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Purification and Partial Characterization of α-Amylase Produced by a Thermo-Halophilic Bacterium Isolate PLS 75

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Abstract

Bio-based industries require stable enzymes in a broad range of environmental conditions. Extremophiles have attracted more interests as the source of such enzymes, one of which is α-amylase. This study aimed to purify and characterize α-amylase produced by a thermo-halophilic bacterium PLS 75 isolated from underwater fumaroles. Ammonium sulfate precipitation results showed that the highest specific α-amylase activity (21.7 U/mg) obtained at 40-60% saturation level, with a purity of 7.7-fold of the crude extract with 16.2% yield. Further purification using DEAE Sepharose column chromatography increased the enzyme purity 11.1-fold of the crude extract with 7.1% yield. Specific activity after column chromatography purification was 31.3 U/mg. The pure enzyme had a low molecular weight of 14 kDa. The enzyme showed the highest activity at 80 °C and pH 5. The activity increased to 126% when in methanol, while decreased when in ethyl acetate and chloroform. The characteristics of α-amylase with low molecular weight, which was active in acidic condition, stable in polar and non-polar solvents, may be used for for specific industrial needs.

How to Cite

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INTRODUCTION

Enzymatic bioprocess in industries has continued to develop in the last two decades because enzyme-catalyzed reactions are specific, selective and easily controlled. The products are also easily purified and the cost of waste processing is cheaper (Schmid *et al.*, 2002). One of important enzymes used in industries is α-amylase, which contributes to 30% of the global enzyme market (Sivaramakrishnan *et al.*, 2006). α-amylases catalyze the hydrolysis of α-1,4 glycosidic bonds in starch molecules and produce dextrins, oligosaccharides and glucose with various chain lengths (Kuriki and Imanaka, 1999). α-amylase has a very broad spectrum of applications, including starch, bioethanol, food, detergent, textile and paper processing industries (Rana *et al.*, 2013). The enzymes can be produced from various sources such as plants, animals and microorganisms, but bacteria remain the main source of the enzymes (Sivaramakrishnan *et al.*, 2006).

Industrial processes sometimes require extreme temperature and pH, thus the use of extremozymes that are active in extreme conditions is preferred (Elleuche *et al.*, 2014). While the exploration of new microorganisms from extreme environments to produce more stable enzymes has been done for many years, studies to find new α-amylases continue being carried out (Krüger *et al.*, 2018). Researches to obtain new α-amylase with better stability are often done by isolating extremophiles from various sources, such as hot spring (Sudan *et al.*, 2018; Wu *et al.*, 2018), deep sea (Jiang *et al.*, 2015), and even honey (Du *et al.*, 2018). Various studies have also been done to improve the stability of the enzyme through genetic engineering, immobilization, chemical modification and protein engineering (Dey *et al.*, 2016).

Owing to the importance of finding new variants of the enzyme, here we purified and characterized α-amylase produced by an extremophilic bacterial isolate from underwater fumaroles in Pria Laot Sabang area, Weh Island, Indonesia. The isolate was thought to have unique metabolisms and produce stable metabolites, as it was isolated from an environment with high temperature and salt concentration. Purification was carried out by ammonium sulfate precipitation, followed by anion exchange chromatography, and checked for its molecular weight. The activity of the pure enzyme was characterized at various temperatures, pH values and organic solvents. The results of this study may be used as the basis of further research to make the enzyme applicable on industrial scale.

METHODS

Microorganism

The microorganism used in this study was a bacterial isolate, previously isolated from a shallow underwater fumarole in the Pria Laot Sabang area, Weh Island, Indonesia, collection number 75 (hereinafter referred to as PLS 75). The isolate was a stock culture of the Biochemistry Laboratory of the Faculty of Mathematics and Natural Sciences Syiah Kuala University. PLS 75 was initially screened on Thermus medium and stored in glycerol. Phenotypic identification showed that PLS 75 belongs to the genera of *Bacillus*.

Production of α-amilase

α-amylase from PLS 75 was produced in a minimum medium with a composition of 0.2% NaCl, 0.4% yeast extract, 0.8% peptone, 0.25% glucose, and dissolved in sterile sea water. Also, 1% starch was added as the inducer. Incubation was carried out at 60 °C, 150 rpm for 30 hours. The crude enzyme was obtained from the supernatant by centrifugation of the fermentation broth at 10000×g for 20 minutes.

Determination of α-amylase activity and protein concentration

The activity of α-amylase was determined using the Dinitrosalysylic Acid method, referring to the standard procedure (Miller, 1959). The method measured the amount of reducing sugars released from 1% starch due to the enzyme activity. Calculation was based on a standard curve of glucose (1-5 mM). One unit (U) of α-amylase activity is defined as the amount of enzyme that produces 1 μmol of glucose per minute under reaction conditions. Determination of protein concentrations was carried out by the Bradford method (Bradford, 1976), using a standard curve of BSA (100-1000 mg/mL). The protein concentrations were used for the determination of the α-amylase specific activity, which is the ratio of α-amylase activity to protein concentration.

Purification of α-amilase

The crude enzyme in the supernatant was precipitated using ammonium sulfate of various concentrations (hereinafter referred to as Fraction), followed by DEAE Sepharose purification. Ammonium sulfate was added to the supernatant at cold temperature to reach saturation levels of 0-20%, 20-40%, 40-60%, 60-80% and 80-100% (Scope, 1982). The mixture was left overnight at

cold temperature and subsequently centrifuged at 10000×g for 15 minutes. The precipitate was then dialyzed using 20mM tris-HCl buffer pH 8, which was replaced periodically until ammonium sulfate was not detected in the buffer solution with the addition of 1mL $BaCl_2$ 0.5M and 1mL 0.1N HCl. Activities and protein concentrations of α-amylase in each fraction were determined as described below. The fraction with the highest specific activity was purified further. All fractions were subjected to SDS PAGE.

Further purification was carried out using DEAE Sepharose fast flow anion exchange chromatography to the fraction with the highest α-amylase specific activity. Before separation, the column was equilibrated with 0.01 M tris-HCl buffer pH 7. Approximately 5 mL of enzyme sample was put on to the column and eluted using 0.01 M tris-HCl buffer pH 7.0 containing 0.15 M; 0.3 M; 0.6 M and 1.0 M NaCl salt gradient. Elution was conducted with a flow rate of 0.9 mL/minute and each 2 mL eluent was collected as a fraction. Activity and protein concentration of the α-amylase in each fraction was then determined.

SDS-PAGE and zymography

Crude extract, ammonium sulfate precipite and DEAE Sepharose purification results with the highest specific activity were subjected to SDS-PAGE. Concentrations of separating gel and stacking gel were 12% and 5% (w/v), respectively. Electrophoresis was carried out in 0.05M phosphate buffer pH 7, for 2 hours at 120 volts and 20 mA. After separation, the gel was stained with Commasie Brilliant Blue R250 or Pierce® Silver Staining kit.

The enzyme with the highest specific activity after DEAE Sepharose purification was also subjected to zymography by renaturating it (without staining) using 2.5% Triton-X in 0.05M phosphate buffer pH 7.0 for 1 hour. The gel was then soaked in 1% starch solution in 20 mM phosphate buffer pH 7.0 for 30 minutes at optimum temperature of α-amylase activity. The gel was then immersed in lugol solution for 15 minutes. The molecular weight of α-amylase was determined by comparing the clear band on the gel to the markers.

Characterisation of α-amilase activity

Characterization of α-amylase activity was carried out at various temperature, pH and organic solvents. The effect of temperature on α-amylase activity was studied at 60, 70 and 80 °C, in phosphate buffer pH 7. The effect of pH on protease activity was carried out at pH 5 (sodium acetate 0.2M), pH 7 (phosphate 0.2M) and pH 9 (glycine-NaOH 0.2M), at optimum temperature. Meanwhile, the effect of organic solvents on protease activity was carried out by mixing methanol, ethyl acetate, chloroform and n-hexane with the substrate to give 50% (v/v) solution, and incubated at optimum temperature and pH.

RESULTS AND DISCUSSION

Ammonium sulfat precipitation

Purification of α-amylase is often done by a combination of several methods. The initial step was mostly done by salt precipitation, followed by chromatograpy methods (Wu *et al.*, 2018; Du *et al.*, 2018; Sudan *et al.*, 2018). Of the five fractions after ammonium sulfate precipitation, Fraction 2 produced the highest activity (42.4 U/mL) and protein concentration (2.09 mg/mL). However, the highest specific activity (21.7 U/mL) was observed in Fraction 3 (Table 1).

The concentration of ammonium sulfate needed to produce optimum α-amylase specific activity varies. Precipitation of α-amylase is commonly carried out at concentrations of 60-80% (Al-Quadan *et al.*, 2011; Sudan *et al.*, 2018), although there is an α-amylase precipitated in only 30% concentration of salt (Wu *et al.*, 2018).

Table 1. Activity of α-amylase and protein concentration after ammonium sulfate precipitation

Ammonium sulfate precipitation	Activity (U/mL)	Protein conc. (mg/mL)	Specific activity (U/mg)
Crude extract	5.0	1.79	2.8
Fraction 1 (0-20%)	24.3	1.53	15.9
Fraction 2 (20-40%)	42.4	2.09	20.3
Fraction 3 (40-60%)	27.2	1.25	21.7
Fraction 4 (60-80%)	23.7	1.19	19.9
Fraction 5 (80-100%)	20.8	1.04	20.1

The surface of proteins from thermophilic bacteria contains plenty of charged residues. Salts thus provide screening effects on the solubility of the proteins. The solubility of some proteins will increase at low salt concentrations (salting in), but precipitate with the increase of the concentrations (salting out). Salt concentrations threshold for salting-in or salting out differ amongst proteins, depending on the type and the amount of amino acids on the protein surface (Hiteshi and Gupta, 2014). This explains why, despite having

the highest activity, Fraction 2 has a lower specific activity compared to Fraction 3.

Although the difference of α-amylase specific activity in Fractions 2-5 was insignificant, Fraction 3 was chosen for further purification using ion exchange chromatography. This selection was supported by SDS-PAGE result, as the crude extract, Fraction 1, and Fraction 5 showed several bands with very low concentrations. Fraction 2 and Fraction 4 also produced several bands but with greater concentrations. Meanwhile, Fraction 3 showed one distinct band at 10-15 kDa, but with fewer proteins of other molecular weights (Figure 1, lane 5).

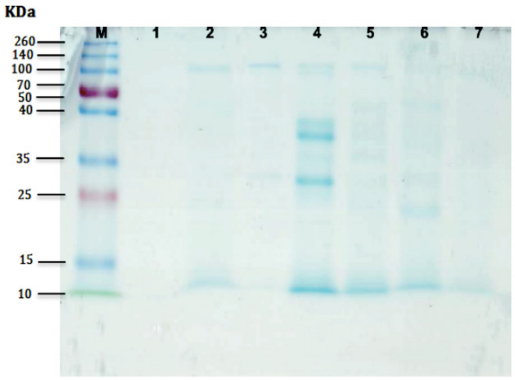

Figure 1. Results of SDS-PAGE from ammonium sulfate precipitation after dialysis. M = Marker, 1 = empty, 2 = crude extract, 3 = Fraction 0-20%, 4 = Fraction 20-40%, 5 = Fraction 40-60%, 6 = Fraction 60-80% and 7 = Fraction 80-100%. Gel staining was done using CBB R250

Purification with DEAE Sepharose

The fractions after DEAE sepharose purification did not show a significant difference in α-amylase activities. Except for flow through, activity ranged from 20.1 - 35.7 U/mL. Some adjacent fractions with similar activity values in the same salt gradient were combined and the activity was determined. Combined fractions were labelled A - I and Fraction H (single fraction 41) showed the highest activity of 35.7 U/mg (Table 2). Fraction H was then used to characterize the α-amylase.

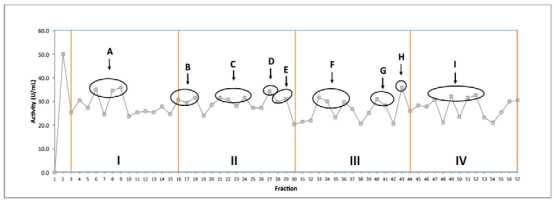

Figure 2. Elution profile of α-amylase from Fraction 3 purified by DEAE Sepharose fast flow. Elution was carried out at a flow rate of 0.9 mL/min using 0.01 M tris-HCl buffer pH 7.0 containing NaCl 0.15 M (I), 0.3 M (II), 0.6 M (III) and 1.0 M (IV).

Evaluation of the purification steps

The comparison of the α-amylase from all purification steps showed that precipitation using ammonium sulfate (40-60%) gave a purity of 7.7-fold of the crude extract, with a yield of 16.2%. Further purification using DEAE sepharose (Fraction H) produced an enzyme with a purity of 11.1-fold of the crude extract, but with only 7.1% yield (Table 3).

The purity and yield of α-amylases from various microorganisms varies despite employing similar purification steps. Purification of α-amylase from *G. thermoleovoran* using Q-Sepharose FF anion exchange chromatography give a purity of 8.3-fold of the crude extract with 9.6% yield (Finore *et al.*, 2011). Meanwhile, α-amylase from *Nesterenkonia* sp. strain F purified with similar methods has a purity of 5.77-fold with 8%

Table 2. The α-amylase activity and protein concentrations after purification with DEAE Sepharose fast flow

Fractions	Combined fraction	NaCl gradient (M)	Activity (U/mL)	Protein conc. (mg/mL)	Specific activity (U/mg)
F4, F6, F7	A	0.15	35.1	1.33	26.4
F14, F15, F16	B	0.3	30.6	1.22	25.1
F19, F20, F22	C	0.3	31.1	1.22	25.5
F25	D	0.3	34.1	1.19	28.6
F26, F27	E	0.6	30.4	1.19	25.5
F31, F32, F34	F	0.6	30.4	1.18	25.9
F38, F39	G	0.6	29.5	1.14	25.8
F41	H	0.6	35.7	1.14	31.3
F45, F47, F49, F50	I	1	31.6	1.20	26.4

Table 3. Comparison of α-amylase and protein concentration of different purification steps

Purification	Vol. (mL)	Protein (mg/mL)	Total protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield %
Crude extract	200	1.79	357.2	5.0	1009	2.8	1.0	100
Fraction 3 (40-60%)	6	1.25	7.5	27.2	163.3	21.7	7.7	16.2
DEAE Sepharose (F41)	2	1.14	2.28	35.7	71.5	31.3	11.1	7.1

yield (Shafiei *et al.*, 2010). Occasionally, a combination of chromatography methods is needed to increase the enzyme purity. For example, combination of exclusion gel (Sephadex G100) and ion exchange (DEAE Sepharose) chromatography increases the purity of about 30% (Al-Quadan *et al.*, 2101), while the combination of Q Sepharose and Superdex 75PG increases the purity by eight folds (Sudan *et al.*, 2018).

This, again, may occur due to differences in the interactions of the purified protein with the substance used for purification, in this case, ammonium sulfate and column matrix. The structure of proteins is largely determined by the amino acids constituting them. The difference in the type and the amount of amino acids on the protein surface will certainly influence the interaction with the purifiying materials.

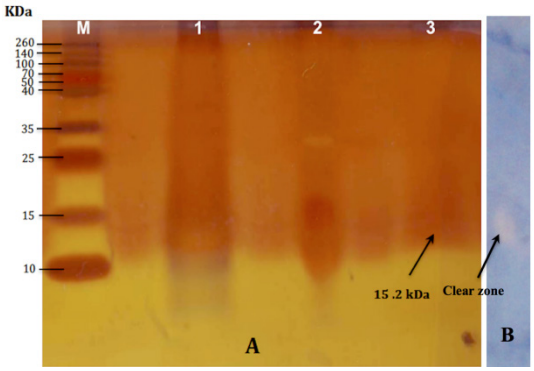

Figure 3. (A) SDS-PAGE results of (1) crude extract, (2) Fraction 3 ammonium sulfate 40-60%, and (3) DEAE sepaharose. (B) Zymographic result. Both gels use the same marker (M). Gel A was silver stained and Gel B was stained using CBB R250.

Molecular weight of α-amilase from PLS 75

SDS PAGE results indicated that Fraction H had a protein band of about 14 kDa. Zymography produced a clear single zone around the same molecular weight, indicating the presence of α-amylase activity, and was a monomeric protein (Figure 3). A majority of α-amylases are reported to have molecular weight greater than 20 kDa (Mehta and Satyanarayana, 2016), and the enzyme with low molecular weight is rare. The relatively low molecular weight of α-amylase from PLS 75 is attractive, as it could simplify the immobilization and protein modification process for better enzyme stability and performance.

Effect of temperature, pH and organic solvent

The effect of temperature on the activity showed that α-amylase from PLS 75 was thermostable, having notable activity in the range of 60 - 80 °C with activity values of 24.5 - 31.7 U/mL (Figure 4A). Maximum activity was shown at 80 °C (30.7 U/mL). Activity above 80 °C was not measured, so that the optimum temperature could not be confirmed. Activity at 60 °C was still around 80% of that at 80 °C.

Optimum temperature for enzyme activity is closely related to the environmental conditions from which the producing microorganisms are isolated. For example, the activity of α-amylase from *Geobacillus* sp. isolated from the sub-seafloor sediments is optimum at 60-65 °C (Jiang *et al.*, 2015). Meanwhile, α-amylase from *B. mojovensis* isolated from hot spring has optimum activity at 80-90 °C (Sudan *et al.*, 2018; Wu *et al.*, 2018).

The effect of pH on the α-amylase activity was tested at three pH values representing acidic (pH 5), neutral (pH 7) and alkaline (pH 9) conditions. The highest α-amylase activity was observed at pH 5 (33.6 U/mL). Activity at pH 7 was about 9% lower than that at pH 5, while activity at pH 9 (20.0 U/mL) was still around 63% of the maximum activity (Figure 4B). The low activity of α-amylase from PLS 75 at high pH indicates that alkaline condition causes changes in the ionic interactions that affect the protein structure, thus the catalytic reaction cannot occur properly.

Acidic α-amylase is not uncommon and several studies have reported α-amylases that are active in an acidic condition. For example, the optimum activity of α-amylase from *Bacillus* strain HUTBS26 is observed at pH 4.4 (Al-Quadan *et al.,* 2011), from *Alicylobacillus* sp. A4 is at pH 4.2

(Bai *et al.*, 2011), from *G. thermoleovoran* is at pH 5.6 (Finore *et al.*, 2011), and from *B. licheniformis* B4-423 is at pH 5.0 (Wu *et al.*, 2018).

Acidic α-amylase is widely used in starch processing industries because of their thermo-acidophilic properties and high conversion rate (Homaei *et al.*, 2015). Starch processing is carried out in several steps, including liquefaction and saccharification. Liquefaction process is done to reduce starch viscosity and usually uses amylases that are active at high temperatures and neutral pH. Meanwhile, saccharification step is done to release reducing sugars and is carried out by enzymatic reaction at pH 4.2-4.5. Therefore, the pH of the liquefied starch must be reduced before saccharification process starts. In production perspective, this step is very time consuming and increases costs (Sharma and Satyanarayana, 2013). Therefore, α-amylase from PLS 75, which has optimum activity at low pH, may be beneficial if being used in the starch industry.

As PLS 75 was a microorganism isolated from sea, its metabolites are expected more adaptive to high salinity compared to those from mesophiles. Adaptation to high salt concentration correlates to their ability to catalyze reactions in organic solvents (Shafiei *et al.*, 2010). In this study, polarity of organic solvents did not correlate to the increase or decrease of enzyme activity. Polar (methanol) and non-polar (n-hexane) solvents increased and maintained the α-amylase activity, respectively. The addition of methanol increased activity to 126% compared to the control, while n-hexane did not cause a change in the activity. In contrast, ethyl acetate (semi-polar) and chloroform (non-polar) reduced the activity to 46% and 59%, respectively (Figure 4C).

Comparison to the activity of α-amylase from other sources also did not show a linear correlation. For example, the activity from *G. thermoleovoran* in non-polar is greater than in polar solvents (Finore *et al.*, 2011). Furthermore, the activites of α-amylase from *B. tequilensis* are comparable when tested in methanol, n-hexane and benzene, with relative activities of 102%, 133% and 99%, respectively (Tiwari *et al.*, 2014). In contrast, α-amylase from *G. thermoleovorans* K1C is tolerance to acetone and benzene, but the activity decreases when in ethanol and methanol (Sudan *et al.*, 2018).

Metabolites from extremophiles require salts to maintain the structure at high temperatures, extreme pH, and organic solvents (Sinha and Khare, 2014). The stability of the structure is contributed by the negative surface of proteins due to the presence of acidic amino acids, especially in halophilic enzymes (Elcock and McCammon, 1998). Nevertheless, there are enzymes from halophiles that still show high activity in the absence of salts and some are stable without excessive negative amino acids on the surface of the structure (Tan *et al.*, 2008).

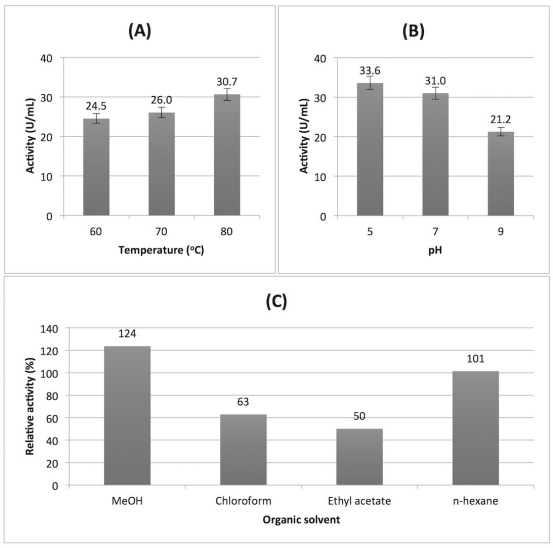

Figure 4. α-amilase activity in various conditions (A) Temperature, measured at pH 7.0; (B) pH, measured at 80 °C; and (C) The present of 50% organic solvent, measured at 80 °C and pH 5.0.

To sum up, the α-amylases from PLS 75 was unique than other reported enzymes. Having a low molecular weight of about 14 kDa, which is rarely reported, it could be more easily immobilised to improve its stability for longer uses. The halophile property is reflected in the enzyme as it showed stability in polar and non-polar solvents, and still had considerable activity in semi polar solvent. As it was reasonably stable across a moderate range of temperature and acidic pH, it would be a good candidate for the use in the starch liquefaction industries.

CONCLUSIONS

The SDS PAGE result showed that α-amylase from PLS 75 is a small protein of about 14 kDa. This enzyme has a good stability because it is active at high temperatures (60-80 °C), acidic to neutral pH and in the presence of methanol. We suggest that complete characterization of the α-amylase should be done, for example by testing the substrate preference, as well as adding metal ions and inhibitor molecules. Enzyme kinetics also needs to be investigated for the determination of K_M and V_{max}.

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