Characterization of Multiple Extracellular Proteases Produced by a *Bacillus subtilis* Strain and Identification of the Strain

Do Thi Bich Thuy
Faculty of Engineering and Food Technology, Hue University of Agriculture and Forestry
102 - Phung Hung, Hue City, Vietnam
Tel: 84-914-091-340 E-mail: dtbthuy@hueuni.edu.vn

Salil Kumar Bose
School of Biological Sciences -Nanyang Technological University
60 Nanyang Drive, 637551, Singapore
Tel: 65-6316-2807 E-mail: skbose@ntu.edu.sg

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Abstract

A *Bacillus subtilis* strain isolated from shrimp by-product in Vietnam produced a mixture of extracellular proteases. The enzyme preparation precipitated by ethanol (EPE) obtained from this strain was characterized. The effect of pH on the protease activities showed that the proteases in this preparation belong to neutral and alkaline protease family. The protease activity of this preparation was decreased by 20% at 70^0C, suggesting that there is at least one thermostable protease in it. The protease activities were also partially inhibited by Chymostatin (45%), Pefabloc (74%), $EDTANa_2$ (22%); this indicated that there were some of proteases that belong to the serine protease family and there was at least one metalloprotease. SDS-PAGE analysis showed that there were four proteases in this preparation having molecular weights 67 kD, 48 kD, 30 kD and 20 kD. These were named EPE-67, EPE-48, EPE-30 and EPE-20 respectively. EPE-48, EPE-30 and EPE-20, but not EPE-67, were found to match with nattokinase by MS analysis. Among these EPE-20 was a thermostable fibrinolytic enzyme. The amino acid sequence at the N-terminal of EPE-20 was about 99% identical to the precursor nattokinase and that of EPE-30 and EPE-48 was about 99% identical to mature nattokinase. There were few differences in the amino acids between the identified sequences and the known sequences in the database. Sequencing analysis of 16S rDNA gene showed 99% identity of the tested strain with the *Bacillus subtilis* in the database. Phylogenetic analysis of this strain showed that it was most closely related to *Bacillus mojavensis* strain KL.

Keywords: *Bacillus subtilis*, Extracellular proteases, Nattokinase

1. Introduction

Production of proteolytic enzymes (proteases) is a normal physiological function of many organisms including bacteria. Besides their use in normal physiology, proteases are used in various industries including pharmaceuticals, detergents, food, and waste processing. Of all industrial enzymes used worldwide, proteases alone constitute nearly 60% (Nascimento and Martins, 2004).

The most common and widely used bacteria for industrial proteases belong to the genus *Bacillus;* the main reason for this is that large varieties of proteases are produced by the *Bacillus*. Among the Bacilli, the most widely used species is *Bacillus subtilis*, mainly because this species is non-pathogenic to humans. *B. subtilis* has recently become more popular source because its genome is completely sequenced, and the biochemical and genetic techniques to study *B. subtilis* are standardized (Ara, *et al.,* 2007*). B. subtilis*, as in the case of many other bacteria and higher organisms, produce several protease enzymes some of which are released in the extracellular medium. These extracellular proteases are easier to extract and purify. The genes of the extracellular proteases are mostly known and sequenced, and several recombinant enzymes have been produced (Ara, *et al.,* 2007; Park, *et al.,* 2004).

One of the authors of the present study has isolated a *B. subtilis* strain in Vietnam from shrimp by-product (shell and head) (Thuy, *et al.,* 2004), which produces four extracellular proteases as detected by SDS-PAGE analysis

(Thuy, *et al.,* 2006). Any single known *B. subtilis* produces one to three proteases, with few exceptions, such as *B. subtilis* 168 (Rao, *et al.,* (1998)). Because production of more than three proteases by a single strain is unusual, we attempted to characterize the proteins and identify the strain.

2. Materials and Methods

2.1 Isolation of the bacterial strain and culture condition

Bacillus subtilis was isolated from shrimp by-product (shell and head) as described by Thuy *et al.* (2004). The samples of shrimp shell (1g) were ground and diluted with 100 mL distilled water. A dilution series (10^{-2} to 10^{-8}) was plated on an agar medium containing 1% peptone, 0.3% meat extract and 0.5% sodium chloride. After incubation at 37°C for 24 h, colonies with different morphology were selected and plated again on the same medium for further purification of the strains. A total of 50 strains were isolated. Three strains selected from eleven strains showed extracellular protease activity by diameter of hydrolyzed protein zone, and are named as C_2, C_3, and C_{10}. According to the results analyzed in the Laboratory of Food & Environmental Microbiology - UCL- Belgium, C_2, C_3, and C_{10} were *Kocuria rhizophila*, *Aeromonas spp*. and *Bacillus subtilis* respectively. The strain was aerobically cultured at 35^0C for 24h in a medium containing 1.0% peptone, 0.3% meat extract, 0.5% sodium chloride, 0.1% yeast extract, and 1.75% soluble starch for optimum production of the extracellular proteases by this strain (Thuy, *et al*., 2004).

2.2 Partial enzyme purification

Stationary phase culture was centrifuged at 15,000 × g for 20 min at 4^0C. The enzymes in the cell-free supernatant were precipitated by 75% ethanol followed by centrifugation at 15,000 × g for 20 min at 4^0C. The precipitate was freeze-dried and named as EPE preparation and stored at 4^0C (Thuy, *et al.*, 2005). On the day of experiment, an aliquot of the EPE preparation was dissolved in a minimal volume of 50 mM Tris-HCl, pH 7.8 and loaded onto a column of Sephadex G-75 equilibrated with the same buffer, at a flow rate 0.7 mL/min. Different fractions of protein peaks were collected and checked for protease activity. The fractions of each peak (purified enzyme) were pooled separately and concentrated by freeze-drying for further studies.

2.3 Determination of molecular mass of the purified enzymes and sample preparation for mass spectrometry analysis

After gel filtration, different fractions (protease activity peaks) were subjected to SDS-PAGE for determining the molecular mass of the enzymes. The SDS-PAGE was performed according to Laemmli (Leammli U.K., 1970) by using 12% acryl amide including 0.1% casein. After running the polyacrylamide gel, it was incubated in 2.5% Triton X-100 solution at 4^0C for 30 minutes to remove SDS. The gel was then incubated in 50 mM Tris-HCl buffer, pH 7.4 at 35^0C for 3 hours, stained with coomassie brilliant Blue R-250 in methanol-acetic acid-water (5 : 1 : 5, v/v), and decolorized in 7% acetic acid. The bands of active proteases were detected on the gel. These bands were cut off and used for Mass Spectrometry analysis.

MALDI TOF/TOF analysis of the trypsin digests was performed on a 4800 MALDI-TOF/TOF (Applied Biosystem, USA). Digested peptides were desalted by using C18 Ziptips (Millipore, USA) and eluted with 70% acetonitrile in 7 µl. About 0.5 µl of the eluent was mixed with 0.5µl of matrix solution (10 mg/ml alpha-cyno-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) and about 0.5 µl of the mixture was loaded onto the MALDI target and dried in ambient environment. MS and MS/MS spectra were collected using the 4000 Series Explorer™ software (Applied Biosystems) and submitted to database search via GPS Explorer™ (Applied Biosystems). Mascot search engine (v.2.2, Matrix Science, London, UK) and NCBI non-redundant database (release date August 22, 2008) were used for protein identification. The search settings were as follows: mass values, monoisotopic; precursor mass tolerance, ± 1 Da; fragment mass tolerance, ± 0.5 Da; enzyme, trypsin; maximum missed cleavage allowed, 1; fixed modification, carbamidomethyl Cys; variable modification, oxidation of Met, phosphorylation of Ser, Thr and Tyr. Results were scored using probability based Mowse score (Protein score is -10 × Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 70 are significant ($p<0.05$)). (David et al 1999) The mass spectrometric experiments were done in the laboratory of Dr. Tang Kai, School of Biological Sciences at Nanyang Technological University.

2.4 Protease Assay

For measuring protease activity, a diluted enzyme solution (EPE) of 0.5 mL was mixed with 1 mL of 2% casein solution (in Tris-HCl buffer, pH 7.5) and incubated for 10 min at 30^0C. The reaction was terminated by adding 2 mL of 5% trichloroacetic acid (TCA) solution to the reaction mixture. The reaction mixture was then centrifuged and the amino acid concentration in the supernatant was measured by the Folin method with tyrosine as the reference amino acid. Blanks were prepared by adding 2 mL TCA before incubation with casein. The hydrolysis

of casein by the protease enzymes in EPE is measured as the protease activity; and one unit of protease activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per min.

2.5 Effect of protease inhibitors on protease activity

The effects of protease inhibitors were studied using antipaindihydrochloride, bestatin, chymostatin, E-64, pepstatin, phosphoramindon, pefabloc SC and EDTA-Na_2. Protease assays were conducted by using substrate casein with different protease inhibitors as indicated. In each experiment, 0.5 mL of EPE (1 mg EPE per mL buffer) was mixed with 1 mL of 2% casein. Different protease inhibitors (5 mM) were added to each reaction. The control sample was without inhibitors. The optical densities at 750 nm (OD) were measured after adding Folin reagent. These experiments were done in the laboratory of Dr. Ho Sup Yoon, School of Biological Sciences at Nanyang Technological University.

2.6 Effect of pH on protease activity

The protease activities of the purified enzyme mixture (EPE) were examined at different pH values. The reaction mixtures including the enzyme and casein in different pH values were incubated at 30^0C for 10 minutes followed by Folin assay to monitor casein hydrolysis by the proteases.

2.7 Effect of temperature treatment on the protease activity

To investigate the effect of temperature treatment, the samples were treated at different temperature between 40 and 80^0C for 10 minutes and then centrifuged to remove the denatured protein. The remaining protease activity in the supernatant was measured at 30^0C as described above (protease assay section).

3. Results and Discussion

3.1 Effect of pH on the protease activity

The protease activity at pH 5 was very little as compared to the activities at neutral and alkaline pHs (Fig. 1). This suggests that the proteases belong to neutral and alkaline protease families. This is consistent with the property of serine protease family. Most of the earlier reports showed that *B. subtilis* proteases belong to serine protease family (Adinarayana, *et al.,* 2003; Jeong, *et al.,* 2001; Suh, *et al.,* 2001; Sung, *et al.,* 2009; Thuy, *et al.,* 2006; Uchida, *et al.,* 2004; Wang, *et al.,* 2006; Wang, *et al.,* 2008). These experiments were done in the laboratory of Dr. Ho Sup Yoon, School of Biological Sciences at Nanyang Technological University

3.2 Effect of temperature treatment on the protease activity

The protease activity of EPE remained practically unchanged up to 60^0C and decreased by 20% at 70^0C (Fig 2). Because *B. subtilis* proteases are rarely heat stable up to 70^0C, we anticipated that there is at least one or even more than one thermostable protease exists in the purified protease mixture EPE. Assuming that these proteases were not just differentially heat sensitive, but were also differentially inhibitor sensitive, we attempted to establish the existence of different proteases in the EPE mixture by examining the effects of various specific inhibitors on EPE as shown below.

3.3 Effect of protease inhibitors on the protease activity

To determine the class to which the proteases in EPE preparation belonged, the effects of different specific inhibitors were studied**.** Protease assays were conducted by using substrate casein with different protease inhibitors separately and the absorbance at 750 nm to measure the final product after adding Folin-Ciocalteau reagent (Table 2). As shown in the Table 2, the activities were inhibited by Chymostatin which is an inhibitor of chymotrypsin by 45% as compared with the control that is without the inhibitors. The activity was also inhibited by Pefabloc (an inhibitor of serine proteases, e.g. trypsin, chymotrypsin, plasmin, and thrombin) and Antipain-dihydrochloride (an inhibitor of serine proteases and cystein proteases) by 74% and 16% respectively as compared with the control. These results indicate that some of proteases in EPE preparation belong to the serine protease family. This important observation needs to be extended to examine the concentration dependence for each inhibitor to indicate further the presence of different proteases in the purified enzyme mixture EPE.

The fibrinolytic enzymes belonging to the metalloprotease require divalent metal for their activities; so their activities can be inhibited by chelating agents such as EDTA (Peng, *et al.*, 2005). As can be seen in Table 2, the activities of EPE preparation were inhibited by EDTA-Na_2 by 22% as compared with the control. This implies that some of proteases in the EPE preparation belong to the fibrinolytic enzymes. The enzymes were further characterized by SDS-PAGE followed by MS.

3.4 Molecular Mass of Purified proteases

After gel filtration of the EPE preparation, different fractions of the peaks were analyzed by 12% SDS-PAGE gel. Four clear bands of potential active protease candidates were observed (Fig. 3). The proteases were called EPE-67, EPE-48, EPE-30 and EPE-20 to correspond their molecular weights of 67 kD, 48 kD, 30 kD and 20 kD respectively. These four bands were cut off for Mass Spectrometry analysis.

3.5 Identification of the candidates by MS

The MS analysis (Table 1) showed that EPE-20, EPE-30 and EPE-48 but not EPE-67 were found to match with nattokinase [*Bacillus subtilis*]. Among them EPE-20 was a thermostable fibrinnolytic enzyme. Previous results (Thuy *et al.,* 2006) reported that EPE-30 was an alkaline protease; while EPE-48 and EPE-20 were neutral proteases. Taken together we suggest that the EPE-48 protease and the EPE-30 protease are neutral nattokinase and alkaline nattokinase respectively; and the EPE-20 protease is a neutral thermostable fibrinolytic enzyme. The EPE-20 protease is suggested to be a new nattokinase because of the two following reasons.

First, although some *B. subtilis* proteases have been reported to be thermostable, their properties, especially the heat sensitivity and the molecular masses, are different from those of the EPE-20 protease: a *B. subtilis* CN2 strain isolated from Vietnamese fish sauce produced a serine protease; this enzyme retained 85% of its full activity after treatment at 60^0C for 30 min, but its molecular mass is 27.6 kD (Uchida, *et al.,* 2004); a serine alkaline protease of *B. subtilis* PE-11 is stable at 60^0C at pH 10, however, it has 15 kD molecular mass (Adinarayana *et al*., 2003); a *B. subtilis* LD-8547 produced a fibrinolytic enzyme that remained 82% and 11% activity after treatment at 60^0C and 70^0C for 60 min respectively but it has 30 kD molecular mass (Wang, *et al.,* 2008); Balaji, *et al.,* (2008) reported a thermostable protease from *B. subtilis* MTCC (9102) that remained stable up to 70^0C, however, it was a keratinase and had the molecular mass in the range from 64 to 69 kD.

Second, the molecular mass of EPE-20 (20 KD) is also different from the other known *Bacillus subtilis* nattokinases (fibrinolytic enzymes), such as, the fibrinolytic enzyme of *B subtilis* DC33 (30 kD) (Wang, *et al.*, 2006), the fibrinolytic subtilisin- like protease of *Bs subtilis* TP-6 (27.5 D) (Kim, *et al.,* 2006), the nattokinase of *B subtilis* YF38 (28 kD) (Liang, *et al.,* 2007), and the nattokinase of *B subtilis* (Strain no: 1A752, *Bacillus* genetic stock center, Ohio, US) (28 kD) (Deepak, *et al*., 2009). An overview of the microbial fibrinolytic enzymes (Peng, *et al.,* 2005) also showed that all of these enzymes have a range of molecular weights from 27 to 44 kD.

On the basis of the above reasons we conclude that the EPE-20 is a new nattokinase. This conclusion prompted us to compare the sequence identities of these proteins (EPE-20, EPE-30 and EPE-48) with the sequences available in the nattokinase database. As shown in Fig. 4, EPE-20 is about 99% identical to the nattokinase precursor. EPE-30 and EPE-48 are about 99% identical with the mature nattokinase. There are a few differences in the amino acids between the identified sequences and the known sequences in the database. Therefore, we conclude that the identified proteins (EPE-20, EPE-30 and EPE-48) with different sizes are probably the precursors or the mature form of the nattokinases.

3.6 Identification of the strain

Because this *B. subtilis* strain produces more than usual number of extracellular proteases which, especially the EPE-20, possess different characteristics despite 99% sequence identity with the database nattokinases, we wanted to determine the phylogenetic location of the strain by using its 16S rDNA. The 16S rDNA gene was successfully amplified from the genomic DNA of the *B subtilis* strain (Fig. 5). The purified PCR product was used for sequencing directly.

Sequencing analysis gave 99% sequence identity of the tested strain with the known sequence of 16S rDNA of *B subtilis* in the database. Phylogenetic analysis showed that the tested strain was closest to *Bacillus mojavensis* strain KL (Fig. 6), a strain that is involved in the hydrocarbon degradation pathway (Roberts, *et al.*, 1994).

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Table 1. Protease identification (ID) Mass Spectrometry (MS) finger printing

Sample for MS	Match to	Score	ID
ETC-67	gi|82749915	1355	Oligopeptide ABC transporter (binding protein) [*Bacillus subtilis* subsp. subtillis str. 168]
ETC-48	gi|14422313	136	Nattokinase [*Bacillus subtilis*]
ETC-30	gi|14422313	2872	Nattokinase [*Bacillus subtilis*]
ETC-20	gi|60687498	1171	Thermostable fibrinolytic enzyme Nk1 [*Bacillus subtilis*]

Mass Spectrometry analysis of the trypsin digests were performed as described in the Materials and Methods. The results were scored using probability based Mowse score (Protein score is –10(log(P)), where P is the probability that the observed match is a random event). Protein score greater than 42 are significant ($p<0.05$).

Table 2. Effect of the protease inhibitors on the protease activity of the EPE

Inhibitors	Specificity of Inhibitors	Residual activity (ABS at 750 nm)	% inhibition
Control	None	1.515	00
Antipain-dihydrochloride	Serine and cysteine protease	1.272	16
Bestatin	Amino peptidase, including amino-peptidase B., leucine aminopeptidase, tripeptide aminopeptidase.	1.344	11
Chymostatin	α-, β-, γ̃δ--chymotrypsin	0.837	45
E-64	Cysteine proteases	1.491	02
Pepstatin	Aspartate proteases like pepsin, renin, cathepsin D, chymosin	1.433	05
Phosphoramidon	Metallo endopeptidases, specifically thermolysine, collagenase.	1.252	17
Pefabloc SC	Serine proteases, e.g. trypsin, chymotrypsin, plasmin, thrombin	0.394	74
EDTA-Na_2	Metalloproteases	1.184	22

0.5 ml of EPE(1 mg EPE per ml buffer) was mixed with 1 ml 2% casein in each experiment. Different protease inhibitor was added to each reaction. The control sample was without inhibitors. The absorbance at 750 nm for the final product after adding Folin-Ciocalteau reagent was measured. The % inhibition was calculated from the absorbance values by using the control absorbance (1.515) as 100% activity, which is 0% inhibition.

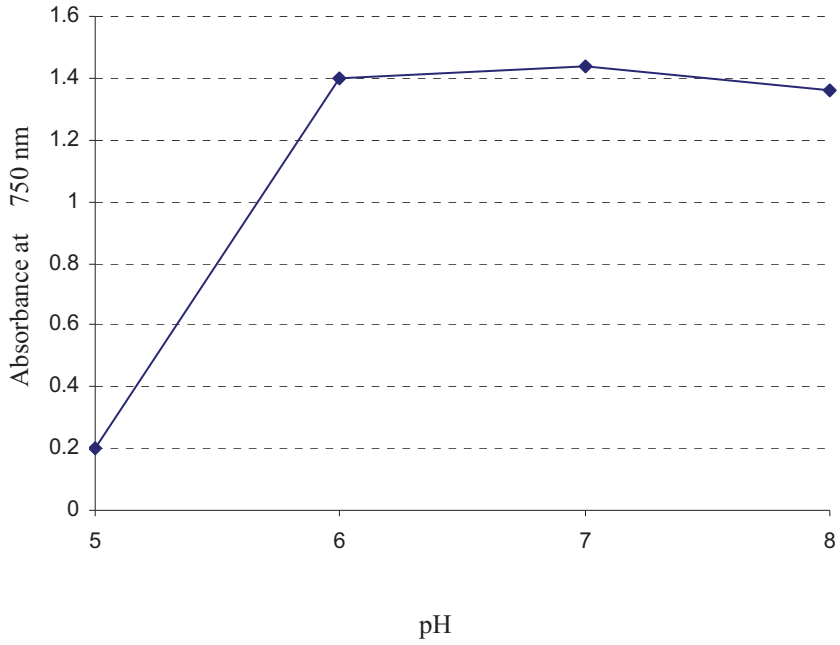

Figure 1. Effect of pH on the protease activities of the EPE

The reaction mixture for each pH contained 0.5 ml of the purified enzyme mixture (EPE 1 mg/ml) and 1 ml 2% casein The pH of the buffers ranged from 5 to 8. The absorbance at 750 nm for the final product after adding Folin-Ciocalteau reagent was measured.

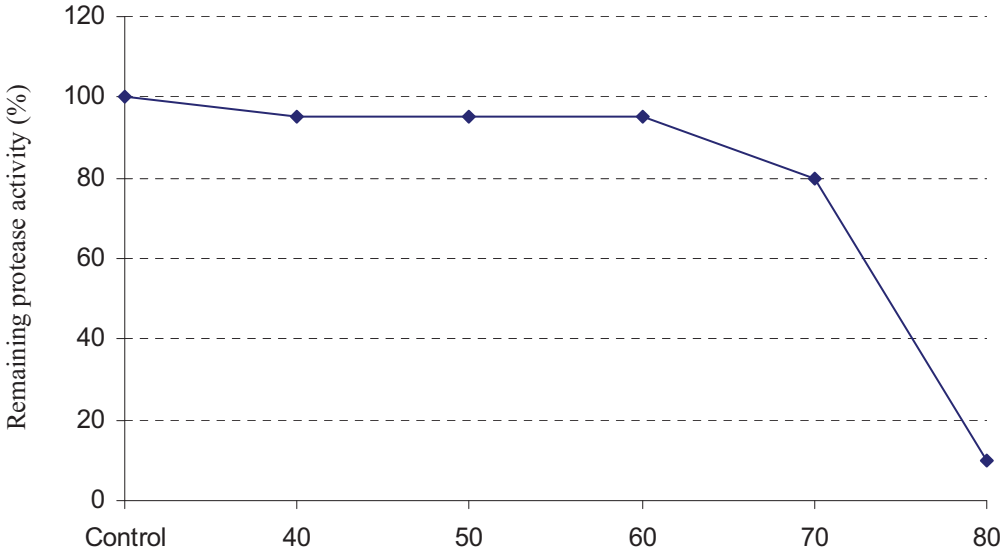

Figure 2. Effect of temperature treatment on the protease activity of the EPE

Each reaction mixture contained 0.5 ml of the purified enzyme mixture (EPE).The reaction mixture was incubated at different temperatures from 40 to 80^0C for 10 minutes. Activity was measured at 35^0C by Folin assay using casein as substrate.

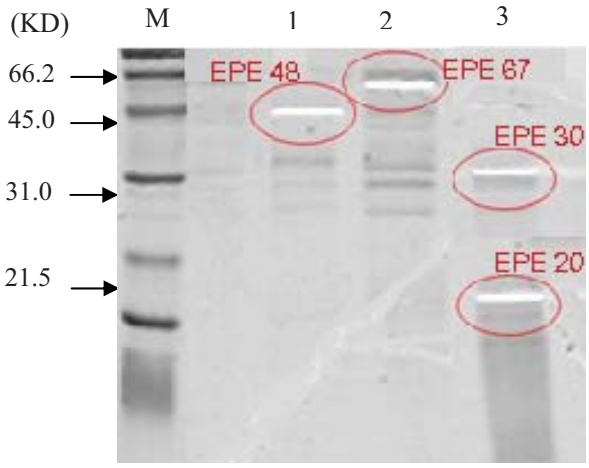

Figure 3. SDS-PAGE patterns of the post-FPLC fractions of EPE

The purified protein mixture (EPE) was run through FPLC which showed three peaks of proteins having protease activity. The fractions from each peak were pooled and concentrated for SDS-P. Lane 1, 2, 3 correspond to these three fractions and the lane M is for the molecular markers. Each of the three fractions revealed several protein bands in the 12% SDS-PAGE. Of these bands only four bands (indicated by circles) showed protease activity in the presence of casein in the gel. These protease active bands were named by their observed molecular weights as (EPE-20, 30, 48, 67)

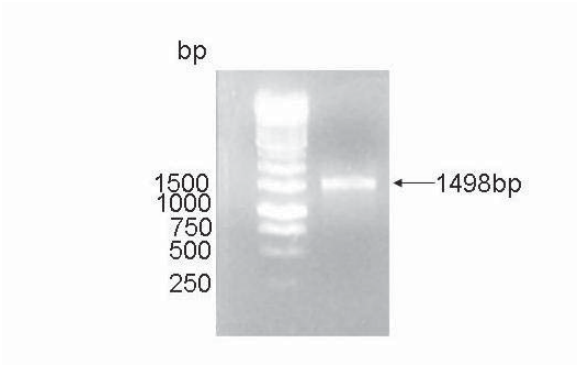

Figure 4. Amplification of 16S rDNA gene from the genomic DNA of the *Bacillus subtilis* strain

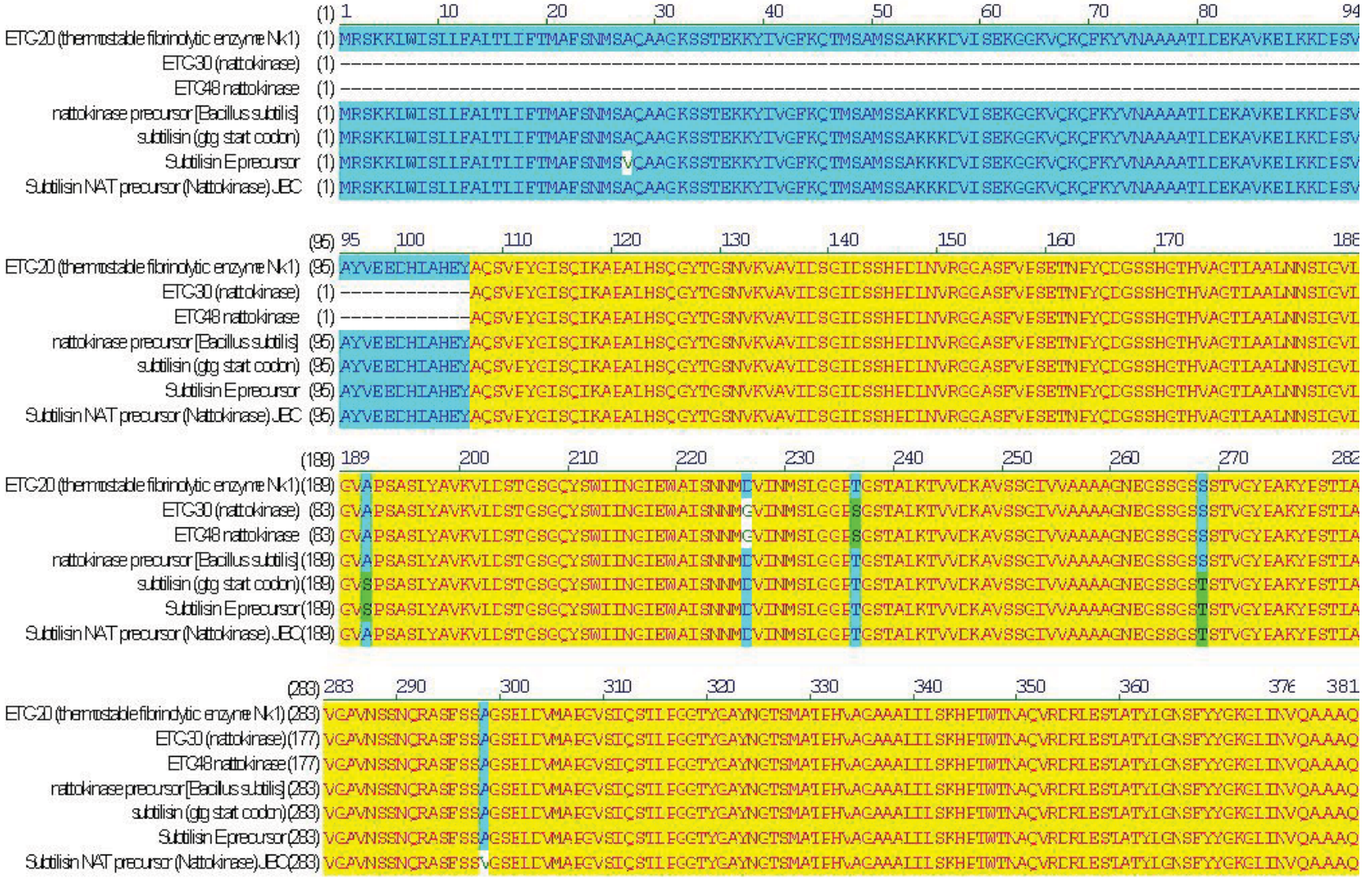

Figure 5. Sequence alignment for the identified nattokinase from MS analysis.
Sequences were analyzed using vector NTI (InforMax). The consensus sequences were highlighted in yellow while non-consensus sequences were indicated in blue.

Figure 6. Phylogenetic neighbor-joining tree obtained by 16S rDNA sequence analysis