



# Characterization of a salt-tolerant aminopeptidase from marine *Bacillus licheniformis* SWJS33 that improves hydrolysis and debittering efficiency for soy protein isolate



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## ABSTRACT

An aminopeptidase was isolated from the marine *Bacillus licheniformis* SWJS33 (BLAP) and purified. According to the tandem mass spectrometry, the enzyme displayed 11% amino acid identity with the aminopeptidase from *Bacillus* (gi|496687392). BLAP exhibited maximum activity at 60 °C and pH 8.0–8.5 and had a molecular mass of 100 kDa. The presence of NaCl enabled 50% improvement of enzyme activity with 10–15% NaCl being the best. The observed inactivation by EDTA and bestatin and activation by Co<sup>2+</sup> and Ag<sup>+</sup> indicated that the obtained enzyme was a metalloaminopeptidase. Such an aminopeptidase could further improve the hydrolysis degree of soy protein isolate hydrolysates catalyzed by papain, Alcalase 2.4 L or Flavourzyme 500MG from 8.5%, 9.5% or 14.4–18.8%, 18.7% or 20.1%, respectively, while decreasing the bitter intensity score of the SPI hydrolysates catalyzed by Alcalase 2.4 L from 3.6 to 0.4.

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## 1. Introduction

Controlled proteolysis of various protein-rich raw materials using proteases to produce protein hydrolysates with desirable bioactivity and flavors has become an important and sustainable approach to add value to local bioresources. Discovering proteases with high efficiency and novel characteristics at low costs from the nature such as marine system, to enrich the existing list of commercial preparations, is one of the strategies for the enzyme industry. Such a demand motivates this current study.

There exist various commercial proteases and most of which are endoprotease except for some in the form of mixtures of endoprotease and exoprotease such as Flavourzyme. Endoproteases have demonstrated high efficiency towards the hydrolysis of aquatic products and animal proteins but unsatisfactory outcomes (including poor protein recovery, low hydrolysis degree and unpleasant taste) for plant proteins such as soybean, wheat gluten and peanut proteins (Chen, Chen, Ren, & Zhao, 2011; Tchobanov, Marinova, & Grozeva, 2011). Exoproteases attack the peptide chain at the ends (i.e. the N- or C-terminus of a protein) through removing a single

amino acid or sometimes a di-/tri-peptide. These enzymes play important roles in improving hydrolysis efficiency as part of the preprocessing steps besides physical methods (Chen et al., 2011), and modifying the flavor of protein-rich products through debittering and monitoring flavor development. For example, aminopeptidases had been used to remove hydrophobic amino acids from the N-terminus of polypeptides (Huang et al., 2015; Li, Lin, Chen, Fu, & Wu, 2015; Stressler, Eisele, Schlayer, Lutz-Wahl, & Fischer, 2013; Wang et al., 2011) and in cheese ripening (Wilkinson, Guinee, O'Callaghan, & Fox, 1994). Though the introduction of aminopeptidases could obviously improve the hydrolysis efficiency, its large scale application is not well realized due to the limited species of commercial aminopeptidase.

Aminopeptidases (EC 3.4.11.) are an important member of exoprotease family, which can catalyze the cleavage of peptide bond linking the terminal amino acid at the free N-terminal end of a polypeptide chain. They can be classified according to their origin, location, preference for specific amino-terminal amino acid substrates, sensitivity to inhibitors, or different requirements for divalent metal ions (Taylor, 1993). Among the existing plant, animal and microorganism sources, microorganisms currently represent the most promising and reliable source for obtaining novel enzymes such as aminopeptidases due to its biochemical diversity, easy cultivation and other advantages such as relatively high

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stability and activity associated with their unique life habitats in the marine environment involving high salinity, high pressure, low temperature and special lighting conditions (Bull, Ward, & Goodfellow, 2000; Stach, Maldonado, Ward, Goodfellow, & Bull, 2003).

The microorganisms that had been reported to produce aminopeptidase were mainly *Aspergillus* (Matsushita-Morita et al., 2010), *Streptomyces* (Wu et al., 2010), *Pseudomonas* (Wu, Zhou, Zhou, Gao, & Tian, 2014), *Lactic acid bacteria* (Tchorbanov et al., 2011) and *Bacillus* (Rodríguez-Absi & Prescott, 1978) genus. *Bacillus licheniformis* is an important industrial microorganism because of its generally regarded as safe (GRAS) status, high growth rate and broad substrates (Voigt et al., 2004). Its ability to produce numerous desirable hydrolytic enzymes, especially alkaline serine protease (subtilisins; annual pure enzyme output: ~500 metric tones) further makes this strain industrially important (Schallmeyer, Singh, & Ward, 2004). However, only a few studies had applied *Bacillus licheniformis* to produce aminopeptidase, e.g. Rodríguez-Absi et al. purified an extracellular aminopeptidase from *Bacillus licheniformis* in 1978 for hydrolyzing dipeptides, aminoacylnaphthylamides, and amino acid amides (Rodríguez-Absi & Prescott, 1978), Pavlova et al. isolated an aminopeptidase from the thermophilic strain of *Bacillus licheniformis* for splitting off the N-terminal leucine in short peptides and hydrolyzing leucinamide (Pavlova, Rotanova, & Zholner, 1988).

In this present study, we aimed at isolating and purifying an aminopeptidase from marine-derived *Bacillus licheniformis* SWJS33. The obtained enzyme was evaluated through standard characterization and its application in plant protein hydrolysis.

## 2. Materials and methods

### 2.1. Materials and chemicals

*Bacillus licheniformis* SWJS33 was newly isolated from the deep-sea mud of South China Sea (kindly provided by South China Sea Institute of Oceanology Chinese Academy of Science) and deposited in the China General Microbiological Culture Collection Center (CGMCC No.7388). DEAE-Sepharose Fast Flow and Superdex 200 pg were purchased from GE Healthcare (Beijing, China). L-Leucine-*p*-nitroaniline (Leu-*p*NA), Glu-*p*NA, Lys-*p*NA, Pro-*p*NA, Met-*p*NA, bovine serum albumin (BSA),  $\beta$ -mercaptoethanol ( $\beta$ -ME), dithiothreitol (DTT), SDS, bestatin hydrochloride, phenylmethanesulphonyl fluoride (PMSF), ethylenediaminetetra-acetic acid (EDTA), pepstatin and soybean trypsin inhibitor (STI) were from Sigma (Beijing, China). Phe-*p*NA, Ala-*p*NA and Arg-*p*NA were from GL Biochem (Shanghai, China). Media components were purchased from Guangzhou Huankai Microbial Technology Co., Ltd. (Guangzhou, China). Soy protein isolate (SPI) was obtained from Guangzhou Honsea Co., Ltd. (Guangzhou, China). Alcalase 2.4 L and Flavourzyme 500MG were purchased from Novozymes (food grade; Beijing, China). Papain was obtained from Baiao Biochemistry Co., Ltd. (food grade; Jiangmen, China). All the other chemicals and solvents were of analytical grade.

### 2.2. Preparation of aminopeptidase

To produce aminopeptidase, the strain *Bacillus licheniformis* SWJS33 was grown in a medium (pH 7.0) consisting of glycerin 3.0 g, glucose 5.0 g, yeast extract 10.0 g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{MgSO}_4$  0.3 g,  $(\text{NH}_4)_2\text{SO}_4$  1.0 g,  $\text{CaCl}_2$  1.0 g, sea salt 10.0 g and distilled water 1.0 L. The culture was incubated at 37 °C with constant shaking at 150 rpm for 48 h to facilitate fermentation. The fermented mixture was then centrifuged at 10,000g and 4 °C for 10 min in a CR22G high-speed centrifuge (Hitachi Co., Tokyo, Japan). The

supernatant was collected as crude *Bacillus licheniformis* SWJS33 aminopeptidase (BLAP). The fermentation of BLAP was performed in duplicate.

### 2.3. Analysis of aminopeptidase activity and protein concentration

The leucine aminopeptidase activity was quantified following the method of Tan et al. (Tan & Konings, 1990) with some modifications. An aliquot (80  $\mu\text{L}$ ) of the diluted sample by Tris-HCl buffer (50 mM, pH 8.5) was mixed with 20  $\mu\text{L}$  of Leu-*p*NA (20 mM). The mixture was subject to heating at 40 °C for 10 min and the reaction was stopped by adding 100  $\mu\text{L}$  glacial acetic acid. A blank was set up through mixing 100  $\mu\text{L}$  glacial acetic acid with the same sample before the substrate addition. The absorbance at 405 nm was measured. One unit of enzyme activity ( $\text{LAP mL}^{-1}$ ) was defined as the amount of enzyme that liberates 1  $\mu\text{mol}$  of leucine-*p*-nitroanilide per minute. Standard curve was plotted using 4-nitroaniline.

The protein concentration was measured based on the Coomassie method using brilliant blue G-250 to bind protein in order to measure the absorbance peak shift (from 465 to 595 nm), as well as using BSA as a known protein standard (Lowry, Rosebrough, Farr, & Randall, 1951). Appropriate dilutions of the test sample were performed.

### 2.4. Aminopeptidase purification

The crude BLAP was precipitated with ammonium sulfate using the fraction at 60% concentration according to the preliminary experiments. The resultant precipitate was collected after centrifugation (10,000g, 4 °C, 20 min) and re-suspended in 20 mM Tris-HCl buffer (pH 8.5). Then it was transferred into dialysis bags (MWCO: 10 kDa) and dialysed against the same Tris-HCl buffer at 4 °C for 48 h (the buffer was changed every 12 h). The dialysed solution was lyophilized (R2L-100KPS, Kyowa Vacuum Engineering, Tokyo, Japan) and stored in a desiccator over silica gel at 4 °C for further use. BLAP purified at this process was usually regarded as food grade enzyme.

Further purification of the dialyzed enzyme was performed using the AKTA pure system (GE Healthcare, USA) fitted with DEAE-Sepharose Fast Flow column (2.6  $\times$  20 cm). The lyophilized enzyme (200 mg) was re-suspended in 4 mL of 20 mM Tris-HCl buffer (pH 8.5) and loaded to the pre-equilibrated column and the system was washed with increasing concentrations of NaCl solutions (0.1, 0.2, 0.3, 0.4 and 0.5 M; in Tris-HCl buffer) at a flow rate of 10 mL/min. Each corresponding eluate fraction (5 mL) was collected. The fractions containing BLAP activity were collected and further loaded onto a Superdex 200 pg column (1.6  $\times$  60 cm). Then elution using the 20 mM Tris-HCl buffer (pH 8.5) was carried out at a flow rate of 1.0 mL/min. Each fraction with 4 mL of eluate was collected and the resulting enzyme was desalted by dialysis and then lyophilized (the enzyme termed “purified BLAP”). Molecular weight of the purified BLAP was analyzed following Laemmli (Laemmli, 1970) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 12% acrylamide under reducing conditions. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250.

### 2.5. Aminopeptidase identification by tandem mass spectrometry

The purified BLAP in the SDS-PAGE was subject to digestion with trypsin (sequencing grade, Promega). The digested peptides were then analyzed on an ABI 4800-plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems Foster City, USA) in the Institute of Life and Health Engineering, Jinan University. The MS spectra were analyzed and the peptide sequence was determined using the Mascot Program and the NCBI BLAST online search tool.

## 2.6. Characterization of purified aminopeptidase

### 2.6.1. Effects of temperature and pH on enzyme activity and stability

**Effect on enzyme activity:** The enzyme activity assay (as Section 2.3) was carried out at pH 8.5 over the temperature range of 20–80 °C to determine the optimum temperature. Then the enzyme activity assay was performed at the detected optimum temperature and different pH values ranging from pH 3.0 to pH 10.0, to determine the optimum pH. To achieve these pH levels, different buffers at 50 mM were used: glycine buffer (pH 3.0), acetate-acetic acid (pH 4.0–5.0), Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O–NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O (pH 6.0–7.0), Tris–HCl (pH 8.0–9.0), H<sub>3</sub>BO<sub>3</sub>–Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O (pH 9.6–10.0).

**Effect on enzyme stability:** The thermal stability of the BLAP was determined by incubating the enzyme at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 30 min, and cooling the system prior to the normal enzyme activity measurement (described as Section 2.3). To study the pH stability, the enzyme was mixed with the buffers (50 mM) of different pHs (3.0–10.0) at 4 °C for 0–48 h prior to the enzyme activity determination. In comparison, a control was set up through leaving the BLAP at 4 °C for the same period before the enzyme activity was measured.

### 2.6.2. Effect of NaCl on enzyme activity

To study the effect of NaCl on aminopeptidase activity, solid NaCl was first added to the Tris–HCl buffer so as to enable the final NaCl concentration in the range of 0–20% in the assay system. Then the enzyme activity assay was conducted as described in Section 2.3.

### 2.6.3. Effects of metal ions on aminopeptidase activity

The effect of metal ions, including Na<sup>+</sup> (NaCl), K<sup>+</sup> (KCl), Li<sup>+</sup> (Li<sub>2</sub>SO<sub>4</sub>), Ag<sup>+</sup> (AgNO<sub>3</sub>·H<sub>2</sub>O), Mg<sup>2+</sup> (MgSO<sub>4</sub>·7H<sub>2</sub>O), Zn<sup>2+</sup> (ZnSO<sub>4</sub>·7H<sub>2</sub>O), Fe<sup>2+</sup> (FeSO<sub>4</sub>·7H<sub>2</sub>O), Mn<sup>2+</sup> (MnSO<sub>4</sub>·H<sub>2</sub>O), Ca<sup>2+</sup> (CaCl<sub>2</sub>), Cu<sup>2+</sup> (CuSO<sub>4</sub>·5H<sub>2</sub>O), Ba<sup>2+</sup> (BaCl<sub>2</sub>·2H<sub>2</sub>O), Cd<sup>2+</sup> (CdCl<sub>2</sub>·H<sub>2</sub>O), Co<sup>2+</sup> (CoCl<sub>2</sub>·6H<sub>2</sub>O), Fe<sup>3+</sup> (FeCl<sub>3</sub>·6H<sub>2</sub>O) and Al<sup>3+</sup> (Al(NO<sub>3</sub>)<sub>3</sub>·7H<sub>2</sub>O) was tested by pre-incubating the enzyme in 5 mM or 1 mM solutions of these ions at 4 °C for 30 min. In comparison, the enzyme in Tris–HCl (pH 8.5) of the same concentrations but in the absence of these ions was kept at 4 °C for 30 min and then subject to the normal enzyme activity assay.

### 2.6.4. Effects of inhibitors

The enzyme was firstly incubated with each of the inhibitors and some reductants ( $\beta$ -ME, DTT, SDS, bestatin hydrochloride, PMSF, EDTA or STI at 5 mM or 1 mM) at 4 °C for 30 min, before being subject to the normal enzyme activity assay (Section 2.3). In comparison, the enzyme in Tris–HCl (pH 8.5) of the same concentrations but in the absence of these inhibitors was kept at 4 °C for 30 min before being subject to the normal enzyme activity assay.

### 2.6.5. Substrate specificity and kinetics parameters

Different commercially available synthetic substrates including Leu-pNA, Phe-pNA, Ala-pNA, Arg-pNA, Glu-pNA, Lys-pNA, Pro-pNA

and Met-pNA were used to determine substrate specificity of the purified BLAP. The effect of substrate concentrations (Leu-pNA; from 0.2 to 4 mM) on the reaction velocity of the purified BLAP was investigated. The  $K_m$  and  $V_{max}$  were determined using the Lineweaver-Burk plot.

## 2.7. The use of BLAP for protein hydrolysis

### 2.7.1. Preparation of SPI hydrolysates

Ten grams of SPI was mixed thoroughly with 90 g of distilled water. The pH of the mixture was adjusted with 2 M NaOH to the pH required by papain, Alcalase 2.4 L or Flavourzyme 500MG for enzymatic hydrolysis (reaction conditions as described in Table 1). For the hydrolysis catalyzed by Papain, Alcalase or Flavourzyme, 0.5 g commercial protease per 100 g SPI was added. For the hydrolysis catalyzed by “Papain 12 h + BLAP 12 h”, “Alcalase 12 h + BLAP 12 h”, or “Flavourzyme 12 h + BLAP 12 h” 0.25 g commercial protease per 100 g SPI was firstly applied for the first 12 h and then 40 LAP food grade BLAP was added to react for another 12 h. The hydrolysis was conducted in sealed glass conical flask in a thermostatic water bath with a stirring speed of 180 rpm. At the end of hydrolysis, the containers with hydrolysates were heated in a boiling water bath for 15 min to inactivate the enzymes. The hydrolysates were then centrifuged in a CR22G high-speed centrifuge at 8000 g and 20 °C for 15 min. The supernatants were collected and stored at –20 °C for further analysis.

### 2.7.2. Determination of the degree of hydrolysis (DH)

DH is defined as the percentage of free amino groups released via cleavage from proteins and can be expressed as the ratio of  $\alpha$ -amino nitrogen to total nitrogen. The amino nitrogen content was determined using a formaldehyde titration method (Nilsang, Lertsiri, Supphantharika, & Assavanig, 2005). The total nitrogen content was determined using the Kjeldahl method (the conversion factor is 6.25 for SPI).

### 2.7.3. Preliminary evaluation on the bitterness of SPI hydrolysates

Bitterness of SPI Hydrolysates were determined quantitatively by 8 people and expressed as quinine hydrochloride equivalent value (Spellman, O’Cuinn, & FitzGerald, 2005). The sensory panel members had been trained to score the intensity of bitterness based on quinine hydrochloride solutions at concentrations of 0.0, 0.1, 0.3, 0.5 and 1.0 mg/100 mL (corresponding to “not bitter at all” (0), “slightly bitter” (1), “bitter” (2), “very bitter” (3), and “extremely bitter” (4), respectively). The different SPI hydrolysate samples were mixed with distilled water with proper stirring to achieve solutions at a protein equivalent of 1.0 g/100 mL and randomly served at room temperature in coded cups to the panelists (the duplicate of each type of test sample was included in the evaluation but as a separate sample with a different code). The panelists were instructed to evaluate the intensity of bitterness, and asked to clean the palate between tasting through rinsing the mouth thoroughly with non-sparkling mineral water. The scores was evaluated by Q-test (an outlier test) and the mean of the remaining scores was recorded.

**Table 1**  
Enzymatic hydrolysis of soy protein isolate.

Group	Enzyme	Temperature °C	pH and time	Degree of hydrolysis (%)	Bitter intensity score
1	Papain	50	7.0, 24 h	8.5	0.6
2	Alcalase	55	8.0, 24 h	9.5	3.6
3	Flavourzyme	50	7.0, 24 h	14.4	0
4	Papain 12 h + BLAP <sup>a</sup> 12 h	50	7.0 for papain, 8.5 for BLAP	18.8	0
5	Alcalase 12 h + BLAP 12 h	50	8.0 for Alcalase, 8.5 for BLAP	18.7	0.4
6	Flavourzyme 12 h + BLAP 12 h	50	7.0 for Flavourzyme, 8.5 for BLAP	20.1	0

<sup>a</sup> Aminopeptidase that produced by marine *Bacillus licheniformis* SJWS33, and is in food grade.

## 2.8. Statistical analysis

The fermentation of BLAP was performed in duplicate and the other tests were conducted in triplicate. Data from sensory evaluation was treated by *Q*-test. Data from chemical analysis was treated by analysis of variance (ANOVA), values followed by different letters (a–f) are significantly different ( $p < 0.05$ ). Analysis was performed using a SPSS package (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

## 3. Result and discussion

### 3.1. Aminopeptidase purification and identification

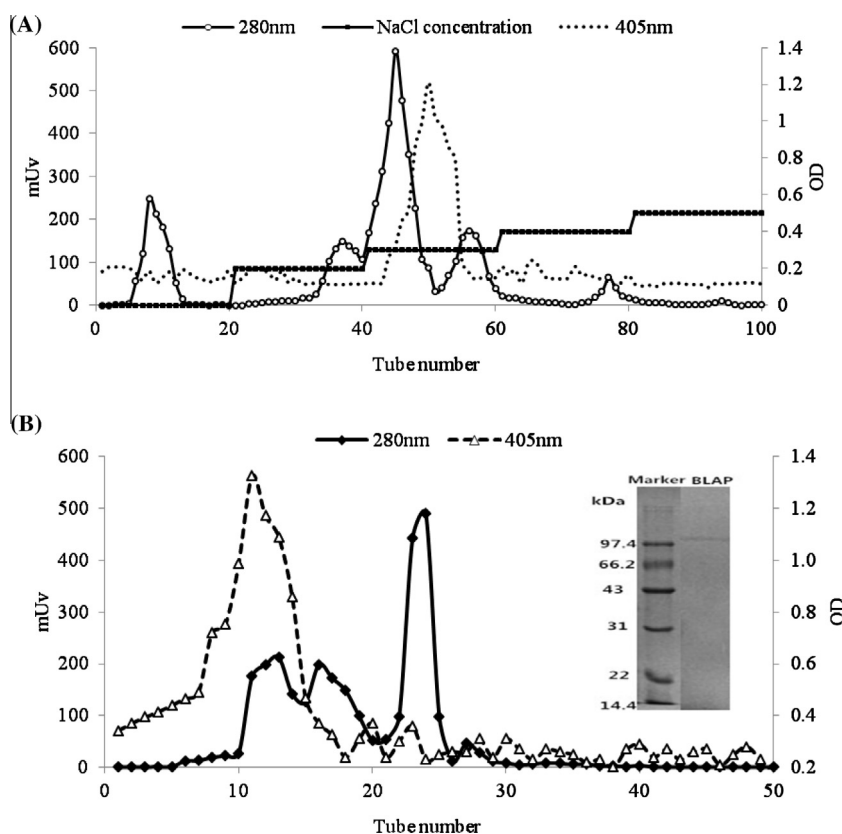
The purification procedures of the BLAP are summarized in Table 2. After fermentation, the supernatant was precipitated with 60% ammonium sulfate. The precipitation was dialysed and lyophilized. The crude enzyme was then purified by DEAE-Sepharose fast flow column and Superdex 200 pg column (Fig. 1). With more purification steps involved, the total protease activity, total protein content and yield decreased while the specific activity and

enrichment factor increased. The BLAP after all the purification steps in this study was enriched 46.1-fold with a specificity activity of 714.1 U/mg protein and a total yield of 6.0%. The purified BLAP had a single 100 kDa band on SDS-PAGE (Fig. 1). Such an apparent molecular weight was different from those of the aminopeptidase from *Bacillus licheniformis* reported by Rodríguez-Absi and Prescott (1978) (43 kDa) and by Pavlova et al. (1988) (60 kDa), but the same as that of the aminopeptidase from Japanese flounder skeletal muscle (100 kDa) obtained by Chen et al. (2012).

The mass fingerprints of the purified BLAP were analyzed by tandem mass spectrometry (spectra not shown). Due to a high signal–noise ratio, seven trypsin-containing fragments were further analyzed by MALDI-TOF/TOF-MS/MS, which were found matching an aminopeptidase from *Bacillus licheniformis* ATCC 14580 (gi|52079505) with the highest protein score 395, under protein score C.I.% 100. Two of the seven identified peptides (i.e. HLDDKASVAL-LIELIR and AGHDIVHGLIGPGIDASHAFER) matched the aminopeptidase fragments from *Bacillus licheniformis* ATCC 14580 with 11% sequence coverage, suggesting that the current BLAP might differ to some extent from the previously reported species in amino acid sequence.

**Table 2**  
Purification procedures and results of aminopeptidase from *Bacillus licheniformis* SWJS33.

	Total protease activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Culture supernatant	2624.8	169.6	15.5	1.00	100.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	1804.4	62.3	29.0	1.87	68.7
DEAE-Sepharose FF	163.2	1.11	147.0	9.50	12.4
Superdex 200 pg	157.1	0.22	714.1	46.1	6.0



**Fig. 1.** Chromatograms of the crude aminopeptidase from *Bacillus licheniformis* SWJS33 after (A) DEAE-Sepharose fast flow purification (5 mL each tube); (B) both DEAE-Sepharose fast flow and Superdex 200 pg purification (4 mL each tube).



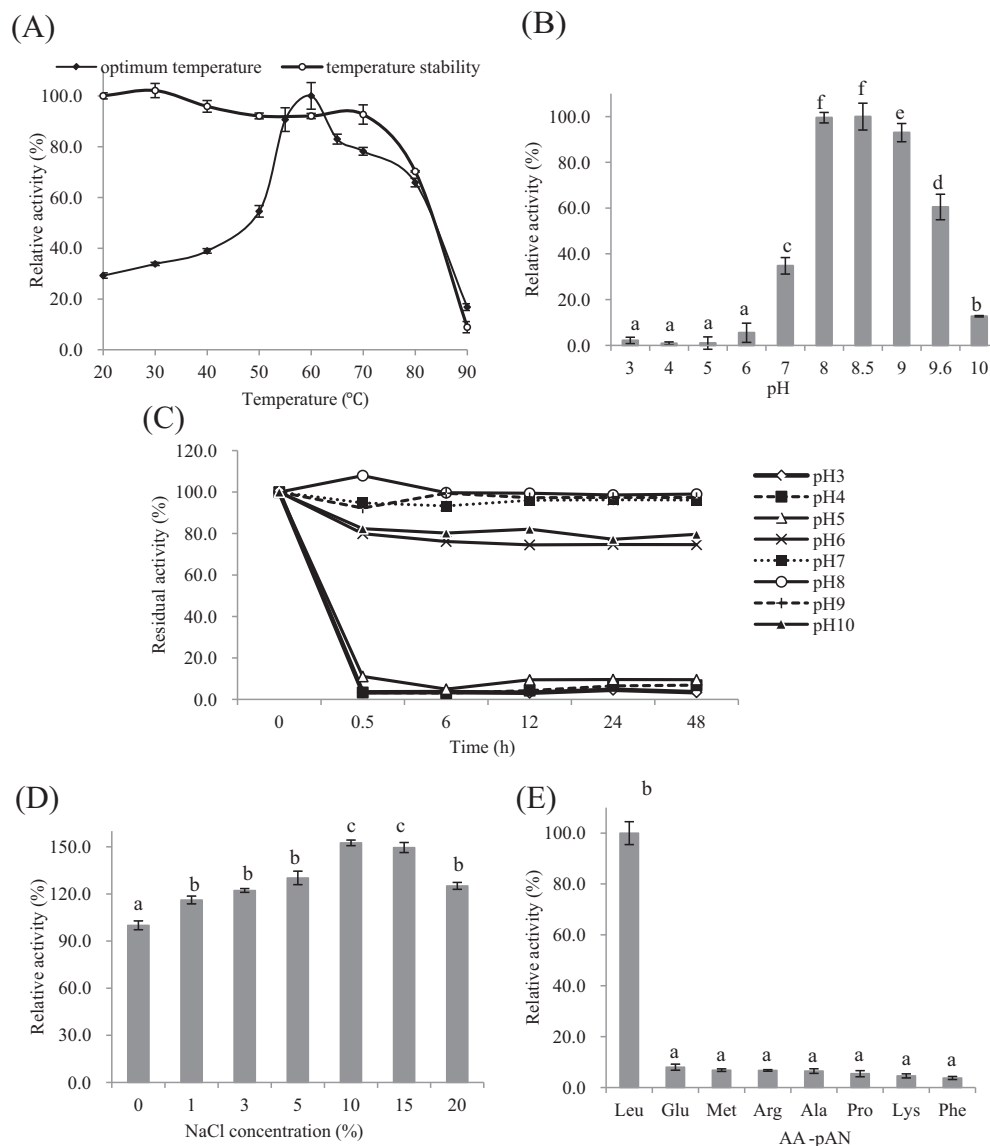
### 3.2. Characterization of purified BLAP

#### 3.2.1. Effects of temperature and pH

The BLAP was found active over a wide range of temperature from 50 to 80 °C (>50%) with 60 °C to be the optimum temperature (Fig. 2A). The lowest relative activity (<20%) occurred at 90 °C for the temperature range examined in this study. The enzyme stability studies revealed that the purified BLAP was rather stable below 70 °C (with >90% of the relative activity retained), and about 60% of the activity was left after holding at 80 °C for 30 min. When the temperature reached 90 °C, the enzyme was almost inactivated (Fig. 2A). These suggested that BLAP belongs to the mesophilic protease family. The optimum temperature of the aminopeptidase secreted by an thermophilic *Bacillus licheniformis* was found as 85 °C with a half-life at 90 °C as 145 min (Pavlova et al., 1988). Aminopeptidases derived from various strains exhibited different thermal stability: The maximal activity of the leucine aminopeptidase from *Bacillus thuringiensis israelensis* was found at 65 °C (Cahan, Hetzroni, Nisnevitch, & Nitzan, 2007), while the aminopeptidase from

marine *Aspergillus flavus* occurring at 45 °C (Sriram, Priyadharshini, & Sivasakthi, 2012); The optimal temperature of the recombinant leucine aminopeptidase II from *Bacillus stearothermophilus* was 55 °C (Wang et al., 2011); The aminopeptidase from psychrophile *Colwellia psychrerythraea* strain 34H, however, had a relatively low optimum temperature (19 °C) (Huston, Methe, & Deming, 2004).

The purified BLAP was most active at pH 8.0–9.0 with the optimum pH at 8.0–8.5 (Fig. 2B). The enzyme activity was negligible below pH 6.0. The stability studies revealed that the enzyme was rather stable at pH 6.0–10.0 buffer with its activity being relatively constant for at least 24 h (Fig. 2C). The enzyme lost most of its activity if it was incubated in buffers with a pH below 6.0 or above 10.0. Most of the previously reported aminopeptidases are alkaline enzyme, including those from thermophilic *Bacillus licheniformis* (pH 8.0–8.3) (Pavlova et al., 1988), *Streptomyces fradiae* var. k11 (pH 8.0) (Matsushita-Morita et al., 2010), *Bacillus thuringiensis israelensis* (pH 10) (Cahan et al., 2007) and *Bacillus stearothermophilus* (pH 8.0) (Wang et al., 2011), while aminopeptidase secreted by *Aspergillus flavus* (Sriram et al., 2012) and *Colwellia*



**Fig. 2.** Characterization of purified aminopeptidase from *Bacillus licheniformis* SWJS33 (BLAP): (A) Effect of temperature on BLAP activity and stability; (B) Effect of pH on BLAP activity; (C) Effect of pH on BLAP stability (standard deviation (SD) was not showed as it would make the main data difficult to be recognized); (D) Effect of NaCl on BLAP activity; (E) Substrate specificity of the BLAP.

psychrerythraea strain 34H (Huston et al., 2004) had a neutral optimum pH.

### 3.2.2. Effects of NaCl

The influence of NaCl in the range of 0–20% (w/v) on the BLAP activity was showed in Fig. 2D. The enzyme was most active at 10–15% NaCl where its activity was increased by 50% comparing to that without NaCl. Further increase the NaCl concentration to 20% led to a drop in enzyme activity (although such an enzyme activity was still higher than that without NaCl). These indicated that the purified BLAP had good salt-tolerance. Aminopeptidases from various sources showed large differences in salt-tolerance. The activity of prolyl aminopeptidase from *Streptomyces aureofaciens* TH-3 (TH-3PAP) in the presence of 1–3 M NaCl was 1.5–2 times higher than that without NaCl (Uraji, Arima, Uesugi, Iwabuchi, & Hatanaka, 2007). Recombinant prolyl aminopeptidase from *Aspergillus oryzae* exhibited almost the same activity at the NaCl concentrations ranging from zero to 4 M (Matsushita-Morita et al., 2010). The X-prolyl dipeptidyl aminopeptidase from *Lactobacillus helveticus* IFO3809 exhibited almost the full activity under 2 M NaCl (Kimura, Nagasawa, Fujii, & Itoh, 2002). The Lys aminopeptidase derived from the black carp muscle retained 58.5% of its initial activity at 3.2% NaCl (Li et al., 2015). The aspartyl aminopeptidase from *Aspergillus oryzae* retained 30% activity at 20% NaCl (Watanabe, Tanaka, Akagawa, Mogi, & Yamazaki, 2007). The salt-tolerant aminopeptidase especially at high levels of salt could potentially be used as an enzyme additive in salty fermented foods such as soy sauce, soy bean paste and fish sauce.

### 3.2.3. Effects of metal ions

Promotion of the enzyme activity was by  $\text{Co}^{2+}$  and  $\text{Ag}^+$  at both 1 and 5 mM, by  $\text{Zn}^{2+}$  and  $\text{Fe}^{3+}$  only at 1 mM (Table 3). Significant inhibition by  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Al}^{3+}$  and  $\text{Cd}^{2+}$  was detected at both concentrations, while only at 5 mM for  $\text{Zn}^{2+}$  and  $\text{Fe}^{3+}$ .  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Li}^+$  at both concentrations had no obvious influence on the enzyme activity. The BLAP activity was increased by 71.6% and 32.5% at 1 mM  $\text{Co}^{2+}$  and  $\text{Ag}^+$ , respectively. The activity of most aminopeptidases was found dependent on metal ions, and  $\text{Co}^{2+}$  commonly exerted positive impact on the enzyme activity, although the underlying mechanism remain unknown. For example,  $\text{Co}^{2+}$  substantially enhanced the activity of the aminopeptidase from *S. fradiae* var. k11 (by 88%) (Wu et al., 2010), aminopeptidase from *Pseudomonas aeruginosa* NJ-814 (by more than 7 times) (Wu et al., 2014), and recombinant aminopeptidase from *Bacillus stearothermophilus* (by more than 26 times) (Wang et al., 2011).

**Table 3**

Effect of different metal ions on the activity of the aminopeptidase from *Bacillus licheniformis* SWJS33.

Metal ion	Relative enzyme activity (%)		Metal ion	Relative enzyme activity (%)	
	5 mM	1 mM		5 mM	1 mM
None	100.0 ± 3.2 <sup>a</sup>	100.0 ± 2.0	$\text{Mn}^{2+}$	82.7 ± 0.4	81.0 ± 1.8
$\text{Na}^+$	101.0 ± 1.3	103.5 ± 0.2	$\text{Ca}^{2+}$	74.3 ± 1.4	81.1 ± 3.2
$\text{K}^+$	99.9 ± 1.1	97.0 ± 0.0	$\text{Cu}^{2+}$	16.3 ± 1.4	36.1 ± 1.6
$\text{Li}^+$	98.8 ± 2.4	99.6 ± 1.5	$\text{Cd}^{2+}$	12.2 ± 0.8	18.7 ± 0.4
$\text{Ag}^+$	124.1 ± 0.6	132.5 ± 3.4	$\text{Co}^{2+}$	157.8 ± 2.0	171.6 ± 4.9
$\text{Mg}^{2+}$	91.2 ± 1.3	92.6 ± 0.8	$\text{Fe}^{3+}$	33.5 ± 1.1	117.6 ± 0.9
$\text{Zn}^{2+}$	46.9 ± 3.7	106.0 ± 3.7	$\text{Al}^{3+}$	4.2 ± 1.2	72.5 ± 0.4
$\text{Fe}^{2+}$	70.6 ± 1.9	82.7 ± 0.2			

<sup>a</sup> Data were presented as means ± standard deviation (SD) for triplicate determinations.

### 3.2.4. Effects of inhibitors

Based on the residual enzyme activity (Table 4), of the compounds that were likely the inhibitors of BLAP included  $\beta$ -ME (at both concentrations), DTT (at both concentrations), SDS (only at 5 mM), Bestatin (at both 1 mM and 5 mM). These results suggest the thiol-dependent and metalloaminopeptidase nature of BLAP. Bestatin was found to inactivate leucine aminopeptidase largely and specifically i.e. reducing the enzyme activity by about 70% (Juan Ye & Ng, 2011). The varied effects of these test compounds on the aminopeptidases were reported extensively, e.g. inhibition on an aminopeptidase from chicken (*Gallus gallus*) intestine (Mane, Damle, Harikumar, Jamdar, & Gade, 2010) whilst stimulation/promotion by reducing agent  $\beta$ -ME but inhibition by *p*-hydroxymercuribenzoate on arginine and lysine aminopeptidase (Yasothornsrikul, Toneff, Hwang, & Hook, 1998).

### 3.2.5. Substrate specificity and kinetics parameters

To evaluate the substrate specificity of the purified BLAP, several commercially available substrates had been examined. Amongst the substrates tested, the enzyme appeared to prefer Leu-pNA the most, while <10% relatively activity was detected for the other examined substrates (Fig. 2E). Therefore, the BLAP prepared in this study was likely a leucine aminopeptidase. The aminopeptidase from *Bacillus licheniformis* was also found having Leu-pNA as the optimal substrate (Wu et al., 2014). In this study, the  $K_m$  and  $V_{max}$  values for the BLAP against Leu-pNA were  $1.85 \mu\text{mol mL}^{-1}$  and  $1.69 \mu\text{mol (mL min)}^{-1}$ , respectively.

### 3.3. Hydrolysis of SPI by BLAP

As an exoprotease, aminopeptidases are mainly used to improve DH and debitter. The combined use of the BLAP and a commercial enzyme (i.e. papain, Alcalase 2.4 L or Flavourzyme 500MG) facilitated 6–10% greater DH compared to the sole use of the corresponding commercial enzyme (Table 1). The obtained results also indicated that even though Flavourzyme 500MG already contains an aminopeptidase, the use of BLAP could further improve the DH. Regarding debittering, further hydrolysis by BLAP after the use of a commercial enzyme generally resulted in reduced bitter taste of SPI hydrolysates (Table 1). For example, the SPI hydrolysates catalyzed only by Alcalase 2.4 L was rather bitter (bitter intensity score: 3.6), whilst the hydrolysate obtained by Alcalase 2.4 L and BLAP had a bitter intensity score of 0.4. The further debittering effect of BLAP could not be demonstrated in the case of papain or Flavourzyme 500MG, as the hydrolysate prepared only by either commercially enzyme already exhibited little or no bitterness. These preliminary results suggested that BLAP could

**Table 4**

Effect of reductants and inhibitors on the aminopeptidase from *Bacillus licheniformis* SWJS33.

Inhibitors	Residual enzyme activity (%)	
	1 mM	5 mM
Blank	100.0 ± 2.6	100.0 ± 1.4
$\beta$ -ME <sup>a</sup>	7.7 ± 0.6	11.3 ± 1.0
DTT <sup>a</sup>	7.6 ± 1.2	11.1 ± 0.6
SDS	110 ± 4.2	23.5 ± 1.2
Bestatin	37.5 ± 1.0	12.3 ± 0.8
PMFS <sup>a</sup>	105.6 ± 2.4	103.8 ± 4.6
EDTA <sup>a</sup>	12.1 ± 1.6	2.4 ± 0.2
STI <sup>a</sup>	109.3 ± 3.5	105.8 ± 3.2
Pepstatin	102.8 ± 0.6	99.2 ± 2.8

<sup>a</sup>  $\beta$ -ME,  $\beta$ -mercaptoethanol; DTT, dithiothreitol; PMFS, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; STI, soybean trypsin inhibitor; Data were presented as means ± standard deviation (SD) for triplicate determinations.

improve the hydrolysis and debittering efficiency for vegetable protein products.

#### 4. Conclusions

The present research produced and purified an aminopeptidase from marine *Bacillus licheniformis* SWJS33. Researches on *Bacillus licheniformis* producing aminopeptidases were not that much and few about its application evaluation. Protein identification showing that BLAP matched the aminopeptidase from *Bacillus licheniformis* ATCC 14580 with 11% sequence coverage and its biochemical characteristics indicated that BLAP had some differences from the related aminopeptidases. And it was able to improve the hydrolysis and debittering efficiency for SPI hydrolysates. As a strain with GRAS, BLAP could be therefore used as an enzyme additive in salty fermented foods. Future studies should be direct towards the effects of BLAP on the free amino acid profiles and the functional properties of various protein hydrolysates.

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