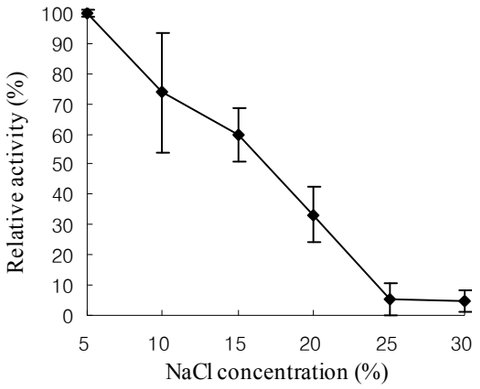

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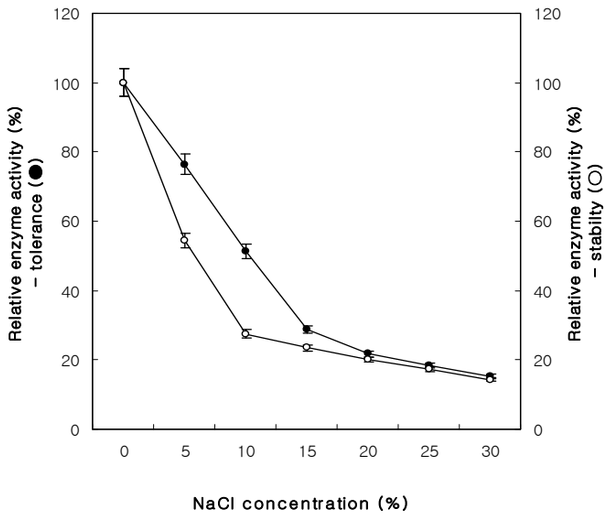

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Fig. 6. Effects of NaCl concentration on the activity of purified *Bacillus subtilis* JM3 protease (A), *Bacillus megaterium* KLP-98 protease (B), and stability and activity of purified *Aspergillus oryzae* LK-101protease (C).

4.5.6 Substrate specificity

The relative activities of the *B. subtilis* JM-3 protease on specific substrates for trypsin-like serine proteases, i.e. BSA, casein, azocasein and L-BApNA, were 120.81, 98.07, 100.00 and 119.24%, respectively. But it did not hydrolyze BTEE, which was the specific substrate for chymotrypsin-like protease. The *B. subtilis* JM-3 protease was therefore presumed to be a trypsin-like serine protease (W.J. Kim and S.M. Kim, 2005).

Substrates with higher specificity for serine and cysteine proteases were also used. *B. megaterium* KLP-98 protease had high affinity to Z-Phe-Arg-NMec (95.56%), which is ideal substrate for cysteine proteases, but also some trypsin-like serine proteases can hydrolyze it. However, *B. megaterium* KLP-98 did not hydrolyze TAME and BTEE, which were model substrates for trypsin and serine protease. Based on the above results, *B. megaterium* KLP-98 protease was, therefore, presumed to be a cysteine protease (Fu et al., 2008).

4.5.7 Effect of inhibitors on enzyme activity

The *B. subtilis* JM-3 protease was strongly inhibited by specific inhibitors for trypsin-like protease, i.e. MTLCK, PMSF, STI and DTT. It was moderately inhibited by inhibitors for chymotrypsin-like proteases, i.e. 2-mercaptoethanol and TPCK. It was not inhibited by inhibitors for cysteine proteases, i.e. NEM and PCMB (Ninojoor, 1985), and inhibitor for metalloproteases, i.e. EDTA. Therefore, the *B. subtilis* JM-3 protease was classified as a trypsin-like serine protease (W.J. Kim and S.M. Kim, 2005).

The *B. megaterium* KLP-98 protease was almost inhibited by NEM, E-64, and egg white cystatin, the specific inhibitors for cysteine protease. Serine protease inhibitors of TLCK and TPCK, aspartic acid protease inhibitor of pepstatin showed no reduction in its activity. Reducing agents such as DTT and 2-mercaptoethanol, and metalloprotease inhibitor, EDTA, even could increase its activity. Based on the results of the substrate specificity and inhibitor studies, *B. megaterium* KLP-98 protease was classified as a cysteine protease (Fu et al., 2008).

EDTA was the strongest inhibitor followed by in order of PMSF, o-phenanthroline, and iodoacetic acid with the inhibition rate of 52, 47, 16 and 8%, respectively. It was moderately inhibited by inhibitors for chymotrypsin-like proteases, i.e. 2,4-dinitrophenol and 2-mercarptoethanol. PMSF is very specific inhibitor for serine protease. Therefore, AOLK-101 protease is classified as a serine protease based on its sensitivity to PMSF. Hence, this indicates that AOLK-101 protease is a serine protease (Hwang et al., 2010).

4.5.8 Kinetic parameters

In order to evaluate the kinetic constants for the purified protease, the initial velocities of the enzyme reactions were determined at various concentrations of the casein substrate. The kinetic constants, K_m and V_{max} values of the protease were calculated from the Lineweaver-Burk plot. The V_{max}/K_m value, which is the physiological or catalysis efficiency value, was calculated. The higher V_{max}/K_m value means the stronger catalytic activity to hydrolyze the substrate. Therefore the protease produced by *B. subtilis* JM3 exhibited the strongest catalytic activity among the three salt –tolerant acid proteases.

Source of protease	K_m (mg/mL)	V_{max} (U/L)	V_{max}/K_m (U mL / L/mg)
Bacillus subtilis JM3	1.75	318	181.6
Bacillus megaterium KLP-98	2.10	285	135.7
Aspergillus oryzae LK-101	1.04	125	119.2

Table 8. Kinetic parameters of the purified protease produced by *Bacillus subtilis* JM3, *Bacillus megaterium* KLP-98, and *Aspergillus oryzae* LK-101 (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Hwang et al., 2010).

5. Applications

The applications of the purified proteases to soybean paste and soy sauce fermentation haven't been investigated yet. However, the characteristics of the proteases indicated their potential application. The specific activities were high, and the kinetic parameters were excellent. These proteases were stable below temperature 30 and 40 °C, respectively. For the fermentation of soybean paste and soy sauce, the temperature was around 30 to 35 °C, thus these proteases should be stable during the fermentation. The optimal pH for the activity of the proteases was 5.5 and 6.5, respectively, and they were also stable near this pH value. During the fermentation of soybean paste and soy sauce, the pH decreased to around 5. Therefore, these proteases will exhibit high activities and remain stable during the fermentation. These proteases showed high activities at 10% NaCl concentration and still remain moderate activities at 20% NaCl concentration. Different categories of the fermented soybean paste and soy sauce have different salt concentrations ranging from 10-21%, thus these proteases will be salt-tolerant enough during the fermentation.

The application of the salt-tolerant acid protease produced by *B. megaterium* KLP-98 to the anchovy sauce processing was investigated (Fu et al., 2008). Anchovy sauce was made by mixing anchovy with 20% NaCl at 30 °C for 2 months. The purified *B. megaterium* KLP-98 protease was lyophilized (15.8 U/mg) and thoroughly mixed with 2 month-ripened anchovy sauce in a ratio of 1 mg/100 g. Samples were stored in screwed boxes in dark at 40 °C for two days and various quality characteristics of the liquid fraction were determined every 12 hours. The degree of hydrolysis (DH), which is defined as the percentage of the free amino group cleaved from protein, was calculated. The yield was determined as the volume of liquid fraction obtained per 100 g fish. The final production was obtained with satisfactory color, flavors and taste (data not shown). The improvement of various parameters related to fish protein hydrolysis was discussed. All parameters of the control samples slightly changed during two days fermentation. However, all the parameters of the fish sauce samples with *B. megaterium* KLP-98 protease were greatly changed. The values of pH, TN, DH of the commercial fish sauces are: Vietnam (5.75, 2590 mg/100 mL, 61.6%), China (6.15, 1490 mg/100 mL, 57.8%) and Korea (5.49, 1270 mg/100 mL, 68.2%) (Park et al., 2001). These values of the anchovy sauces in this study are 5.17, 1252 mg/100 mL, 57.4%, which are slightly lower than the commercial products; however, the fermentation period decreased from one year of the commercial production to 2 months and 2 days.

The principle of fish sauce fermentation is somewhat similar to the soybean product fermentation. The successful application of the *B.* megaterium KLP-98 protease to anchovy sauce fermentation indicated the potential application of salt-tolerant acid protease to soybean paste and soy sauce fermentation.

6. Conclusions

From the collective information on salt-tolerant acid proteases, we conclude that proteases are important enzymes naturally purified from variety of halotolerant microorganisms. A few salt-tolerant acid proteases, i.e. proteases produced by *Bacillus subtilis* JM3, *Bacillus megaterium* KLP&98, and *Aspergillus oryzae* LK&101, have a remarkable production and possess excellent properties such as high specific activity, excellent temperature and pH stability and salt-tolerant ability, which enlighten their potential applications in sauce bean paste and soy sauce fermentation.

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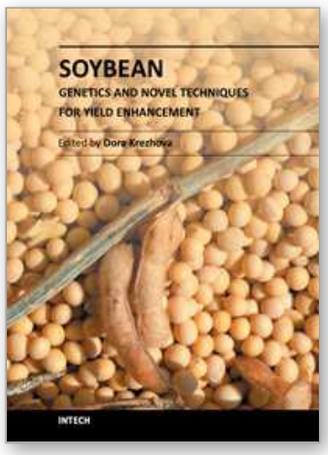

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This book presents the importance of applying of novel genetics and breading technologies. The efficient genotype selections and gene transformations provide for generation of new and improved soybean cultivars, resistant to disease and environmental stresses. The book introduces also a few recent modern techniques and technologies for detection of plant stress and characterization of biomaterials as well as for processing of soybean food and oil products.

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