Protein Expression and Purification 110 (2015) 145–150

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

# Functional expression and molecular characterization of *Culex quinquefasciatus* salivary α-glucosidase (MalI)

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ARTICLE INFO

*Article history:*
Received 18 December 2014
and in revised form 20 February 2015
Available online 5 March 2015

*Keywords:*
α-Glucosidase
MalI
Mosquitoes
Expression
*Pichia pastoris*

ABSTRACT

Salivary α-glucosidases (MalI) have been much less characterized when compared with midgut α-glucosidases, which have been studied in depth. Few studies have been reported on the partial characterization of MalI, but no clear function has been ascribed. The aim of this study is to purify and characterize the recombinant *Culex quinquefasciatus* (CQ) α-glucosidase expressed in *Pichia pastoris*. The cDNA encoding mature *Cx. quinquefasciatus* α-glucosidase gene with polyhistidine tag (rCQMalIHis) was successfully cloned into the expression vector, pPICZαB, designated as pPICZαB/*CQMalIHis*. The activity of recombinant rCQMalIHis expressed in *P. pastoris* could be detected at 3.75 U/ml, under optimal culture conditions. The purified rCQMalIHis showed a single band of molecular weight of approximately 92 kDa on SDS–PAGE. After Endoglycosidase H digestion, a single band at 69 kDa was found on SDS–PAGE analysis, suggesting that rCQMalIHis is a glycoprotein. Additionally, tryptic digestion and LC-MALDI MS/MS analysis suggested that the 69 kDa band corresponds to the *Cx. quinquefasciatus* α-glucosidase. Thus, rCQMalIHis is a glycoprotein. The rCQMalIHis exhibited optimum pH and temperature at 5.5 and 35 °C, respectively. The catalytic efficiency ($k_{cat}/K_m$) of the purified rCQMalIHis for maltotriose is higher than those for sucrose, maltotetraose, maltose and p-nitrophenyl-α-glucoside, indicating that the enzyme prefers maltotriose. Additionally, the rCQMalIHis is significantly inhibited by D-gluconic acid δ-lactone, but not by $Mg^{2+}$, $Ca^{2+}$ and EDTA. The rCQMalIHis is strongly inhibited by acarbose with $IC_{50}$ 67.8 ± 5.6 nM, but weakly inhibited by glucose with $IC_{50}$ 115.9 ± 7.3 mM.


## Introduction

An α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is an enzyme catalyzing the hydrolysis of the α-glucosidic bond at the non-reducing end of the glucose moiety. In insects, gut α-glucosidase plays an important role in sugar digestion, supplying monosaccharides to metabolic pathways [1]. Like other insects, not only mosquito midgut but also salivary α-glucosidases can digest oligosaccharides from nectar to supply monosaccharides for metabolism [2–5]. Some reports suggest that mosquitoes fed with sucrose show increased longevity, fecundity, and available energy [6,7]. Sucrose is very important for their survival, and its digestion into monosaccharides requires the pivotal enzyme, α-glucosidase. There have been few reports on isolation and characterization of α-glucosidases from both mosquito salivary gland and midgut. Three isozymes of midgut *Anopheles aquasalis* α-glucosidases were isolated and characterized, but their roles in hydrolysis of sucrose and other glycosides have not been yet determined [5]. The recombinant midgut *Culex pipiens* (Cpm1) and *Cx. quinquefasciatus* (Cqm1) α-glucosidases could be expressed in *Escherichia coli*, but while only low activities were detected, these enzymes were defined as receptors of insecticidal binary (Bin) toxin from *Bacillus sphaericus* [8,9]. Recently, the recombinant midgut *Anopheles gambiae* α-glucosidase (Agm1) was also found to show high affinity with Cry11Ba toxin of *Bacillus thuringiensis* subsp. jegathesan [10]. In *Anopheles darlingi*, *Aedes aegypti* and *Aedes albopictus*, salivary α-glucosidases (MalI) were accumulated in the proximal portion of the lateral lobes [2–4]. An *Ae. aegypti MalI* gene was also sequenced and characterized for its expression patterns [11]. However, there is little information on the molecular characterization of mosquito salivary α-glucosidase. Recently, a

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http://dx.doi.org/10.1016/j.pep.2015.02.018
1046-5928/

study on control mites showed that inhibition of α-glucosidase activities by acarbose had a suppressive effect on their growth rate [12]. Thus, inhibitors targeting mosquito α-glucosidases could be used to delay glucose production, affecting mosquito growth and development, and are of interest as for potential insecticides.

As mentioned, while midgut α-glucosidases have been studied in depth, particularly in the *Culex* genus, salivary α-glucosidases (MalI) from this genus have not been thoroughly characterized. Although MalI has been proposed to be involved in the solubilization and intracellular digestion of disaccharides [3,5], so far there has been no report on its functional expression and molecular characterization. In addition, the recombinant MalI may be useful in searching for specific salivary α-glucosidase inhibitors, which may later be applied to study functions of MalI and vector control. Since the small amount of salivary α-glucosidases in mosquitoes is not sufficient to allow molecular characterization and inhibitor screening, functional expression of the MalI is required for the production of its high amounts. In this study, recombinant expression of functional MalI is required for its characterization.

## Materials and methods

### *Cloning the full length cDNA encoding Cx. quinquefasciatus CQMalIHis*

Using TRIZOL reagent (Invitrogen, USA), total RNA was extracted from *Cx. quinquefasciatus* females. With total RNA extract as a template, the first strand cDNA was synthesized by reverse transcription. Total cDNA was used as template for PCR-amplified α-glucosidase gene (*CQMalI*) by using KOD hot start polymerase (Novagen). Specific primers were designed from the sequences of salivary of *Cx. quinquefasciatus* α-glucosidase (NCBI Reference Sequence: XP_001866573); forward primer: 5′-ATGAAGACC TTCGCACCCCTGCTGG-3′ and reverse primer: 5′-TTATGCCTCGTA CACG ACGACCGG-3′. The PCR-product of *CQMalI* gene was amplified, an A-overhang was added before ligation into pGEM®-T easy vector, and designated as pGEM-T/*CQMalI* extracted from positive transformants, and then sequenced at Macrogen Inc., Korea.

### *Construction of the pPICZαB plasmid harboring CQMalIHis*

A *CQMalI* gene without signal peptide, which was designed with polyhistidine tag at C-terminus, was amplified with a PCR instrument using the pGEM-T/*CQMalI* as a template together, with designed forward primer containing the *Xho*I site: CQagluXhoF (5′-CCCCCC**CTCGAG**AAAAGAGAGGCTGAAGCTCTGCAATGGTGGGAG CACGG-3′), and reverse primer containing polyhistidine tag and *Xba*I site: CQHXbaR (5′-GGGGGG**TCTAGA**TAATGATGATGATGAT GATGTGCCTCGTACACGACGACCGG-3′). The PCR product was cloned into pPICZαB vector. The construction of the recombinant plasmid, designated as pPICZαB/*CQMalIHis,* was confirmed by DNA sequencing at Macrogen Inc., Korea.

### *Transformation and selection of Pichia pastoris clones secreting rCQMalIHis*

Transformation of recombinant plasmid pPICZαB/*CQMalIHis* into *P. pastoris* GS115 was performed according to the method of Invitrogen. The pPICZαB/*CQMalIHis* (5–10 μg) was linearized by *Sac*I, and then electroporated into the competent cells. Positive transformants were screened from YPDS plates containing 150 μg/ml Zeocin. Initially, each colony harboring pPICZαB/*CQMalIHis* was inoculated in 400 ml BMGY medium and then cultivated in a shaking incubator at 30 °C and 250 rpm, until $A_{600}$ reached 5. The cells were harvested and resuspended in 200 ml BMMY medium. To maintain induction, methanol at 0.5% was added to the culture medium every 24 h for five days. The cell-free supernatants (CFS)[1] were collected for α-glucosidase activity assay and protein profiles analyzed by SDS–PAGE. The *P. pastoris* harboring pPICZαB was used as a control.

### *Enzyme assay and protein determination*

Two assay methods were used for measuring α-glucosidase activity. First, the pNP assay was used to study the glycone specificity. The assay was performed by incubating the enzyme solution with 2 mM p-nitrophenyl-α-D-glucoside (pNPαGlc) for 10 min at 37 °C in 50 mM sodium acetate pH 5.0. After terminating the reaction by adding 500 mM $Na_2CO_3$, the absorbance of released p-nitrophenol was measured at 400 nm. One unit of enzyme was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min at pH 5.0 and 37 °C.

The second method is used to investigate the natural substrates or aglycone specificity. The enzyme solution was incubated with 2 mM of each substrate for 10 min at 37 °C in 50 mM sodium acetate pH 5.0. The reaction was terminated by boiling for 5 min and the glucose release was determined by a glucose oxidase/peroxidase assay, as described previously [13]. One unit of enzyme was defined as the amount of enzyme releasing 1 μmol of glucose per min at pH 5.0 and 37 °C. Protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad, USA) or measured by a NanoDrop ND-1000 spectrophotometer.

### *Purification of the rCQMalIHis*

All purification procedures were performed at 4 °C. The CFS (200 ml) containing rCQMalIHis was precipitated with 80% ammonium sulfate and harvested. The pellet was resuspended in 20 mM sodium phosphate, pH 7.4 (buffer A) containing 300 mM NaCl. The dialyate (27.5 ml) was then loaded onto a Nickel-Sepharose column (1 × 4 cm) equilibrated with buffer A containing 300 mM NaCl. The column was first washed with buffer A containing 300 mM NaCl and then eluted with a stepwise gradient of 3 CV of 100, 200, and 300 mM imidazole in buffer A containing 300 mM NaCl. Eluted fractions containing enzyme activity (5 ml) were pooled and concentrated by using ultrafiltration through a centricon membrane with molecular weight cut off of 10 kDa. The concentrated sample (1.5 ml) was then applied onto the first Sephacryl S-200 column (1.5 × 90 cm) run in buffer A containing 200 mM NaCl. Only fractions containing high enzyme activity were pooled and applied onto the second Sephacryl S-200 column (1.5 × 90 cm) with the same conditions.

### *SDS–PAGE and Native-PAGE analysis*

SDS–PAGE was carried out according to Laemmli's method [14] and stained by coomassie brilliant blue R-250. To detect the α-glucosidase activity band on 8% Native-PAGE run at 4 °C, the gel was soaked in 50 mM sodium acetate buffer pH 5.0 containing 2 mM 4-methylumbelliferyl(4-MU)-α-D-glucoside at 37 °C for 15 min, before being visualized.

### *Native molecular weight determination*

Gel filtration was performed in an AKTA system (GE, USA) by using a Superdex200 10/30 column (GE, USA) run in buffer A containing 150 mM NaCl. Fractions of 1 ml were collected at a flow rate of 0.3 ml/min. Relative molecular weight was calculated by

[1] *Abbreviations used:* CQ, *Culex quinquefasciatus*; CFS, cell-free supernatants; pNPαGlc, p-nitrophenyl-α-D-glucoside; EndoH, Endoglycosidase H.