Gene Cloning and Characterization of Pcal_0222, α-Amylase from Pyrobaculum calidifontis

Sadaf Ashraf1, Masood Ahmed Siddiqui1*, Kanwal Nisa1, Samar Ali1 and Naeem Rashid2

1Department of Chemistry, Biotechnology Research Laboratory, University of Balochistan, Quetta 87300, Pakistan
2School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan

INTRODUCTION

Carbohydrates are most common and widespread biomolecules in nature and among them starch, a polymer composed of repeating subunits of α-D-glucose residues, is the second most abundant homopolysaccharide on our planet after cellulose (Kujawski et al., 2002). Green plants are the main source of starch that is utilized by various organisms as energy source (Janeček, 1997). Amylose and amylopectin are two types of α-D-glucose polymers found in starches. Amylose is an unbranched polymer of α-D-glucose monomers which are linked through α-1,4 glycosidic bonds. Due to the presence of α-1,6 glycosidic linkage in addition to α-1,4 glycosidic linkage amylopectin exhibits branched polymer shape (Guzman-Maldonado and Paradez-Lopez, 1995). Starch are mixture of two polymers and the ratio of (amylose to amylopectin) depends on various factors such as the origin, plant species, growth conditions, plants organs and their age etc. (Swinkels, 1985; Hii et al., 2012).

Starch hydrolyzing industry converts starch to obtain various useful syrups such as glucose, fructose and dextrose, and sugar alcohols such as erythritol, sorbitol and mannitol. Formerly, industry was using mild acid for the hydrolysis of starch but nowadays, due to the discovery of thermostable and more robust carbohydrates hydrolyzing enzymes, the acid has been replaced by these enzymes.

Normally four different types of enzymes including endoamylase, exoamylase, transferase and debranching enzyme, are used for the complete hydrolysis of starch. Endoamylases, such as α-amylases, hydrolyze α-1,4 glycosidic linkage of the inner part of starch while exoamylases such as glucoamylases and α-glycosidases hydrolyze α-1,4 and α-1,6 linkages on the outer part of the polymer (Van Der Maarel et al., 2002). Enzymes transferases such as amylomaltases and cyclodextrin glycosyltransferases act on donor molecule to cleave α-1,4 glycosidic linkage and transfer part of it to an acceptor molecule to form a new glycosidic linkage. Debranching enzymes such as pullulanases act on α-1,6-glycosidic bond in starch, pullulan, amylopectin and other related oligosaccharides (Van der Maarel et al., 2002; Hii et al., 2012; Rehman et al., 2018).

A large number of wild type and recombinant α-amylases have been purified from all the three domains of life including bacteria, archaea and eukaryotes. Pyrobaculum calidifontis is a hyperthermophilic archaeon isolated from Philippines (Amo et al., 2002). In this study we report on cloning, purification and characterization of a highly thermostable α-amylase from this hyperthermophilic archaeon.

MATERIALS AND METHODS

Chemicals, reagents and enzymes

In current report, all the chemicals and reagents such as restriction enzyme (endonuclease), cloning kit for PCR, kit for DNA extraction, Phusion DNA polymerase for
PCR, DNA ligase, protein and DNA markers used were of analytical grade and bought from Life Sciences Fermentas (USA), New England Biolabs, Inc. (Nebraska, USA), or Sigma (St. Louis, Mo., USA). Chemicals for the Growth of hyperthermophilic archaeon *P. calidifontis* strain were purchased from Nacalai Tesque Inc. (Kyoto).

**Plasmids and bacterial strains**

Plasmid pET101/D-TOPO from Invitrogen (Thermo Fisher Scientific, California, USA) was used as cloning vector. One Shot chemically competent *E. coli* TOP10 cells were used for the cloning of amyl gene and for the expression of cloned gene One Shot™ BL21 Star™ (DE3) cells were used. All reactions and culture were performed at 37°C.

**Culture medium for *P. calidifontis* growth**

*P. calidifontis* cells were cultivated according to previously reported culture medium for the growth of *P. calidifontis* (Amo *et al.*, 2002). The medium in distilled water contained tryptone 1%, yeast extract 0.1% and sodium thiosulphate 0.3%. The cells growth temperature was kept at 90°C.

**Gene cloning into pET101**

For the amplification of *Pcal_0222* gene and insertion of amplified gene in pET101 plasmid, the forward primers Amyl F: 5’- CACCATGTGCGTAGGGAAGTGAGGAG-3’, and a reverse primer Amyl-R: 5’- CTATATAAA- GATTTCAGGAGCCGCCC-3’ were designed according to the available sequence of open reading frame, *Pcal_0222* and pET101 plasmid as previously published by (Shuman, 1991, 1994). The thermal cycler (Gene Amp PCR System 24000, Perkin Elmer, Foster, Calif) was used for the amplification of gene. Phusion DNA polymerase (New England BioLabs inc.) was used for polymerase chain reaction (PCR), under the following conditions. 2 min at 100°C; 25 s at 95°C; 30 s at 55°C and 55 s at 74°C (32 cycles). The amplified PCR product was cleaned, ligated into TOPO pET101 vector to obtain pET101-0222. Commercially available chemically competent *E. coli* cells (One Shot TOP10) were transformed with pET101-AMYL plasmid.

**Electrophoretic analyses**

DNA samples including PCR amplified *Pcal_0222* gene product were analyzed by performing 1.0% agarose gel electrophoresis. The gels were soaked in ethidium bromide solution for staining and DNA bands were visualizing under the UV light. 0.1% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed for the analysis of protein. Protein samples were stained with Coomassie brilliant blue (CBB).

**Production and purification of recombinant *Pcal_0222* protein**

Recombinant *Pcal_0222* protein was produced using *E. coli* BL21 Star™ (DE3) cells. The bacterial cells were transformed with pET101-AMYL plasmid, the cells were grown with shaking at 37°C for 13 hours in 100 μg/mL ampicillin containing Luria-Bertani broth (LB) culture medium. LB medium containing same amount of ampicillin (100 μg/mL) was freshly prepared. 1% preculture was added into culture medium and cells growth was continued. At 660 nm Optical density (OD) of the culture medium was measured hourly to obtain the required OD 0.5 for the purpose of induction of gene expression. 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction. The incubation was continued at 37°C with shaking and after 6 h of induction, the incubation was stopped and *E. coli* cells were collected by centrifugation (6,000 × g, 06 min). The harvested cells were washed with 50 mM sodium acetate buffer (pH 5.5). The pellet was obtained and resuspended in 50 mM sodium acetate buffer (pH 5.5). The cells were lysed by sonication on ice and then to obtain the soluble and insoluble fraction, the lysed cells were centrifuged at 15,000 × g for 20 mins at 4°C. The gene expression for soluble and insoluble fractions was confirmed by SDS-PAGE. The insoluble fraction was solubilized in sodium acetate buffer (50 mM) and (pH 5.5) containing 20% (v/v) glycerol with continuous stirring at room temperature for 06 hrs. The sample was centrifuged at 15,000 × g for 30 mins. The glycerol-amylose containing supernatant was purified according to modified previously reported method (Rashid *et al.*, 2010). The sample was filtered through 0.45 μm pore size containing membrane. The filtrate was loaded on a commercially available (GE Healthcare, UK) prepacked ion exchange chromatography Resource S column. Fast protein liquid chromatography (FPLC) system was used for liquid chromatography. Linear gradient of 50 mM sodium acetate buffer (pH 5.5) containing 1 mol/L NaCl was used to elute the proteins form column at 1 mL/min flow rate. The fractions collected and were checked for amyrase activity. The active fractions were combined, concentrated and applied on a gel filtration column Sephadex G 75 column (2.6 x 70.0 cm). 50 mM sodium acetate buffer (pH 5.5) was used as elution buffer containing 50 mM/L NaCl.

**Molecular mass determination**

The molecular mass of recombinant *Pcal_0222* protein was determined by both SDS-PAGE and gel filtration column. For SDS-PAGE, the pre stained protein calibration kit (Invitrogen) containing 12 marker with
molecular mass 260–3.5 kDa was used. The gel was stained with Coomassie Blue R-350. Protein calibration kit containing cytochrome c (12.3 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), and bovine albumin (67 kDa) was used for gel filtration column.

Enzyme assay

The α-amylase activity was calculated by the amount of reducing sugar liberated during hydrolysis of soluble starch (1%) in 50mM acetate buffer (pH 5.5). The reaction mixture with substrate was incubated at 85°C for 15 min. Reaction mixture without substrate was used as control experiment. The amount of reducing sugar liberated was calculated by the previously reported dinitrosalicylic acid (DNS) method (Miller, 1969). The amount of enzyme that released 1 μmol of reducing sugars in one minute was defined as one unit of enzyme activity.

RESULTS AND DISCUSSION

Sequence analysis of Pcal_0222 gene

The full length of Pcal_0222 gene (GenBank accession number: Pcal_0222), encodes 529 amino acids containing polypeptide with a calculated molecular mass of 58 kDa exhibiting a theoretical isoelectric point as 7.05. A putative signal polypeptide containing 21 amino acids from Met1 to Ala21 was identified using Signal P4.1, indicating the mature protein contains 508 amino acids with calculated molecular mass of 56 kDa. The deduce amino acid sequence of Pcal_0222 exhibited the highest identities with putative α-amylases from Pyrobaculum ferrireducin (NC_016645.1) 62%, and with Pyrobaculum arsenaticum (NC_009376.1), Pyrobaculum oguniense (CP003316.1) and Pyrobaculum islandicum (NC_008701.1) 60% each. It has identities with Pyrobaculum aerophilum (NC_003364.1) 59% and with Thermoproteus tenax (NC_016070.1) 40%. The deduced amino acid sequence (Fig. 1) has four highly conserved regions indicating Pcal_0222 belongs to GH family 13 (Van Der Maarel et al., 2002). The conserved regions I, II, III and IV contain the amino acids DVVNH, GFRLDV AHG, EHVGN and FLENHDTD, respectively. In region I (DVH), region II (RD), region III (E) and region IV (HD) are the conserved amino acids found in α-amylases.

Expression of Pcal_0222 gene and purification of recombinant protein

The chromosomal DNA was obtained by growing the cells of hyperthermophilic archaeon P. calidifontis according to the procedure as described in experimental section. For the amplification of Pcal_0222 gene, forward and reverse primers were constructed. The amplified Pcal_0222 gene was ligated in pET101 vector to obtain pET101-0222. To know the exact amplified Pcal_0222 gene sequence, both strands of the gene were sequenced and no any nucleotide base mutation was observed in both strands. Thus, for the expression of Pcal_0222 gene the E. coli competent BL21 Star™ (DE3) cells were transformed with pET101-0222 plasmid. A number of white colonies were observed on the selection plates. For gene expression a random colony was selected and heterologous gene expression was achieved with induction of 0.1 mM IPTG.
The expression of Pcal_0222 gene in E. coli indicated that ≈95% recombinant protein was produced in insoluble protein aggregates.

Usually the insoluble aggregates do not exhibit enzyme activity (Hockney, 1994; Thomas and Baneyx, 1997) but insoluble aggregates of Pcal_0222 exhibited significant (13 U mg⁻¹) α-amylase activity indicating that the protein was not completely misfolded (Linden et al., 2000). Due to complications in using denaturizing agents for solubilization of proteins (Rudolph and Lilie, 1996) or heating up to certain extent (Dong et al., 1997) we used the glycerol extraction method (Rashid et al., 2010). When the glycerol-Pcal_0222 containing supernatant was applied to ion exchange column (Resource S), Pcal_0222 was eluted from the column with 0.4 mol/L NaCl in 50 mmol/L sodium acetate buffer (pH 5.5). The active fractions after Resource S column were combined, concentrated and further purified by gel filtration column. When purity of the recombinant protein was analyzed by SDS-PAGE, production of 56 kDa recombinant protein was observed (Fig. 2). Approximately 87-fold purification was achieved with a final yield of 290%. The specific activity of purified Pcal_0222 was 1131 U/mg. The purification steps and corresponding activities are summarized in Table I.

### Table I. Purification of recombinant α-amylase from P. calidifontis expressed in E. coli.

| Purification steps   | Total protein (mg) | Total activity (Units) | Specific activity (Units/mg) | Yield % | Purification (fold) |
|----------------------|--------------------|------------------------|-------------------------------|---------|---------------------|
| Centrifugation       | 60.0               | 780                    | 43                            | 100     | 1.0                 |
| Solubilization       | 3.2                | 2922                   | 913                           | 374     | 70                  |
| Resource S column    | 2.3                | 2413                   | 1050                          | 309     | 81                  |
| Gel filtration       | 2.0                | 2262                   | 1131                          | 290     | 87                  |

**Enzymatic properties**

The effects of pH and temperature on amylase activity were determined. The buffers used for pH determination were 50 mM sodium acetate; Tris-HCl and potassium phosphate. The soluble starch was used as substrate for the determination of amylase activity at different pH ranges. The enzyme exhibited highest activity at pH 5.5, the rapid decrease in activity was observed above or below 5.5 (Fig. 3A). Highest activity was observed at 85°C when measured at pH 5.5. Pcal_0222 retained more than 50% activity at 65 and 95°C (Fig. 3B).

In order to know the effect of metal ions, the enzyme activity was measured in 50 mM Na-acetate buffer at pH 5.5 and 85°C in the presence of chloride salt of various metal ions at a final concentration of 1 mM. It was noticed that metal ions are not essential for the activity of Pcal_0222 (Table II). In fact, the activity was reduced in the presence of metal ions. Cu²⁺, Mn²⁺, Ni²⁺ and Zn²⁺ exhibited 47, 86, 82 and 73% inhibitory effect on the activity while Hg²⁺ inhibited the activity completely. Other metals ions Ca²⁺, Co²⁺, Mg²⁺ and Sr²⁺ did not significant affect the enzyme activity.

### Table II. Effect of metal ions on amylase activity.

| Metal ion | Relative activity (%) |
|-----------|-----------------------|
| None      | 100                   |
| Ca²⁺      | 109                   |
| Co²⁺      | 102                   |
| Cu²⁺      | 47                    |
| Hg²⁺      | 02                    |
| Mg²⁺      | 99                    |
| Mn²⁺      | 86                    |
| Ni²⁺      | 82                    |
| Sr²⁺      | 100                   |
| Zn²⁺      | 73                    |

![Fig. 2. The 0.1% SDS-15% polyacrylamide gel electrophoresis (SDS-PAGE) demonstrating the purified α-amylase. Lane 1, Novex™ Sharp Pre-stained Protein Standard; lane 2, cells carrying pET101-AMYL plasmid insoluble fraction; lane 3, cells carrying pET101-AMYL plasmid soluble fraction; lane 4, purified recombinant α-amylase.](image)
CONCLUSION

PcaL_0222, a homologue of α-amylase from hyperthermophilic archaean \textit{P. calidifontis}, is a true α-amylase enzyme which exhibits highest activity at 85°C and pH 5.5. PcaL_0222 is a metal ion independent enzyme. These features make it a good candidate for its use in starch industry.

ACKNOWLEDGEMENTS

The research was partially supported by grant from the University of Balochistan project (No. UBRF-17/033).

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Amo, T., Paje, M.L.F., Inagaki, A., Ezaki, S., Atomi, H. and Imanaka, T., 2002. \textit{Pyrobaculum calidifontis} sp. nov., a novel hyperthermophilic archaean that grows under atmospheric air. \textit{Archaea}, 1: 113-121. https://doi.org/10.1155/2002/616075

Dong, G., Vieille, C., Savchenko, A., and Zeikus, J.G., 1997. Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from \textit{Pyrococcus furiosus} and biochemical characterization of the recombinant enzyme. \textit{Appl. Environ. Microbiol.}, 63: 3569-3576. https://doi.org/10.1128/AEM.63.9.3569-3576.1997

Guzman-Maldonado, H., and Paredes-Lopez, O., 1995. Amylolytic enzymes and products derived from starch: a review. \textit{Crit. Rev. Fd. Sci. Nutr.}, 35: 373-403. https://doi.org/10.1080/10408399509527706

Hii, S., Tan, J.S., Ling, T.C. and Ariff, A.B., 2012. Pullulanase: Role in starch hydrolysis and potential industrial applications. \textit{Enzyme Res.}, 2012: 921362. https://doi.org/10.1155/2012/921362

Hockney R.C., 1994. Recent developments in heterologous protein production in \textit{Escherichia coli}. \textit{Trends Biotechnol.}, 12: 456-463. https://doi.org/10.1016/1072-7795(94)90021-3

Janecek, Š., 1997. α-Amylase family: Molecular biology and evolution. \textit{Prog. Biophys. mol. Biol.}, 67: 67-97. https://doi.org/10.1016/S0079-6107(97)00015-1

Kujawski, M., Ziobro, R. and Gambus, H., 2002. Raw starch degradation by pullulanase. \textit{Acta Sci. Pol. Technol. Aliment.}, 1: 31-35. http://www.food. actapol.net/issue2/volume/4_2_2002.pdf

Linden A., Niehaus F. and Amranikian G., 2000. Single-step purification of a recombinant thermostable α-amylase after solubilization of the enzyme from insoluble aggregates. \textit{J. Chromatogr.}, 737: 253-259. https://doi.org/10.1016/S0378-4347(99)00364-3

Miller, G.L., 1969. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. \textit{Anal. Chem.}, 31: 426-428. https://doi.org/10.1021/ ac60147a030

Rashid, N., Ahmed, N., Haider, M.S. and Haque, I., 2010. Effective solubilization and single-step purification of \textit{Bacillus licheniformis} α-Amylase from Insoluble Aggregates. \textit{Folia Microbiol.}, 55: 133-136. https://doi.org/10.1007/s12223-010-0020-y

Rehman, H., Siddiqui, M.A., Qayyum, A., Bano, A. and Rashid, N., 2018. Gene expression in \textit{Escherichia coli} and purification of recombinant type II pullulanase from a hyperthermophilic archaean, \textit{Pyrobcaculum calidifontis}. \textit{Pakistan J. Zool.}, 46: 1381-1386. https://doi.org/10.17582/journal. pizj/2018.50.4.1381.1386

Rudolph, R. and Lilie, H., 1996. \textit{In vitro} folding of inclusion body proteins. \textit{FASEB J.}, 10: 49-56. https://doi.org/10.1096/fasebj.10.1.8566547

Shuman, S., 1991. Site-specific interaction of vaccinia virus topoisomerase I with duplex DNA. Minimal DNA substrate for strand cleavage in vitro. \textit{J. Biol. Chem.}, 266: 11372-11379.

Shuman, S., 1994. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. \textit{J. Biol. Chem.}, 269: 32678-32684.

Swinkels, J.J.M., 1985. Sources of starch, its chemistry and physics in starch conversion technology (eds. G.M.A. Vaan Beynum and J.A. Roels) Deckker, New York, NY, USA. pp. 115-145.

Thomas J.G., and Baney, F., 1997. Divergent effects of chaperone overexpression and ethanol
supplementation on inclusion body formation in recombinant *Escherichia coli*. *Protein Expr. Purif.*, **11**: 289-296. https://doi.org/10.1006/prep.1997.0796

Van Der Maarel, M.J., Van Der Veen, B., Uitdehaag, J.C., Leemhuis, H. and Dijkhuizen, L., 2002. Properties and applications of starch-converting enzymes of the alpha-amylase family. *J. Biotechnol.*, **94**: 137-155. https://doi.org/10.1016/S0168-1656(01)00407-2