Characterization of thermostable aminoacylase from *Geobacillus* sp. strain SZN

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Abstract. Aminoacylase (EC 3.5.1.14) hydrolyzes N-acetylated amino acids to produce amino acids. Although thermostable aminoacylase has been commercially produced since 2004, there was a knowledge gap in the field of understanding aminoacylase thermostability from a structural point of view. This study investigated the physical and structural properties of the purified thermostable aminoacylase SZN. The spectropolarimetry data for structural determination has indicated a gradual decrease of α -helix from 36 to 27.6%, followed by tremendous disorientation of the structure at the transition of temperatures from 60 to 70°C (27.6 to 19.5%). In contrast, the percentage of β -sheet has increased steadily over the tested temperatures. The α -helix, where notable metal binding and catalytic residues are located, was totally weakened at temperatures above 70°C, thus resulted in loss of activity. The loss of the α -helical structure could further explain drastic deterioration of activity at temperatures beyond 70°C. The activity of aminoacylase SZN was enhanced by divalent metal ions, such as Mn^{2+} and Cu^{2+} , and inhibited by detergent Triton-X-100. As a conclusion, the isolated aminoacylase SZN was characterized as a thermostable enzyme based on the α -helical structure integrity and functional stability in high temperatures. This enzyme could be used as an alternative enzyme for bioindustries in view of its activity enhancement in high temperatures and stability in various tested inhibitors.

Keywords: aminoacylase; *Geobacillus* sp.; secondary structure; thermostable enzyme; α -helix

INTRODUCTION

Global amino acid market demand was 7.5 million tons in 2016 and was expected to reach a volume of more than 10 million tons in 2020 (Research & Market, 2015). The market of amino acids is anticipated to reach USD 30.8 billion by the end of 2024. This can be attributed to various factors such as growing consumer awareness and the rising demand for healthy and functional foods. The search for new local thermostable

aminoacylase is needed in the global industry, especially in the industry that focusing on productions of amino acids. Enzymes from extremophiles are more attractive compared to other common enzymes because they can perform industrial processes even under harsh conditions, under which common proteins are completely denatured. In common cells, enzymes will be denatured and unfolded when the

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temperature increased beyond a specific point. The rate of molecular movement and reaction increased when the temperature increased, but at the same time, there is a progressive inactivation caused by denaturation of the enzyme protein (Robinson, 2015). This is because the three-dimensional structures of proteins are damaged resulted from breakage of hydrogen and disulphide bonds that are responsible to maintain the enzyme structure. Protein unfolding may cause the enzyme unable to catalyze the reactions appropriately because it had lost its fundamental shape which is associated with its specific affinity and its substrate binding site. This work was emphasized on studying thermostable aminoacylase SZN secondary structure stability and its distortion at high temperatures. Furthermore, providing a better understanding of the adaptation of thermostable aminoacylase at high temperatures, from the structural point of view is our subsequent objective.

MATERIALS AND METHODS

Source of bacterium

The water samples (59°C) were collected from Ulu Slim Hot spring in Perak (GPS coordinate: 3.8988° N, 101.4979° E). A thermophile from *Geobacillus* species, identified as a new strain known as strain SZN, was isolated (Adenan *et al.*, 2018), and the aminoacylase extracted from the bacteria cells was applied throughout this work to study its biochemical properties and characteristics.

Aminoacylase assay

Aminoacylase activity was determined in 0.5 mL assay mixtures containing 0.2 mL of aminoacylase enzyme in 50 mM Tris-HCl (pH7.5) and 0.3 mL of 10 mM of N-acetyl-L-methionine (NAMET) substrate. The 0.5 mL assay mixtures were shaken for 30 min with shaking at 200 rpm, 60°C. The reaction was stopped using 0.5 mL of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation method with 4000 rpm for 1 min. Then, 1 mL supernatant was mixed with 0.25 mL of 3% ninhydrin solution and 0.25 mL of 250 mM acetic-cyanide (pH5.0) buffer. The mixture was boiled for 15 min and cooled to

ambient temperature by the addition of 1.5 mL of 50% (v/v) isopropanol. The liberated L-methionine was measured using Biomate 3S UV visible spectrophotometer (Thermo Fisher Scientific, USA) at absorbance wavelength of 570 nm (A_{570}). L-methionine amount was determined from standard curve that was prepared according to Moore and Stein (1948).

Preparation of cell extract

The crude cell extract for the assay was prepared from 1 L of the culture growth at 60°C overnight in LB broth. The supernatant of the culture was removed by centrifugation at 12,000 rpm and 4°C for 10 min. The precipitated cells were washed with 50 mM Tris-HCl (pH7.5). The cells were then resuspended in 50 mL of 50 mM Tris-HCl (pH7.5) and disrupted by using ultrasonic disruptor UD-200 at 60 W for 4 min intermittently in an ice bath. Cell extract was obtained by centrifugation at 12,000 rpm for 10 min at 4°C. The cell extract was subjected to aminoacylase assay at a different range of temperatures (30, 40, 50, 60, 70, 80, and 90°C) for characterization, and at 60°C for standard assay.

Aminoacylase purification steps

Ion exchange chromatography (IEX)

A flow rate of 0.7 mL/min was used during crude enzyme sample injection into XK16 column containing 10 mL of DEAE-Sephacrose Fast Flow resin (GE Healthcare, Sweden). Prior to sample injection, the resin was pre-equilibrated with 50 mM Tris-HCl (pH 7.5), as the binding buffer. The column was then washed with 10 column volumes (cv) of the binding buffer followed by ascending linear gradient elution with 25 cv of NaCl varying from 0 to 1.0 M. Eluate size was set at 2.0 mL per fraction, in which all fractions were subjected to aminoacylase assay and dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to observe the protein size and enzyme purity. Fractions with high enzyme activity and less contaminants (unwanted proteins) were pooled and subjected to gel filtration purification step by using Sephacryl (S-200) HR resin (GE Healthcare, Sweden).

Gel filtration chromatography

The fractions from IEX with high aminoacylase activity were pooled and concentrated up to 0.8%

of the total column volume (80 mL resin) by using molecular weight cut-off spin column (10.0 kDa) (Milipore, USA) at 10,000 rpm, 4°C. The concentrated sample was then loaded into S-200 column with flow rate 0.2 mL/min using the peristaltic pump. The sample was eluted using 50 mM Tris-HCl (pH 7.5) buffer. Eluate size was set at 1.0 mL per fraction, in which all fractions were subjected to aminoacylase assay and SDS-PAGE to observe the protein size and purity. The protein content was determined by the Bradford (1976) method using the Bio-Rad assay reagent and Bovine serum albumin as the standard. The protein concentration from each step of purification was monitored by measuring their absorbance at 280 nm.

Thermal characterization of aminoacylase SZN

Assessment of aminoacylase secondary structure stability by circular dichroism (cd)

Mechanisms of protein stability in various temperatures were analyzed using JASCO J-810 Circular Dichroism Spectropolarimeter (Tokyo, Japan). By utilizing several parameters pre-programmed in Spectra Manager software (JASCO, Japan), the generated CD spectra can be used for analysis in secondary structure changes as well as thermal denaturation. Secondary structures estimation was determined by CD spectra obtained at wavelength 190-240 nm. The enzyme sample was transferred into a completely closed 10.0 mm cell containing 1.0 mg/mL native enzyme solutions, which was heated at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100°C at 1°C/min heating rate. Data pitch, bandwidth, response and scanning speed were programmed as 0.1 degree, 1 nm, 8 seconds and 1 degree per minute.

Thermal denaturation analysis

The measurement of thermal denaturation (T_m) was acquired from ellipticity at 222 nm of a completely closed 10.0 mm cell containing 1.0 mg/mL native enzyme solutions, which was heated at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100°C with 1°C/min heating rate. Data pitch, bandwidth, response, and scanning speed were programmed as 0.1 degree, 1 nm, 8 seconds and 1 degree per minute, respectively. T_m is determined at the midpoint of sigmoidal melting curves.

Effect of temperature on aminoacylase activity

Investigation on hydrolysis of *N*-acetyl-L-Methionine (NAME^T) by aminoacylase SZN at temperatures 30, 40, 50, 60, 70, 80, and 90°C was conducted at a shaking rate of 200 rpm for 30 min. Aminoacylase activities that exhibited in various temperatures were compared in relative to activity at 60°C that served as control (100%).

Effect of temperature on enzyme stability

Pre-incubation of the enzyme in temperatures of 5, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C for 30 min in 200 rpm water bath shaker was carried out prior to aminoacylase assay, in order to determine the enzyme temperature stability. Activity at 5°C was selected as a standard (100%).

Effect of exposure time at 60°C on aminoacylase SZN

The half-life of the enzyme was examined by pre-incubating the protein at 60°C from 0-36 hours in a water bath shaker. Samples were removed at intervals of 4 h for measurement of activity, in which the activity at 0 min is a control (100%) of the experiment.

Other characterization of thermostable aminoacylase SZN

Effect of pH on aminoacylase activity and stability

The optimum pH of aminoacylase SZN activity was determined by the enzymatic hydrolysis of NAME^T in 50 mM of different buffers. The buffering systems used were sodium acetate (pH 4, pH 5, pH 6), potassium phosphate (pH 6, pH 7, pH 8), Tris-Cl (pH 8, pH 9), sodium tetraborate (pH 9, pH 10, pH 11), and sodium hydrogen phosphate (pH 11, pH 12). The substrate was resuspended with buffers at various pHs with ratio 1:3 (v/v). For pH stability test, aminoacylase was pre-incubated with buffers of tested pHs at ratio of 1:3 (v/v) at 60°C and 200 rpm for 30 min followed by enzyme assay. Enzyme activity in 50 mM phosphate buffer (pH 8.0) was further chosen as control for this experiment.

Effect of metal ions on aminoacylase activity

In this study, the enzyme was pre-treated with 0, 1.0 and 10.0 mM of chloride (Cl⁻) metal ions, such as Na⁺, K⁺, Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ for 30 min at 60°C before subjected to enzyme assay.

Effect of inhibitors on aminoacylase activity

Denaturing and reducing agents, such as β -mercapthoethanol, Triton-X-100, Tween 20, sodium dodecyl sulphate (SDS) and dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), were used at final concentrations of 5.0 and 10.0 mM to treat the protein at 60°C for 30 min. Inhibitor-free mixture was considered to be the negative control in the colorimetric assay.

RESULTS***Purification of aminoacylase from Geobacillus sp. strain SZN***

Aminoacylase SZN was successfully purified using Ion Exchange DEAE Sepharose Fast Flow chromatography (IEX) (Figure 1) and Sephacryl S-200 Gel Filtration chromatography (GF) (Figure 2). The purification results are summarized in Table 1. The enzyme purity was obtained at 14.04 fold, compared to crude, with final yield at 16.53% utilizing only two steps of chromatography, caused its specific activity of the purified enzyme increased to 66.4 U/mg at the final step compared to specific activity from crude

which was 4.73 U/mg. The protein sample from each purification step was analyzed by SDS-PAGE and the purity of aminoacylase SZN from each purification step can be observed in Figure 3, shows that the purified protein was resolved almost as single band at ~42 kDa.

Characterization of aminoacylase strain SZN***Assessment of aminoacylase secondary structure stability by CD***

The protein stability in various temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C) was analyzed using JASCO J-810 CD Spectropolarimeter (Figure 4) and estimated the percentage of α -helix and β -sheet secondary structures by using Perry Freeshell server (Raussens *et al.*, 2003) (Table 2). The results showed that the α -helix was still maintained its original structure even heated up to 60°C. At 10-40°C, the percentages of α -helix and β -sheet were maintained in a range of 34-37% and 16-19%, respectively. The α -helix was appeared at 28-32% and β -sheet at 19-23% when temperatures increased to 50-60°C. At 100°C, aminoacylase SZN significantly lost its α -helical conformation and changed to β -sheet as each of the conformation was recorded at 12% and 33%, respectively.

Figure 1. Ion-Exchange Column Chromatography of cell extracts from *Geobacillus* strain SZN by using DEAE Sepharose Fast Flow column. Each step in the protein purification protocol: FT, flowthrough; W, washing unbound; E, elution and WAE, wash after elution, is separated by dashed lines. The gradient blue dashed line indicates the gradient concentration of NaCl in elution steps. Absorbance at 280 nm and aminoacylase activity was indicated by (■) and (▲), respectively.

Table 1. Purification table of aminoacylase from *Geobacillus* strain SZN by Ion Exchange and Gel Filtration S-200 purification steps.

Purification step	Total activity (U)	Total protein content (mg)	Specific activity (U/mg)	Enzyme purity (fold)	Yield (%)
Crude	2130	450	4.73	1	100
IEX*	635	32.5	19.5	4.12	29.8
S-200 ⁺	352	5.3	66.4	14.04	16.53

*Ion Exchange Chromatography

⁺Gel Filtration Chromatography **Figure 2.** Sephacryl S-200 Gel Filtration Chromatography of pooled fractions of protein from IEX. Absorbance at 280 nm and aminoacylase activity was indicated by (▲) and (■), respectively.*Thermal denaturation analysis*

Melting point analysis is an assessment of the dissociation-characteristics of protein secondary structure during heating. As the temperature raised, the helical structures begin to dissociate, leading to a rise in the absorbance intensity at 222 nm. The temperature at which 50% of protein denatured is known as the melting point. The result was found to be parallel with its aminoacylase activity optimal temperature, where the enzyme started to lose 50% of the activity at above 80°C (Figure 5).

Effect of temperatures on aminoacylase activity and stability

The aminoacylase SZN relative activity was increased gradually from 37.6% to 100% at the temperatures from 30-60°C and decreased from 66% to 27% when increased temperatures from

70 to 90°C. The aminoacylase SZN optimum temperature was detected at 60°C (Supplementary Material 1a). Stability of aminoacylase SZN from 5°C to 70°C was supported by its relative aminoacylase activity in Supplementary Material 1b in which a significant lost (more than 50%) was observed when the temperature increased to 80°C and above. At 70°C, the stability of aminoacylase SZN was 73% (Supplementary Material 1b).

Effect of exposure times at 60°C on aminoacylase SZN

An in-depth investigation on the half-life of aminoacylase SZN was carried out at 60°C. The half-life for the enzyme was deduced at 16 h (Supplementary Material 1c). Relative activity of aminoacylase SZN was reduced from 47% to 28% when incubated at 20, 24, 28 and 32 h.

Table 2. Estimated percentage of secondary structures by using Perry Freeshell Software when protein was incubated at various temperatures.

Secondary structure (%)	Temperature (°C)									
	10	20	30	40	50	60	70	80	90	100
α -helix	36.0	37.1	33.8	35.0	31.8	27.6	19.5	16.4	15.8	12.4
β -sheet	17.2	16.9	18.9	16.3	19.3	23.3	27.8	27.7	31.5	33.1
Random	31.0	30.5	31.5	31.5	32.2	33.2	35.7	36.2	36.5	37.6

Figure 3. SDS-PAGE of aminoacylase SZN purification. Line 1, 2, 3, and 4 at top of the gel are samples from Unstained Protein Molecular Weight Marker (Fermentas, Canada), crude enzymes, IEX, and gel filtration, respectively. Components with their respective sizes (kDa), found in standard protein marker are: β -galactosidase, 116.0; bovine serum albumin, 66.2; ovalbumin, 45.0; lactate dehydrogenase, 35.0; REase Bsp98I, 25.0; β -lactoglobulin, 18.4 and lysozyme, 14.4. **Figure 4.** Far UV spectra of aminoacylase at various incubation temperatures. **Figure 5.** CD spectra for determination of thermal denaturation point of aminoacylase. A curve that indicates the transition state of the α -helical structure was formed when the temperature raised from 70-80°C. The temperature of 80°C at approximate midpoint of the sigmoidal curve has defined as thermal denaturation point of aminoacylase.

Other characterization of thermostable aminoacylase SZN

Effect of pH on aminoacylase activity and stability

In Supplementary Material 2a, the relative activity appeared low at pH 4 (22.4%) and accelerated to optimum activity (100%) when pH increased to 8. However, the relative activity was significantly dropped after pH 9 where the relative activity lost almost 80% at pH 12, indicating that aminoacylase SZN works more efficiently at neutral pH environment. To test its stability in broad range of pH, aminoacylase SZN was added to different buffers with different pHs. At pH 4, the relative activity of the enzyme was 59%. As the pH increased from 5 to 9, the relative activity was restored to 81% at pH 9 and 100% at pH 8. Further increase of pH 10 and pH 11 had exhibited declination of the activity until 46% and totally lost at pH 12.

Effect of metal ions on aminoacylase activity

Aminoacylase is a metalloenzyme, from M20 family that contained with at least one zinc ion for

each subunit enzyme assisting in catalytic process. Treatment of aminoacylase SZN with 1 mM and 10 mM Zn^{2+} did inhibit the activity, but strongly enhanced the thermostability, suggesting that additional Zn^{2+} may occur at the second metal binding site and induce a possible alteration in enzyme conformation to improve thermostability (Tanimoto *et al.*, 2008). Our results showed that 1 mM metal ion reduced the aminoacylase activity, whereas the 10 mM Cu^{2+} , Mg^{2+} , Mn^{2+} and Na^{+} increased the aminoacylase activity to 107, 153, 160 and 136%, respectively (Supplementary Material 2c). We postulate that increased concentration of divalent ions, such as Mg^{2+} and Mn^{2+} , may result in enhancement of activity, probably due to increased stability in enzyme-substrate and product complexes (Knappe *et al.*, 2017; Rahman *et al.*, 2011).

Effect of reducing and denaturing agents on aminoacylase activity

Tween 20 and Triton X-100 demonstrated inhibition effects at concentrations of 5 and 10 mM, where the relative activity was decreased from 90.7 to 85.8% and 73 to 66.4%, respectively. At 10 mM, inhibition effects occurred in enzyme assay containing PMSF and SDS with its relative activity decreased to 91.6% and 85.4%, respectively. Treatment of aminoacylase SZN with EDTA at 5 and 10 mM did not inhibit the activity due to weak affinity of EDTA to Zn^{2+} (Tanimoto *et al.*, 2008). This observation was supported by Sakanyan *et al.* (1993) who reported that the activity of aminoacylase from *B. stearothermophilus* can only be diminished at 50 mM EDTA.

DISCUSSION

Aminoacylase enzyme is classified into the M20A family of metallopetidases, in which zinc is needed as an essential metal for catalytic activity (Lindner *et al.*, 2003). Based on our reported data regarding amino acid sequence alignment of aminoacylase SZN with *Pyrococcus horikoshii* and several other species, the conserved regions of metal binding residues (Glu139 and Glu140), and catalytic residue (Arg261), were located at α -helical structure (Adenan *et al.*, 2018). Similarly,

Tanimoto and colleagues (2008) also revealed that the positions of metal binding site residues for *Pyrococcus horikoshii* were found at His106, Glu139, Glu140 and His164 and catalytic residues at H198 and A260. These findings are essential to understand the aminoacylase thermostability at structural point of view, as significant loss of α -helical structure was observed at temperatures beyond 70°C, and thus, preventing the metal binding and catalytic residues to be folded into its catalytically active conformation. Moreover, the melting point of aminoacylase SZN was recorded at 80°C (Figure 5), which was in agreement with the observation obtained from Supplementary Material 1a.

Thermostability of aminoacylase SZN may be assisted by existence of metal ion as ligand during catalysis reaction. This was supported by finding of α -helix at the predicted catalytic and metal binding sites which is known to contribute to high portion of hydrogen bonds. More than 50% α -helix structure was predicted by using SOPMA (Deleage & Geourjon, 1995) tool in ExPASy for computation of secondary structures α -helix, β -strand, and random coils, that is inferred to play a crucial role in maintaining the active conformation (Adenan *et al.*, 2018). Native and active proteins are held together by a delicate balance of non-covalent forces among the secondary structures (hydrogen bonds, ion pairs, and hydrophobic and Van der Waals interactions). When high temperatures disrupt these non-covalent interactions, proteins become unfold and inactive.

The inhibitory effect of divalent ions, such as Zn^{2+} and Ni^{2+} , was clearly observed on aminoacylase SZN. These ions have been reported to inhibit the proteolytic activities of elastase strain K (Rahman *et al.*, 2011), ME-4 (Cheng *et al.*, 2009) and PseA (Gupta *et al.*, 2005) which also happened to be metalloproteases. The highest half-life of aminoacylase was reported by Hollingsworth (2002), who found that the stability of aminoacylase from *Thermococcus litoralis* was 25 h at 70°C. Aminoacylase SZN can withstand a broad range of inhibitors and did not exhibit significant effects on its stability in detergent especially Tween 20. Thus, the aminoacylase SZN can be potentially applied in industries, including pharmaceutical and food and beverage industries. Similar characteristics were reported for protease

from *Bacillus horikoshii*, whereby the enzyme has shown excellent potential as an additive in feed and detergent industries due to its stability in SDS, oxidizing agents and organic solvents (Joo & Choi, 2012). Characterization on aminoacylase SZN activity and stability in different pHs has shown that the enzyme optimum activity is ranging from pH 6-8, similar to those optimum pHs for *P. furiosus*, *Streptomyces mobaraensis* and *Deinococcus Radiodurans* R1 at pH 6.5 (Story *et al.*, 2001), pH 7.5 (Koreishi *et al.*, 2009) and pH 8 (Lin *et al.*, 2007), respectively.

CONCLUSION

The wild-type aminoacylase SZN was purified in two steps, namely ion exchange and gel filtration chromatography, with approximately 14-fold of specific activity compared to crude sample (Table 1). From this work, the structural properties of thermostable aminoacylase from *Geobacillus* sp. have unveiled the protein folding and unfolding mechanism involving α -helical structure in high temperatures. Thus, the findings reported in this communication provide insights into characteristics, adaptation mechanism and behaviour of enzymes in various temperatures, especially at the estimated α -helical structure of aminoacylase SZN active site, which is known to assist the enzyme stability at high temperatures.

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