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Contributed Paper

Molecular Cloning and Sequencing of Raw Starch Degrading Gene from *Laceyella sacchari* LP175 and Its Functional Expression in *Escherichia coli*

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ABSTRACT

Raw starch degrading enzyme (RSDE) produced by *Laceyella sacchari* LP175 was purified 14.7 fold to a 40.5 % yield. The first 15 N-terminal amino acids were sequenced and showed 100% homology with α-amylase from *Laceyella* sp. DS3 and *Thermoactinomyces vulgaris*. The RSDE gene was functionally annotated with the *Laceyella sacchari* strain GS1-1 available genome, which showed the presence of a putative gene of 1362 bp encoding 453 amino acids. The RSDE gene was amplified from *Laceyella sacchari* LP175 genomic DNA and cloned for expression in *Escherichia coli*, which showed the highest activity on raw cassava starch at pH 6.5 and a temperature of 50 °C. Homology structure analysis revealed the presence of three domains that are conserved among the structures of GH13 α-amylases, where the active and binding sites both play an important role in starch hydrolysis. The recombinant LsA175 could hydrolyze raw cassava starch at below gelatinization temperature, and showed higher efficiency for hydrolysis than commercial α-amylase (Termamyl®) at 50 °C. This shows the possibility for application of recombinant LsA175 at an industrial level, particularly in terms of energy consumption savings.

Keywords: α-amylase, *Laceyella sacchari* LP175, molecular cloning and expression, homology structure, raw starch degrading enzyme

1. INTRODUCTION

The application of raw starch degrading enzyme (RSDE) is currently of interest in various starch processing industries. The use of RSDE enables the hydrolysis of starch granules at a lower temperature than that of conventional α-amylase at 90 °C [1]. Indeed, RSDE could hydrolyze raw starch granules at below gelatinization temperature without the heating process, which reduces energy consumption and operational costs [2]. The energy required in the gelatinization step of conventional processes corresponds to 10-20 % of fuel value in ethanol processing [3]. RSDE is therefore of interest in terms of energy consumption savings in various starch conversion processes [4, 5]. Currently, two companies - Genecor International Inc. and Novenzyme technology - have developed RSDE as commercial products, as Stargen™ and BPX™, respectively [1, 2]. The thermophilic filamentous bacterium *Laceyella sacchari* LP175, isolated from soil in Thailand, is a potent strain that has shown high efficiency in raw starch degrading enzyme production. RSDE produced by this strain showed simultaneous liquefaction and saccharification of ungelatinzed cassava starch with glucoamylase at 50 °C [5]. Recently, ElSayed *et al.* [6] purified, characterized and investigated the structure of α-amylase from *Laceyella* sp. DS3. Nevertheless, the properties of RSDE which against raw starch from this strain has not yet been reported.

In addition, the isolation, cloning and expression of the RSDE gene would be helpful in terms of understanding its structure-function relationships for further developments and engineering [7]. The expression of recombinant protein in *E.coli* has shown several advantages, e.g., easy to genetically manipulate, simple fermentation, easy to scale up, fast growth and cost effectiveness, as compared to the expression of recombinant proteins in other hosts [8].

In this work, we purified and characterized the RSDE produced by *L. sacchari* LP175. We identified the N-terminal sequence of mature RSDE. We isolated the putative RSDE gene encoding an α-amylases belonging to the GH13 by genome mining. We further subjected to clone and to express the RSDE gene (*lsa*175) in *E. coli* strain. The analysis of 3D homology structure were able to localize on newly isolated RSDE from *L. sacchari* LP175 the three structural domains that function as the catalytic domain and substrate binding site and that are well-conserved among the structures of GH13 α-amylases [9], as well as the raw starch binding domain (SBD) for adsorption on raw starch granules, which play active role in the catalysis of native starch granules [1, 9-10]. We thus provide some clue concerning its evolutionary relationship, mechanism and function for improving their properties in the future.

2. MATERIALS AND METHODS

2.1 Substrates, Bacterium, and Enzyme Production

Various types of starch used in the study as mentioned in the experiments and results, were obtained from Sigma (USA) except cassava starch obtained from local factory as mentioned in previous was used as substrate for enzyme production which was prepared according to the method of Lomthong *et al.* [11].

The bacterial strain of *L. sacchari* LP175 which previously isolated from soil in Thailand [11], was used for enzyme production and source of genomic DNA for PCR amplification. RSDE production was carried out in a made-to-order 3 L water

jacket glass airlift fermenter 185 mm in diameter and 632 mm in height, equipped with a controller (B.E. Marubishi Thailand, Co., Ltd) modified from Miura *et al.* [12] and Maneeboon *et al.* [13] using a 2.0 L working volume of the optimized medium: 4.93 g/L cassava starch, 2.8 g/L yeast extract, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, and 1.0 g/L $CaCl_2$ (pH 6.5) [11]. The fermentation was operated at an aeration rate of 0.5 vvm and controlled the temperature by the circulation of tap water in water bath of 45 °C through the water jackect for 36 h [5].

2.2 Activity Assays

The activity of RSDE was determined following the method of Lomthong *et al.* [11] by analyzing the released reducing sugars from hydrolysis of 2.0 % raw cassava starch at 50 °C for 60 min, using the DNS method as described by Miller [14]. One unit of RSDE activity was defined as the amount of enzyme releasing 1 μg of glucose equivalent per min under standard assay conditions.

The activity of α-amylase was estimated by starch-iodine method, which measuring the changes in the color intensity of starch-iodine solution at 660 nm as described by Fuwa [15]. One unit of α-amylase activity was defined as the amount of enzyme that could degrade 1 mg of starch per minute under standard assay conditions.

Protein concentration was determined by using a Bio-Rad Protein Assay Kit, following the method of Bradford, using bovine serum albumin as standard and measured at wavelength of 595 nm [16].

2.3 Purification of RSDE Produced from *L. sacchari* LP175

Unless otherwise stated, the purification procedure was carried out at 4 °C. The 36-h culture in 2.0 L working volume airlift fermenter was centrifuged (Boeco U-32, Germany) at 8,000 rpm, 4 °C for 10 min. The obtained clear supernatant was then freeze dried using a lyophilizer (FD50; Epsilon Co., Ltd., Bangkok, Thailand). The freeze-dried powder obtained was dissolved in 0.1 M phosphate buffer (pH 6.5) as the crude enzyme, which was then dialyzed overnight with 50 mM Tris-HCl buffer pH 8.0. The dialyzed enzyme (5 mL) was then applied to an anion exchange column (HiTrap Q HP, 5 mL), previously equilibrated with 50 mM Tris-HCl, pH 8.0. Proteins were eluted with a linear gradient of 0-1.0 M NaCl in the same buffer at a flow rate of 1 mL/min with the FPLC system Akta purifier (GE Healthcare, Amersham Pharmacia Biotech). The fractions showing activity on raw starch were pooled, concentrated and the buffer exchanged by washing them three times with 0.1M phosphate buffer pH 6.5 using Amicon® Ultra-15 Centrifugal Filter Units with 30 kDa cutoff (Merk Millipore, Bedford, MA, USA) and were then adsorbed with 5 % (w/v) of raw cassava starch for 60 min in a batch operation. The pellets of starch were collected and washed with 0.5 M NaCl in 0.1 M phosphate buffer pH 6.5. The pellets were further incubated at 55 °C for 10 min in 0.1 M phosphate buffer pH 6.5. The suspension solution, after incubation at 55 °C, was separated by centrifuge at 10,000 rpm for 10 min at 4 °C.

2.4 Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Zymogram

The purification process was followed by SDS-PAGE using a Bio-Rad™ system [17]. Zymographic analysis were carried out following the protocol of Dojnov *et al.* [18] and Ji *et al.* [19] : the gel from SDS-PAGE was incubatied in 1% Triton X-100 solution

at room temperature for 45 min to remove the SDS and restore the enzyme activity. It was then washed with water and incubated in 1% soluble starch at 50 °C for 60 min. α-Amylase activity appeared as clear bands on a dark background after soaking the gel in a staining solution (1.3% (w/v) I_2, 3% (w/v) KI). The molecular mass of the purified RSDE was determined by SDS-PAGE as compared with the protein standards of the Precision Plus Protein™ Standards (10- 250 kDa) [18]. When necessary 250 µl of 25% TCA were added to 1 mL of sample containing RSDE, and kept at 4 °C for 30 min. The precipitated protein was centrifuged at 10,000 rpm for 15 min at 4 °C before resuspention for analysis.

2.5 N-Terminal Amino Acid Sequencing

N-terminal amino acid sequence of the purified RSDE from *L. sacchari LP175* was determined by applying the single band of purified RSDE excised from SDS-PAGE to the automatic microsequencer Procise Protein Sequencing System Model 494 connected to a PTH-amino acid analyser Model 140 of Perkin Elmer Applied Biosystems (Foster City, CA, USA) at Protein Sequencing Service of PISSARO Platform (Rouen, France).

2.6 Cloning of RSDE in *E. coli* Expression Vector pMALC5x

The cloning of the RSDE from *L. sacchari* LP175 (*lsa*175) was carried out using In-Fusion® HD Cloning Kit (Takara, USA), following the instructions of the manufacturer. The primers were designed following the functional annotation of the 42 assembled contigs resulting from the whole genome sequencing of *L. sacchari* strain GS 1-1 [20]. First, using MetaGene program [21] we predicted Open Reading Frames (ORF), gene and protein sequences from the 42 contig sequences. Protein sequences were further annotated using dbCAN web server [22] dedicated to automated carbohydrate-active enzyme annotation. A single protein sequence encoded by a gene belonging to the contig 26 (ASZU01000026) was predicted as amylase from the family GH13. A sequence similarity was found between the previous results of the N-terminal amino acid sequencing of *L. sacchar*i LP175 amylase as detailed above and the amino acid sequence of the *L. sacchari* strain GS 1-1 predicted amylase. 2 potential ATG starting codons were found upstream the sequence encoding *L. sacchari* LP175 mature amylase (-105 and -87). The 29 or 35 amino-acids (depending on the strating Met_1 or Met_2) of the *L. sacchari GS 1-1* sequence located upstream to the N-terminal sequence of *L. sacchari* LP175 (Met_1KGRGCMet_2KKGALLSGLVCLLSAV LTAPTAVA) were predicted to correspond to a signal peptide targeting the protein to the secretory pathway and cleaved when the protein is secreted [23]. We thus designed the primers for cloning according to the nucleotide sequence of *L. sacchari* strain GS 1-1 predicted amylase starting from the nucleotide position downstream to the cleavage site. Primers to amplify the *lsa*175 gene from *L. sacchari* LP175 were : forward (5'-GGATTTCACATATGCTTTCTCCTG CGGATTGGCAAGAGA-3') and reverse (5'-TTAATGATGATGATGATGATGTT TGGTGAATATCTTTACTTCTTTG-3'). PCR amplification included an initial denaturation step at 98 °C for 30 s. This was followed by 20 cycles (30 s at 98 °C, 30 s at 55 °C, and 1 min at 72 °C), and a final additional 5 min extension at 72 °C. A linearized vector of pMAL-c5X was prepared by amplification using forward primer 5'-CATCATCATCATTAAGTCGA CGGATCCGAATTCCCTGC-3'and reverse primer 5'-ATCCGCAGGAGAAA

GATATGTGAAATCCTTCCCTCGATC-3' with PCR conditions including initial denaturation step at 98 °C for 1 min, followed by 20 cycles (98 °C for 30 s, 60.5 °C for 30 s and 72 °C for 3 mins) and a final 5 min extension step at 72 °C. Primers were designed as to encode a fusion protein consisting of MBP-LsA175-His_6, with a maltose binding protein (MBP) at the N-terminal and a tag of 6 histidines at the C-terminal of the protein (underlined parts of primers). The amplified products were infused using an In-Fusion® HD Cloning Kit. The recombinant vector was then transformed into *E. coli* DH5Q competent cells. The recombinant plasmid was extracted from the *E. coli* DH5Q using a Quick Lyse Miniprep Kit (QIAGEN) and sequencing of the *lsa*175 gene was done by GATC using universal primers for pMAL-c5X vector including pMalE (TCAGACTGTCGATGAAGC) and GATC-pMALreverseprimer-1441053 (TGTCCTACTCAGGAGAGCGTTCAC) as forward and reverse primers, respectively.

Primers used for verification of the entire RSDE encoding gene on *L. sacchari* LP175, especially the end of the gene encoding C-terminal region that was included in our primers for cloning, were designed using sequences localized average 100 bp upstream and downstream of putative mature amylase on the contig 26 of *L. sacchari* strain GS 1-1 (Supplemental Figure S1) : forward (5'-GAGAGGATGCA TGAAAAAGGGAG-3') and reverse (5'- GG TGCTCAGTGCACTGGGTGGATCAC-3'). PCR amplification included an initial denaturation step at 98 °C for 30 s. This was followed by 20 cycles (30 s at 98 °C, 30 s at 62 °C, and 1 min at 72 °C), and a final additional 5 min extension at 72 °C. The resulting PCR products were cloned by using a Zero Blunt® TOPO® PCR cloning kit (Invitrogen, USA), and plasmid DNA containing inserts were sequenced by using standard protocols as the Manufacturer's instructions described.

Phylogenetic tree was constructed using the neighbour-joining method with a bootstrap value of 1,000 replicates using MEGA 7.0 software [24]. The sequence alignment was performed using the MUSCLE program embedded within the MEGA 7.0 software.

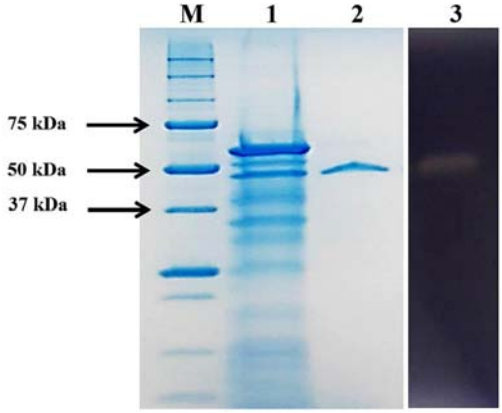

Figure 1. The SDS-PAGE chromatogram of the native RSDE from *L. sacchari* LP175; 1: crude cell extract containing the enzyme; 2: purified enzyme after HiTrap Q and raw starch adsorption; 3: zymogram of purified RSDE on 1 % soluble starch at 50 °C for 60 min.

2.7 Homology Modeling

The homology model of amylase was built by SWISSMODEL [25] using *Anoxybaciilus* Q -amylase (PDB access code: 5A2A) as a template [9]. The quality of the amylase model was validated by PROCHECK [26]. Structures and coordinates of maltose and calcium ions were transferred from PDB access code: 5A2B. The structures were visualized and analysed using Discover Studio Viewer 2016 [27].

2.8 Expression of Recombinant RSDE in *E. coli*

For expression of the recombinant protein (LsA175), the recombinant plasmid was transformed to *E. coli* BL21 (DE3) competent cells and then plated on LB plates containing ampicillin (100 µg/mL). A single colony harbouring recombinant plasmid was grown in 5 mL of LB media supplemented with 100 µg/mL ampicillin and incubated overnight at 37 °C in a shaking incubator. On the next day, the culture was transferred into 50 mL of Terrific Broth (TB) [28], supplemented with the 100 µg/mL ampicillin, and bacteria were grown until they reached mid-logarithmic phase (2.0 Optical density at 600 nm). Finally, *E. coli* cells were then induced for protein expression by the addition of 0.5 mM of IPTG and incubated at 20 °C, 150 rpm overnight. The crude enzyme was obtained by lysis of *E. coli* cells: the pellet was freezed at -80 °C for 2 h, completely thawed and re-suspended in lysis buffer (0.1 M phosphate buffer pH 6.5); then Lysonase™ Bioprocessing Reagent (Merck, USA), was added at a ratio of 2 µL for 6 mL of lysis buffer, and the suspension was incubated at 25 °C for 1 h before centrifugation at 10,000 rpm for 30 min at 4 °C to remove insoluble cell debris.

2.9 Purification of Recombinant LsA175

The recombinant protein LsA175 was purified from the crude intracellular fraction of *E. coli* harbouring plasmid pMALC5x-LsA175 obtained from cell lysis by using immobilized metal affinity chromatography (IMAC). The *E. coli* crude extract was pre-equilibrated at pH 8.0 and 300 mM NaCl and then applied to the column containing 5 mL of resin (TALON® Superflow™, GE Healthcare), previously equilibrated with 20 mM Tris buffer (pH 8.0) containing 300 mM NaCl. The column was washed with 5 times column volumes of the same buffer. The sample was then eluted with each 2.5 times column volumes of 5-200 mM imidazole in 20 mM Tris buffer, pH 8.0, containing 300 mM NaCl. The buffer from elution fractions were exchanged by washing them three times with 0.1M of the phosphate buffer pH 6.5 using Amicon® Ultra-15 Centrifugal Filter Units with 30 kDa cutoff (Merk Millipore, Bedford, MA, USA).

To cleavage of the fusion protein, 100 µL of the fusion protein MBP-LsA175-His_6 at 1 mg/mL was mixed with 0.05 Units of Factor Xa protease (Sigma, USA). Tubes were incubated at room temperature overnight, and checked for complete cleavage using SDS-PAGE. To separate the protein of interest from MBP after protease cleavage, the fusion protein cleavage mixture was dialyzed with 50 mM Tris-HCl buffer pH 8.0. The dialysed solution was applied to Q Sepharose (HiTrap Q HP, 1 mL) anion exchange columns previously equilibrated with 50 mM Tris-HCl, pH 8.0. Proteins were eluted with a linear gradient of 0-1.0 M NaCl in the same buffer, at a flow rate of 1 mL/min, on the FPLC system Akta purifier (GE Healthcare). The buffer from pooled elution fractions was exchanged with 0.1 M phosphate buffer pH 6.5 using Amicon Ultra-15 Centrifugal Filter Units (Amicon, Beverly, MA, 30 kDa cutoff).

2.10 Hydrolysis of Raw Cassava Starch

The crude recombinant enzyme was used to hydrolyze raw cassava starch compared with commercial α-amylase (Termamyl®, Novo Nordisk Ferment Ltd., Dittingen, Switzerland) at the same α-amylase activity (20 U/mL). The reaction mixture (in a total of 50 mL) in a 250 mL Erlenmeyer flask contained 5 g of raw cassava starch, 25 mL of 0.2 M phosphate buffer (pH 6.5) and

25 mL of the crude recombinant enzyme or commercial α-amylase. The mixture was incubated at 50 °C in an incubating shaker at 150 rpm for 6 h. At designated time intervals, samples were taken for determination of liberated reducing sugars.

3. RESULTS AND DISCUSSION

3.1 Purification of RSDE from *L. sacchari* LP175

Crude RSDE solution obtained by dissolved the freeze-dried powder of *L. sacchari* LP175 was purified to homogeneity using anion exchange chromatography (Q Sepharose, HiTrap Q HP, 5 mL) and a raw starch adsorption method. Several reports have described the use of anion exchange chromatography columns for purification of RSDE from bacteria [1, 29]. Since the pI of RSDE from *L. sacchari* was 5.3 [20], this form of chromatography is suitable for purification purposes. The adsorption of enzyme on raw starch granules is used to separate amylase from other proteins. Here, RSDE from *L. sacchari* LP175 was adsorbed to raw cassava starch at 4 °C, with most other proteins being washed off and with no affinity for starch, as indicated by only one protein band on the gel chromatogram being observed with an estimated molecular weight of 50 kDa, corresponding to the active band on the soluble starch-based zymogram (Figure 1, lane 3)). RSDEs are well known to contain raw starch-binding domains that can bind to substrates at low temperatures with specific affinity [1]. The specific activity changed from 164.4 to 2416.7 U/mg protein, corresponding to a 14.7-fold increase, with 40.5 % yield (Table 1). Najafi and Kembhavi [30] reported a 78% yield with a specific activity of 327 U/mg, and a 163-fold purification being obtained by slowly swirling insoluble corn starch with a crude α-amylase from marine *Vibrio* sp at 4 °C for 60 min, as one step of an alternative affinity method.

Table 1. Summary of purification of the raw starch degrading enzyme of *L. sacchari* LP175 using anion exchange chromatography and raw starch adsorption.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude enzyme	716.7	4.36	164.4	1	100
Dialysis enzyme	623.3	2.93	212.7	1.3	86.9
HiTrap Q HP	413	0.17	2385.3	14.5	57.6
Adsorption	290	0.12	2416.7	14.7	40.5

3.2 Cloning and Expression of RSDE in *E. coli*

The sequence of the first 15 N-terminal amino acids showed 100% homology with α-amylase from *Laceyella* sp. DS3 (accession number A0A0P1P9M5) and α-amylase from *T. vulgaris* 94-2A (amyE (TV1), accession number Q60051), which confirmed that RSDE from *L. sacchari* LP175 was α-amylase. This latter was already cloned and expressed in *B. subtilis* [31]. However, the ability of α-amylase from *T. vulgaris* 94-2A (amyE (TV1)) on raw starch digestion has not yet been reported. To confirm the sequence of mature LsA175 RSDE from *L. sacchari* LP175, we took advantage of our annotation of RSDE from whole genome sequence of *L. sacchari* strain GS 1-1, and designed primers for genomic DNA amplification localized average 100 pb upstream and downstream of gene encoding mature amylase on the contig 26 of *L. sacchari* strain GS 1-1

(Supplemental Figure S1). The *lsa*175 gene encoding mature LsA175 without the signal peptide, obtained by sequencing the amplification product, corresponds to 1362 bp. The nucleotide sequence was kept in the GenBank with the accession number KX816333. There were some differences in nucleic sequences from *L. sacchari* LP175 and *L. sacchari* strain GS 1-1, but most were silent, only leading to four differences in protein sequences including D169, E174, A177 and Q350; the entire codon is underlined if it results in translation change, as shown in Supplemental Figure S1.

The nucleotide sequence displayed 98 % identity with amyE (TV1) of *T. vulgaris* 94-2A (GenBank ID X69807.1) and AmyLa of *Laceyella* sp. DS3 (GenBank ID LN901326.1). The mature protein consisted of 453 amino acids had a predicted molecular weight of 51.8 kDa, as determined by the ExPASy server (http://web.expasy.org/protparam). LsA175 is very close to the amylases encoded by amyE (TV1) and AmyLa, with 99.12 and 98.45 % identity, respectively.

The phylogenetic analysis of α-amylases in this study was selected from a total of 41 subfamilies (as of Dec 2016) are found in the database of Carbohydrate-Active enZyme (http://www.cazy.org/) which exhibit α-amylase activities as reported among subfamily 1, 5, 6, 7, 15, 19, 24, 27, 28, 32 and 36 [9, 32-33] to construct using the neighbour-joining method with representative α-amylases from each subfamily, as well as LsA175 from the studied bacterial strain, is presented in Figure 2. The tree indicates that LsA175 from the *L. sacchari* LP175 clusters with amyE (TV1) of *T. vulgaris* 94-2A (GenBank ID X69807.1) and AmyLa of *Laceyella* sp. DS3 (GenBank ID LN901326.1); however, this cluster forms a phylogenetically coherent cluster separated from all existing GH13 subfamilies; this suggests that LsA175 from *L. sacchari* LP175 is also distinct from other α-amylases, as well as amyE (TV1) and AmyLa, which were distinct from the existing GH13 subfamilies (Figure 2). However, the evolutionary tree also illustrates that the cluster of LsA175 from *L. sacchari* LP175 was close to the cluster of two *Anoxybacillus*-amylases (ASKA and ADTA), which was reported as a novel group of the α-amylase family GH13 [33, 34].

3.3 Structural Analysis of the 3D Homology Structure of LsA175

The amino acid sequence of LsA175 α-amylase showed 42.63 % identity with α-amylase from *Anoxybacillus* (PDB access code: 5A2A), which is a member of the GH13 family. The amino acid sequence alignment between LsA175 amylase and *Anoxybaciilus* α-amylase (ASKA) showed conserved and mutated amino acids and secondary structure of helices and β-strands in tree domains of GH13 α-amylases, as shown in Figure 3. The catalytic triad residues (Asp-Glu-Asp) are conserved and located at D198, E222, and D287, while the mutated amino acids are underlined (Figure 3). The Ramachandran plot of the LsA175 model from PROCHECK showed that 86.9 % of the residues were in the most favored region, and 12.1 % of the additional allowed regions, indicating a good quality of homology structure. The homology model of LsA175 contained three domains that are conserved among the structures of GH13 α-amylases (Figure 4A). Domain A (residue 30-147, 195-396) contained a TIM barrel fold with the active site. Domains B (residue 148-194) and C (residue 397-453) contained all-beta-fold which are substrate and maltose binding site, respectively.

There were two different residues in the active site of LsA175 compared with the *Anoxybacillus* α-amylase - Y143D and F178A.

The former is located at domain A, which may relate to the function of the active site, while F178A is located at domain B, which may play a role in the mode of substrate binding [9]. The catalytic triad residues (Asp-Glu-Asp) that are important for the hydrolysis of the substrate, are conserved, and are located at D198, E222, and D287 (Figure 4B). The hydrogen bonding of Tyrosine 143 to maltose did not occur in *Anoxybacillus* α-amylase when aspartic acid (D125) was substituted in LsA175. Furthermore, two out of four calcium binding sites that were found in *Anoxybacillus* α-amylase were lost due to the mutation of glutamic acid in those binding site to other residues. At the first calcium binding site (as described by Chai *et al.* [9] the longer loop was found with two glycine insertions (G30, G31) together with the mutation of E109 to D91, E110 to G92, and N92 to K74 which meant that this region could no longer bind to the calcium ion. In the other calcium binding site at the fourth position, all three glutamic acids (283, 382, and 400) were found to be mutated to arginine (E261), tyrosine (Y362) and valine (V380), indicating that the calcium binding site in this region was also lost. Calcium-binding sites are well known for their important role in α-amylase thermostability and overall function [9]. The addition of calcium ions to the reaction can increase enzyme activity [1, 9]. In this study, two calcium binding sites were found in the LsA175 structure, suggesting that they may be involved in substrate catalysis. This corresponds to previous investigations of Lomthong *et al.* [11], who found that addition of calcium ions enhanced RSDE activity of *L. sacchari* LP175.

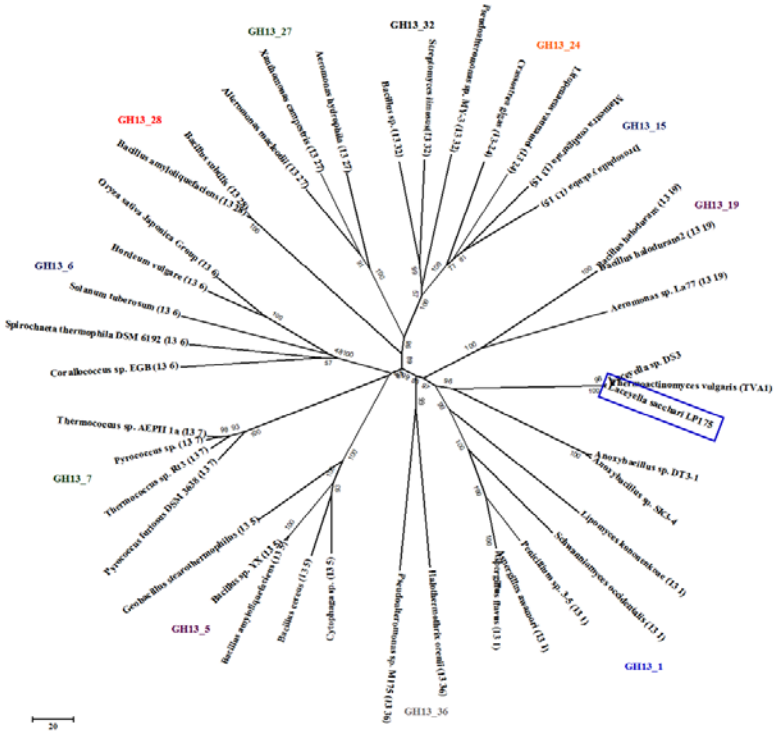

Figure 2. The phylogenetic analysis of α-amylases construction using the neighbour-joining method with representative of α-amylases from each subfamily within the GH13 family. Representative were chosen from a total of 41 subfamilies in GH 13 family which exhibit α-amylase activities.

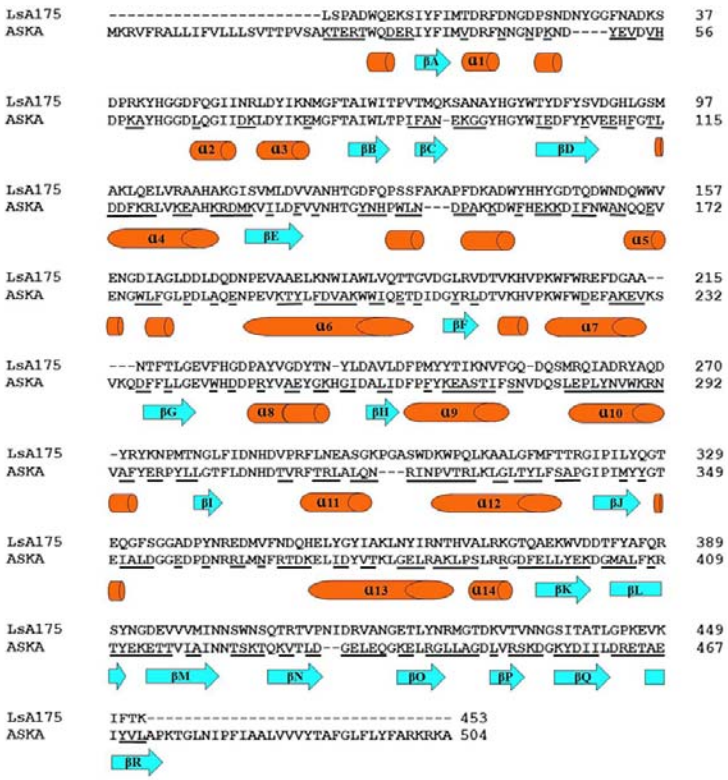

Figure 3. Comparision of alignment between LsA175 mature amylase and *Anoxybacillus* α-amylase (accession number JF932307). Cylinders and arrows represent helices and strands of secondary structure, respectively.

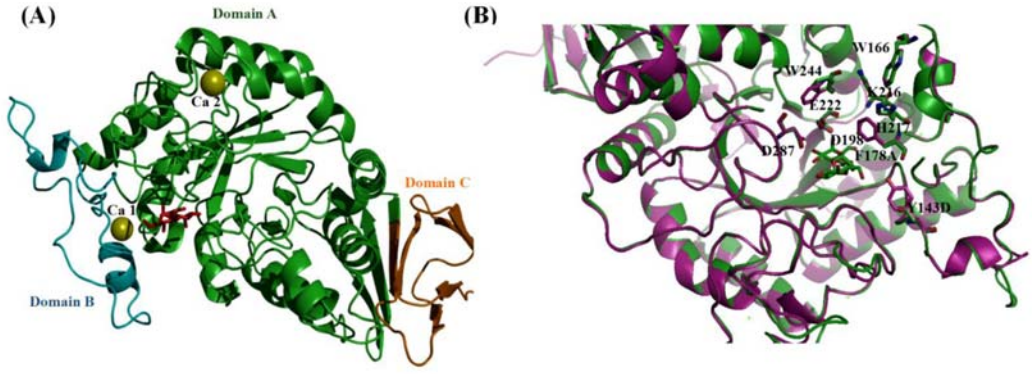

Figure 4. 3D homology model of LsA175 α-amylase. The three domains are shown in different colours, domain A (green), domain B (blue) and domain C (orange) contained the active site, calcium binding site, and substrate binding site, respectively. The maltose (red) was located in the active sites (A). Superposition of the LsA175 (green) and *Anoxybacillus* α-amylase (Magenta, PDB entry 5A2A) showed different residues, Y143D and F178A, in the binding site of substrates. All the important residues in the binding site were labelled (B). The conserved catalytic triad (D198, E222, and D287) was found in both LsA175 and *Anoxybacillus* α-amylase.

3.4 Expression of Recombinant RSDE in *E. coli*

To confirm the functional analysis of RSDE gene from *L. sacchari* LP175, recombinant LsA175 was expressed in *E. coli* BL21 (DE3). Primers were designed as to create a fusion protein between the maltose binding protein (MBP) at the N-terminal of mature protein, and a six histidines tag at the C-terminal of the fusion protein. MBP has been recognized as one of the most effective solubilizing agents used to increase the expressability and solubility of recombinant proteins expressed in *E. coli*, as it prevents from aggregation [35]. The recombinant strain produced RSDE, as visualised by halo zone formation on a raw starch agar plate (Figure 5). The maximum activity on hydrolysis of raw cassava starch swas at pH 6.5 and temperature at 50 °C which were similar properties with RSDE in the native strain (*L. sacchari* LP175). In comparing to other *Thermoactinomyces* 's amylases in the same cluster of amylase subfamily, The amyE (TV1) protein showed high active at pH 4.8-6.0 and temperature at 62.5 °C using ethylidene-pNP-G7 (E-pNP-G7) as the substrate [31], AmyLa from *Laceyella* sp. DS3 showed optimum at pH 7 and temperature at 50 °C [6]. But both of them are not previously reported on raw starch hydrolysis.

The findings of this study suggest that the LsA175 gene from *L. sacchari* LP175 can be expressed in *E. coli*, thus providing an alternative choice for enzyme production at an industrial level in terms of improving enzyme production in a stirrer fermenter, and with respect to avoiding stability loss during storage from the effect of protease in the native strain. Bioinformatics analysis of LsA175's structure also provides relevant information for optimizing the production and catalytic efficiency of recombinant enzymes through enzyme engineering.

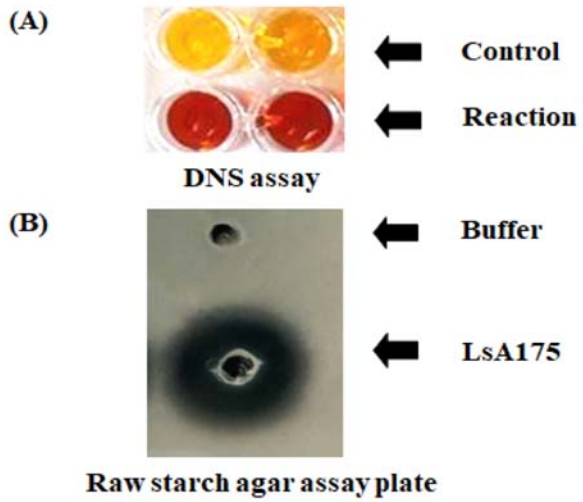

Figure 5. Raw starch degrading activity of LsA175 on raw starch hydrolysis by DNS method (A) and raw starch agar assay plate (B).

3.5 Purification of Recombinant Enzyme

The purified recombinant protein was obtained by one step of IMAC method, as shown in Figure 6A. The molecular weight of recombinant MBP-LsA175 presents about 92.5 kDa, correspondence to the mass of 51.8 kDa for mature RSDE from *L. sacchari* LP175 fused to the MBP, which has a molecular mass of 42.5 kDa. The specific activity of the recombinant MBP-LsA175 on raw cassava starch was 2532 U/mg protein. To confirm the molecular weight and specific activity of the recombinant enzyme, cleavage of the MBP from the fusion protein was done by digestion with factor Xa protease. As shown on SDS-PAGE in Figure 6B, two bands were released, which corresponded to about 50.4 kDa and about 42.5 kDa. The recombinant RSDE in the digestion solution was purified using anion exchange chromatography; Recombinant LsA175 had a molecular mass corresponding to 50 kDa, which is the same as the MW of the native protein, as confirmed by SDS-PAGE (Figure 6B). The summary of purification of the recombinant enzyme is shown in Table 2. The specific activity of recombinant LsA175 after cleavaged the MBP was 4211 U/mg protein, corresponding to a 108-fold purification, with a purification yield of 35% of the activity, which increased 1.74-fold as compared with specific activity of the native protein.

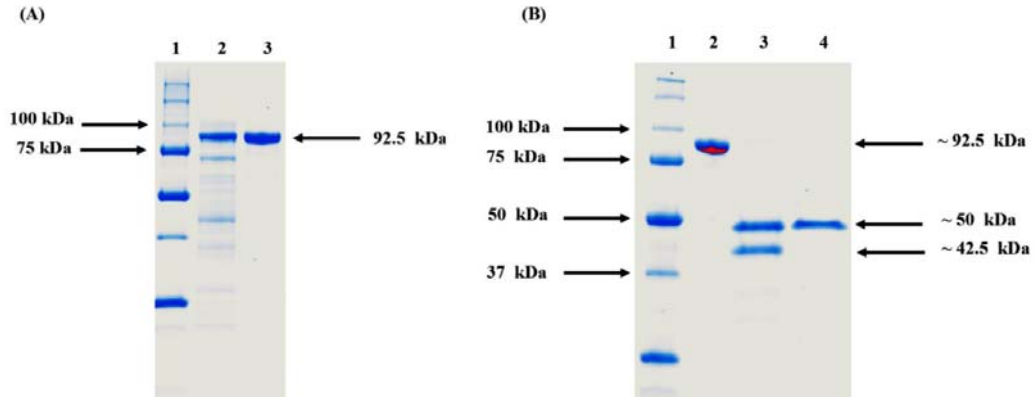

Figure 6. The SDS-PAGE chromatogram of the recombinant enzyme expressed by *E. coli* BL21. Step of purification by IMAC(A), 1: protein marker; 2: crude *E. coli* extract; 3: IMAC purified recombinant enzyme. The SDS-PAGE chromatogram of the purified enzyme after cleavage by factor Xa protease(B): 1: protein marker; 2: purified recombinant MBP-LsA175-His_6 fusion protein; 3: proteins in the reaction after cleavage with factor Xa protease and 4: purified LsA175 protein after ion exchange chromatography.

Table 2. Purification summary of recombinant raw starch degrading enzyme LsA175 from *E. coli* BL21 using IMAC purification and anion exchange chromatography.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude	1479	37.98	38.9	1	100
Affinity Chromatography	633	0.25	2532	65	42.8
HiTrap Q HP	518	0.123	4211	108	35

3.6 Hydrolysis of Raw Cassava Starch

The liberated reducing sugars at different times are shown in Figure 7. The recombinant LsA175 showed higher efficiency for hydrolysis of raw cassava starch than commercial α-amylase (Termamyl®) at 50 °C. Raw starch degrading enzyme have known that contained raw starch binding site at the structure which could adsorb to the surface of granule without the high temperature heating [1-2, 5]. In addition LsA175 had the optimum activity at 50 °C while commercial α-amylase activity usually was at 90-95 °C [36]. This showed the possibility for application of recombinant raw starch degrading enzyme (LsA175) in starch processing industries to reduce the energy consuming and operational costs.

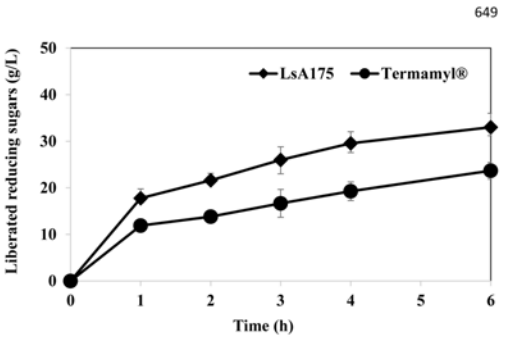

Figure 7. Liberated reducing sugars of raw cassava starch hydrolyzed by the recombinant LsA175 and commercial α-amylase (Termamyl®) at 50 °C.

4. CONCLUSIONS

The purified raw starch degrading enzyme from *L. sacchari* LP175 is highly efficient for starch hydrolysis in an uncooking process when compared to the conventional α-amylase enzyme. Our findings indicate that RSDE produced by *L. sacchari* LP175 is a potent candidate for applications in the starch industry. The expression of the raw starch degrading gene in *E. coli* could provide important information regarding the evolutionary relationship, structure and mechanism of raw starch hydrolysis; this will be helpful for applications in the future, in terms of reducing energy consumption and operational costs at an industrial level.

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