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Gene Expression in *Escherichia coli* and Purification of Recombinant Type II Pullulanase from a Hyperthermophilic Archaeon, *Pyrobaculum calidifontis*

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MAS designed the study. HuR, MAS, AQ and AB conducted the experimental work. NR, MAS and HuR wrote the article.

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Hyperthermophile, *Pyrobaculum calidifontis*, Debranching enzyme, Type II pullulanase, Amylopullulanase.

ABSTRACT

Type II pullulanase also known as amylopullulanase hydrolyzes α-1,6 glycosidic bonds in pullulan and α-1,6 as well as α-1,4 glycosidic bonds in starch. Most of the previously reported type II pullulanases are metal ion dependent and hard to purify, to homogeneity, due to low level of expression or the proteolytic machinery of the host. Herein we report expression in *Escherichia coli* and purification of metal ion independent type II pullulanase from *Pyrobaculum calidifontis*. Thermophilic origin and metal ion independency of the enzyme reported in this study make it a potential candidate for starch hydrolyzing industry.

INTRODUCTION

Starch is one of the most abundant homopolysaccharide on our planet and is a polymer of α-D glucose residues (Kujawski *et al*., 2002). Starch is synthesized in most green plants as energy reserve, and is used as energy source for various microorganisms, higher plants and animals (Janeček, 1997). Starch is composed of two glucose polymers; one is amylose and second is amylopectin. Amylose is a linear polymer of approximately 6000 α-D-glucose residues which are linked through α-1,4 glycosidic bonds. Amylopectin is a branched polymer that in addition to α-1,4 glycosidic linkage also contains α-1,6 glycosidic linkage (Nigam and Singh, 1995).

Formerly starch processing industry was using mild acid for the hydrolysis of starch but nowadays, due to the discovery of more efficient thermostable starch hydrolyzing enzymes, the starch hydrolyzing industry has switched to the enzymes for starch hydrolysis to make beneficial products such as maltodextrin, dextrose, glucose and fructose syrups and sugar alcohols such as mannitol, sorbitol, erythritol *etc*.

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The pullulanases (E.C. 3.2.1.41) degrade pullulan, a linear α-glucan that contains repeating subunits of triose sugar (maltotriose) linked by α-1,6 glycosidic bond, into different types of saccharides. Pullulanases are also known as debranching enzymes due to their capability to hydrolyze α-1,6 glycosidic linkages in starch and other branched polysaccharides (Van der Maarel *et al*., 2002; Hii *et al*., 2012).

On the basis of reaction end products and substrate specificity five types of pullulanases have been reported till now, among them two are called pullulanases (pullulanase types I and pullulanase type II) while the other three are known as pullulan hydrolases (pullulan hydrolase types I, pullulan hydrolase type II and pullulan hydrolase type III). Type I pullulanase, hydrolyzes α-1,6 glycosidic bonds in pullulan, amylopectin and other branched chain polysaccharides but is unable to act on α-1,4 glycosidic bonds in substrates (Rudiger *et al*., 1995; Kim *et al*., 1996; Koch *et al*., 1997; Bertoldo *et al*., 1999; Ben-Messaoud *et al*., 2002; Han *et al*., 2013). The end product of reaction catalyzed by type I pullulanase is maltotriose (Wasko *et al*., 2011). Type II pullulanase also known as amylopullulanase (APU) hydrolyzes α-1,6 glycosidic bonds in pullulan and starch but cannot hydrolyze α-1,4 linkage in pullulan and surprisingly can hydrolyze α-1,4 glycosidic linkage in starch (Melasniemi, 1988; Saha *et al*., 1988; Spreinat and

Antranikian, 1990; Mathupala *et al*., 1993; Ramesh *et al*., 1994). The third and fourth types are pullulan hydrolase I (neopullulanase) and pullulan hydrolase II (isopullulanase) (Duffner *et al*., 2000; Niehaus *et al*., 1999; Matzke *et al*., 2000) hydrolyze α-1,6 glycosidic bonds in pullulan while fifth type is pullulan hydrolase III has the ability to degrade α-1,4 and α-1,6 glycosidic linkages in pullulan (Niehaus *et al*., 2000; Ahmed *et al*., 2014).

Nowadays the enzymes from hyperthermophiles are widely used in industries (Sabir et al., 2017) and especially are replacing the use of other amylolytic enzymes in starch industry. However, these enzymes are Ca^{2+} dependent for their activity and adding the Ca^{2+} in reaction may inhibits the activity of other enzymes involved in reaction processes or choke the heat exchangers. Previously we have cloned and expressed Pcal_1616 (*apu*) gene for the production of Ca^{2+} independent type II pullulanase from *Pyrobaculum calidifontis* strain VA1 (Siddiqui *et al*., 2014). *P. calidifontis* strain VA1 is an obligate heterotrophic and hyperthermophilic archaeon that was isolated from a terrestrial hot spring in Philippines (Amo *et al*., 2002). Unfortunately in our previous report the recombinant type II pullulanase could not be purified (Siddiqui *et al*., 2014). We report here the purification and activity assays of type II pullulanase from *P. calidifontis* strain VA1.

MATERIALS AND METHODS

Chemicals, restriction enzymes and reagents

All the chemicals used in this study work were of molecular biology or analytical grade and were obtained from Sigma (St. Louis, Mo., USA). All restriction endonucleases, *Phusion* DNA polymerase, PCR cloning kit, (DNA) extraction and ligation kits, DNA markers and protein markers were obtained from New England Biolabs, Inc., Nebraska, USA.

Growth conditions for Pyrobaculum calidifontis

Routine cultivation of hyperthermophilic archaeon *P. calidifontis* was performed according to previously described method (Amo *et al*., 2002) in a medium containing 1% tryptone, 0.1% yeast extract and 0.3% sodium thiosulphate in distilled water at 90°C.

Host bacterial strains, plasmids and media

Plasmid pET101/D-TOPO (Invitrogen) was used as expression vector. One Shot TOP10 chemically competent *E. coli* F$^-$ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BG) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G was used for cloning while *E. coli* BL21 Star™ (DE3) F$^-$ *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm rne 131* (DE3) was used for overexpression of the cloned gene. All *E. coli* strains were cultivated at 37°C.

Cloning of Pcal-apu *gene*

Based on the DNA sequence of *Pcal_1616* and pET101 plasmid as described by Shuman (1991, 1994). The forward primers Apu-F1: 5'-CACCATGAAGATCCTCGCCCTCCTC-3', and a reverse Apu-R1: 5'- TATAGATTGTCGCGGCCTCC-3' primer for the amplification of *apu* gene were constituted. The gene amplification was performed by polymerase chain reaction (PCR) using Phusion DNA polymerase (New England BioLabs Inc.) as follows: 3 min at 98°C; 30 s at 95°C, 30 s at 53°C and 1.0 min at 74°C (30 cycles) using thermal cycler (Gene Amp PCR System 24000, Perkin Elmer, Foster, Calif). The resulting amplified PCR product of *apu* gene was ligated into TOPO pET101 vector and the resulting plasmid was named pET101-APU. One Shot TOP10 chemically competent *E. coli* cells were transformed using pET101-APU plasmid.

DNA sequencing

The gene fragment cloned in pET101 vector was sequenced using an automated DNA sequencer (Beckman Coulter 121 CEQ8000; Beckman Coulter Inc., Fullerton, CA).

Gene expression and purification of protein

For expression, *E. coli* BL21 Star™ (DE3) cells were transformed using pET101 plasmid harboring *apu* gene. Cells harboring recombinant vector pET101-APU were cultured at 37°C for 16 hours in Luria-Bertani broth (LB) medium containing (100 µg/mL) ampicillin antibiotic. This overnight grown preculture was added (1%) into freshly prepared LB medium containing (100 µg/mL) ampicillin and cells growth was continued at same temperature (37°C) with shaking at 100 rpm. The optical density (OD) was measured hourly at 660 nm and when it reached to 0.4 then the gene expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The incubation was continued at 37°C for further 4 h. The cells were obtained by centrifugation at 8,000 × g for 08 min and then washed with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet obtained was resuspended in 50 mM Tris-HCl buffer. The cells were sonicated and centrifuged at 15,000 × g for 15 min to obtain the soluble and insoluble fractions. The *apu* gene expression was confirmed by SDS-PAGE and enzyme activity assay. The soluble fraction was kept in shaking water bath at 85°C for 20 min and then again centrifuged at 15,000 × g for 15 min to separate soluble and insoluble fractions. The soluble fraction obtained after heat treatment was precipitated with the addition of 75% ammonium sulfate. The sample was centrifuged at 20,000 x g for 20 min, the pellet obtained after centrifugation was dissolved in 10 mL of 20 mM potassium phosphate buffer (pH 7.4) containing 0.5 M KCl and 40 mM imidazole and dialyzed against same buffer. The dialyzed sample

was used for immobilized metal affinity chromatography (IMAC) by using His GraviTrap (Amersham Biosciences) column. The sample was applied, and the His tag protein was eluted from the His GraviTrap column with 20 mM potassium phosphate elution buffer (pH 7.4) containing 0.5 M KCl, and 0.5 M imidazole. The elution buffer was exchanged with Tris-HCl (pH 8.0) utilizing Amicon Ultra-4 30K (Millipore) by centrifugation at 4,000 × g for 15 min, the collected fractions were stored at 4°C for initial examination of activity.

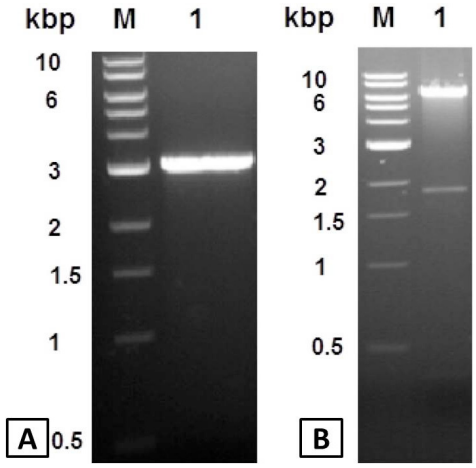

Fig. 1. DNA fragments analysis by ethidium bromide stained 1% agarose gel. **A**, PCR amplified type II pullulanase gene. Lane M, Kilobase DNA marker; Lane 1, 3.1 kbp PCR amplified *apu* gene. **B**, Analysis of recombinant pET101-APU plasmid. Lane M, standard; Lane 1, pET101-APU plasmid cut with *Hin*dIII restriction enzyme.

Enzyme assay

Type II pullulanase activity was determined according to previously reported method (Bernfeld, 1955) by measuring the amount of reducing sugar released during enzymatic hydrolysis of 1% pullulan or soluble starch in 125 µL 50mM acetate buffer (pH 5.6) with further addition of 125 µL diluted recombinant enzyme and incubation at 90°C for 15 min. A control without addition of enzyme was used. The amount of reducing sugar released was determined by the dinitrosalicyclic acid method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugars in one minute under standard assay conditions.

For the purpose of thermostability determination of APU in the presence or absence of Ca^{2+}, the purified protein was incubated at 90°C for 5 h. Aliquots were withdrawn at every 30 min intervals of time and assayed for the activity at 90°C for 15 min. The activity of untreated enzyme at 90°C for 5 h was considered as 100%.

Activity staining

The amylopullulanase activity was confirmed on SDS-PAGE. The red pullulan 5 mg/mL was incorporated in polyacrylamide gel. After performing electrophoresis, the gel was incubated at 58°C for 4 h in 50 mM phosphate buffer (pH 7.0). After flooding with Lugol's iodine, yellowish white band against the red background on gel was appeared.

Effects of metal ions on amylopullulanase

The effects of various metal ions on the amylopullulanase enzyme activity were studied. The purified amylopullulanase (2.0 U/mL, final concentration) was incubated with 5 mM metal salts at 60°C for 15 min. Samples were then withdrawn, and activity of enzyme was measured at 90°C.

RESULTS AND DISCUSSION

Gene cloning

The cells of hyperthermophilic archaeon strain *P. calidifontis* were cultivated as described in experimental section and chromosomal DNA was extracted. For the amplification of type II pullulanase gene (*apu*), sequence specific primers were designed according to the procedure described by Shuman (1991, 1994) by using topoisomerase I produced by *Vaccinia* virus. In this system to work additional bases (CACC) were added to forward primer to join the overhanging (GTGG) bases in the pET101 vector created by topoisomerase I. In the reverse primer stop codon was not added for the purpose to add 6xHis tag in PCR product. The polymerase chain reaction (PCR) was performed which resulted in the amplification of approximately 3.1 kbp DNA fragment (Fig. 1A). The PCR product harboring *apu* gene was isolated from agarose gel (Fig. 1A), gene cleaned and ligated in pET101 vector to obtain pET101-APU and *E. coli* One Shot TOP10 competent cells were transformed with pET101-APU. In order to confirm the insertion of 3.1 kbp PCR amplified fragment, pET101-APU plasmid was digested with *Hin*dIII restriction enzyme. Two DNA bands were generated, a 1.9 kbp part of the inserted gene and the other 6.9 kb fragment containing 5.7 kbp vector and 1.2 kbp of the other part of the gene, indicating that the recombinant pET101-APU contained the *apu* gene (Fig. 1B). Complete nucleotide sequences of both strands of inserted *apu* gene were determined and no variation in both strands was observed. The result of gene sequence confirmed that it encodes type II pullulanase.

Heterologous expression of apu gene and purification of APU protein

For the expression of *apu* gene the pET101-APU plasmid was extracted. The *E. coli* competent BL21 Star™

(DE3) cells were transformed with pET101-APU and a number of colonies were appeared on selection plates. A colony was selected randomly for gene expression. Heterologous gene expression was achieved with 0.1 mM isopropyl-β-D-galactopyranoside in 6 h. According to SDS-PAGE analysis approximately 111 kDa recombinant protein was produced (Fig. 2A).

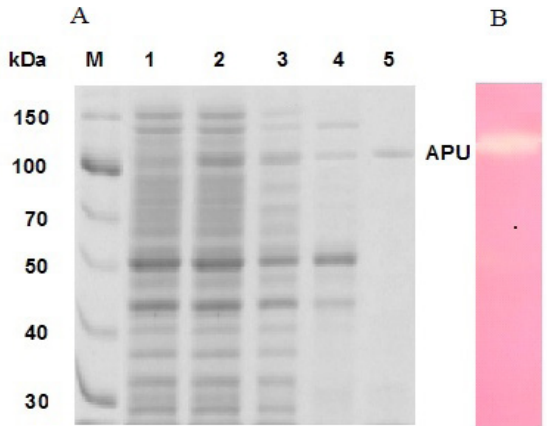

Fig. 2. Coomassie brilliant blue stained SDS-PAGE demonstrating the production of type II pullulanase. **A**, Lane M, protein marker; Lane 1, cells carrying pET101 vector; Lane 2, cells carrying pET101-APU plasmid; Lane 3, soluble fraction of the sample in lane 2; Lane 4, soluble fraction after heat treatment of sample in lane 3; Lane 5, purified recombinant type II pullulanase. **B**, Activity of purified type II pullulanase.

Previously recombinant amylopullulanase from *P. calidifontis* could not be purified when *apu* gene was expressed in *E. coli* BL21 (DE3) cells culture (Siddiqui *et al*., 2014), similar to a number of reports on APU enzymes that could not be purified due to low gene expression or proteolytic effect (Duffner *et al*., 2000; Chen *et al*., 2001). In the current expression and purification system, the *apu* gene was cloned in pET101/D-TOPO plasmid that contains polyhistidine (6xHis) tag that facilitates the purification of recombinant fusion protein with metal chelating resin. The expression was also performed in competent BL21 Star™ (DE3) cells that contain *rne*131 mutation responsible for the enhancement of expression capabilities of competent BL21 Star™ (DE3) cells. After gene expression, the soluble fraction was heat treated at 85°C for 20 min and then again soluble and insoluble fractions were separated by centrifugation. The heat treatment removed most of the heat-labile host protein. The soluble fraction obtained after heat treatment was precipitated with the addition of 75% ammonium sulfate. The sample was centrifuged at 20,000 x g for 20 min, the pellet obtained after centrifugation was dissolved in 10 mL of 20 mM potassium phosphate buffer (pH 7.4) containing 0.5 M KCl and 40 mM imidazole and dialyzed against same buffer. The soluble fraction was used for immobilized metal affinity chromatography by using His GraviTrap (Amersham Biosciences) column. The purified APU protein eluted from GraviTrap column was analyzed by SDS-PAGE (Fig. 2A). The purification steps and the amount of enzyme activity of APU are summarized in Table I.

Table I.- Purification of recombinant type II pullulanase.

Purification steps	TP (mg)	TA (units)	SA (units/mg)	Yield (%)	Purification (fold)
Crude extract	1208	6548	5.4	100	1.0
Heat treatment	677	6210	9.2	94.8	1.7
$(NH_4)_2SO_4$ppt	120	5980	49.8	91.3	9.2
Affinity chromatography	42.5	3113	73.2	47.5	13.6

TP, total protein; TA, total activity; SA, specific activity.

Activity staining

The purified amylopullulanase activity was determined by activity staining on SDS-PAGE as described in materials and methods section. Lugol's iodine forms dark blue/black color with the interaction of amylose and amylopectin, the yellowish white band against the red background on gel appeared (Fig. 2B) indicating that the APU enzyme was active and converted the starch into product.

Effects of metal ions on amylopullulanase activity

The effect of various metal ions on the purified APU enzyme activity was studied. The metal ions such as Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} have inhibitory effect on APU activity while Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} did not effect on the APU enzyme activity (Table II).

Table II.- Effect of metal ions on type II pullulanase.

Metal ion	Concentration (mM)	Specific activity (Units/mg)	Relative activity (%)
None	-	73.2	100
Ca^{2+}	5	75.3	104
Sr^{2+}	5	72.5	99
Ba^{2+}	5	71.4	97
Mg^{2+}	5	73.0	99
Co^{2+}	5	64.8	88
Mn^{2+}	5	62.9	86
Ni^{2+}	5	56.6	77
Zn^{2+}	5	50.5	69
Cu^{2+}	5	41.4	57
Na^{+}	5	75.4	103
K^{+}	5	74.0	101

The Ca^{2+} usually increases the activity and thermostability of Ca^{2+} dependent enzymes. In order to know the role of Ca^{2+} on APU, the enzyme was incubated with or without 0.5 mM Ca^{2+} for 5 h at 90°C and activity was determined as described in materials and methods section. In both cases no detectable difference was observed in the activity as well as thermostability of enzyme (Fig. 3).

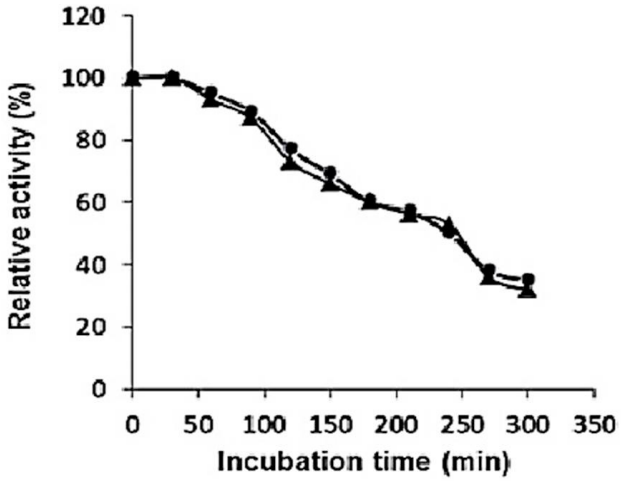

Fig. 3. Thermostability of purified recombinant type II pullulanase in the presence (●) or absence (▲) of Ca^{2+}.

CONCLUSION

We have purified type II pullulanase (APU) from a hyperthermophilic archaeon, *P. calidifontis*, by using gene cloning in pET101 cloning vector and purification of APU was achieved with immobilized metal affinity chromatography technique. As APU is Ca^{2+}-independent thermostable enzyme therefore, purified APU is a good candidate to replace Ca^{2+}-dependent enzymes being used in starch industry.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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