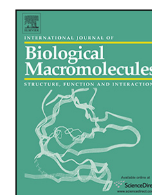
International Journal of Biological Macromolecules 76 (2015) 230–235

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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



Identification of essential tryptophan in amylomaltase from *Corynebacterium glutamicum*

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ARTICLE INFO

Article history:
Received 16 December 2014
Received in revised form 25 February 2015
Accepted 25 February 2015
Available online 4 March 2015

Keywords:
Amylomaltase
Chemical modification
N-bromosuccinimide
Site-directed mutagenesis
Essential tryptophan

ABSTRACT

This work aims to identify essential tryptophan residue(s) of amylomaltase from *Corynebacterium glutamicum* (*Cg*AM) through chemical modification and site-directed mutagenesis techniques. The recombinant enzyme expressed by *Escherichia coli* was purified and treated with *N*-bromosuccinimide (NBS), a modifying agent for tryptophan. A significant decrease in enzyme activity was observed indicating that tryptophan is important for catalysis. Inactivation kinetics with NBS resulted in pseudo first-order rate constant (k_{inact}) of 2.31 min^{-1}. Substrate protection experiment confirmed the active site localization of the NBS-modified tryptophan residue(s) in *Cg*AM. Site-directed mutagenesis was performed on W330, W425 and W673 to localize essential tryptophan residues. Substitution by alanine resulted in the loss of intra- and intermolecular transglucosylation activities for all mutated *Cg*AMs. Analysis of circular dichroism spectra showed no change in the secondary structure of W425A but a significant change for W330A and W673A from that of the WT. From these results in combination with X-ray structural data and interpretation from the binding interactions in the active site region, W425 was confirmed to be essential for catalytic activity of *Cg*AM. The hydrophobicity of this tryptophan was thought to be critical for substrate binding and supporting catalytic action of the three carboxylate residues at the active site.

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1. Introduction

4-α-Glucanotransferase (4α-GTase) belongs to the α-amylase family; it catalyzes the intramolecular and intermolecular transglucosylation reaction of α-1,4-glucan. The two main types of enzymes in this group are cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) and amylomaltase (AM, EC 2.4.1.25). AM catalyzes a similar reaction to CGTase, but it is an intracellular enzyme which forms large-ring cyclodextrin (LR-CD) as the major cyclization product. A similar enzyme to AM in plants is called disproportionating enzyme (D-enzyme). AM and D-enzyme are classified in the glycoside hydrolase GH77 while CGTase and α-amylase are part of the GH13 family. GH77 enzymes are efficient 4α-GTases with remarkably low hydrolytic activity compared to GH13 enzymes [1].

Amylomaltases have many applications. Firstly, they are used for the production of LR-CDs with a degree of polymerization (DP) of 17 up (DP ≥ 17) [1,2]. LR-CDs can form complexes with organic [1] or inorganic molecules [3,4], which have potential applications in food science, pharmaceuticals and biotechnology [5]. Secondly, they are used in the synthesis of functional oligosaccharides, such as a prebiotic isomalto-oligosaccharide (IMO) as neutraceuticals with health benefits [6]. The use of AM for the synthesis of maltooligosyl-sucrose, an anticariogenic oligosaccharide, has also been reported [7]. Recently, AMs have been used in the production of a thermoreversible starch gel to be used as a substitute for gelatin or agar in food products [6].

Up to now, the three dimensional structures of only six AMs have been determined [8–15]. In *Thermus aquaticus*, the core of the catalytic cleft that contains seven conserved residues includes three catalytic residues, Asp293, Glu340 and Asp395, and four binding residues, Tyr59, Asp213, Arg291 and His394 [14]. These seven residues possibly form the minimum necessary active site environment for the enzymes in α-amylase family [14]. In addition to the catalytic core, two loops (250s and 460s) containing many important hydrophobic residues lie close to the active center of AMs were reported. A unique long extended 250s loop in *Thermus* AM partially covers the active site, and was proposed to be important for the binding of substrates and dissociation of products. This loop is conserved in the GH77 family but is absent in the GH13 and GH57 families of α-amylase [14,15]. A Trp258 residue in the

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http://dx.doi.org/10.1016/j.ijbiomac.2015.02.035
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250s loop, which exists only in the amylomaltase group and not in other glycoside hydrolases or α-amylase, has been suggested to be responsible for reaction specificity and for governing the product ring size. The mutated W258G exhibited no hydrolytic activity and showed neither cyclization nor coupling activity, suggesting that Trp258 plays an essential role in catalytic activity of *Thermus* AM [10].

Our research group has characterized a novel AM from a mesophilic *Corynebacterium glutamicum* (*Cg*AM) with amino acid sequence identity of 28% as compared to the extensively studied AM from a thermophilic *T.* aquaticus (*Ta*AM) [16]. *Cg*AM has a size of 84 kDa. In view of differences in the amino acid sequence and enzyme properties in comparison to *Ta*AM, our preliminary investigation on the essential residues for *Cg*AM activity was explored using chemical modification. This modification technique is useful for studying structure–function relationship of proteins when the three-dimensional structures are not known. From preliminary result, we found that enzyme inactivation by the modification of carboxylic amino acids known to be catalytic residues of most enzymes in α-amylases including AMs yielded a moderate loss of *Cg*AM activity. This finding was in agreement with the three-dimensional structural information of a few *Thermus* AMs in that they contain two unique loops that are closed and partially cover the active site cleft consisting of the three catalytic acidic side chains [14]; thus, these catalytic residues should be well protected from inactivation by group-specific reagents. In contrast to the partial activity loss resulted from carboxylate modification in *Cg*AM, modification of tryptophan led to an almost total loss of enzyme activity. Thus, the aim of this work was to identify the essential tryptophan residue(s) of *Cg*AM through the combined use of chemical modification and site-directed mutagenesis techniques.

2. Materials and methods

2.1. Chemicals

N-bromosuccinimide (NBS), soluble potato starch, ampicillin, β-D-1-thiogalacto-pyranoside (IPTG) and acarbose were purchased from Sigma Chemical Co. (St. Louis, USA). Standard LR-CD (CD22–CD50) was a product of Ezaki Glico Co. Ltd. (Japan). Glucose was from BDH (England) while maltose and maltotriose were from Fluka (Switzerland). Pea starch was kindly provided by Emsland-Stärke GmbH (Emlichheim, Germany). Glucose oxidase kit was a product of Human Biochemical and Diagnostics mbH (Germany). *Pfu* DNA polymerase was form Promega (USA). All other chemicals and solvents used were of analytical grade.

2.2. Gene construction of mutated AMs

Mutated *CgAMs* were constructed by site-directed mutagenesis. The *CgAM* gene in the pET-19b [16] was used as a template DNA, and the following synthetic oligonucleotides were constructed as primers for mutagenesis work at W330, W425 and W673.

W330A_FWD: CTTATTGATTTCGCCACC<u>GCG</u>TGCGCGGACCGCGAAACTG and
W330A_REV: CAGTTTCGCGGTCCGCGCA<u>CGC</u>GGTGGCGAAATCAATAAG
or W425A_FWD: CAGCAGGGCCAAGAC<u>GCG</u>TCCCAGCCACCATGG and
W425A_REV: CCATGGTGGCTGGGA<u>CGC</u>GTCTTGGCCCTGCTG
or W673A_FWD: GGATATGTATCCCAAC<u>GCG</u>TGTATCCCACTG and
W673A_REV: CAGTGGGATACA<u>CGC</u>GTTGGGATACATATCC

The underlined letters were coded for the mutated residues. The PCR program was 2 min at 95 °C, 16 cycles of: 1 min at 95 °C, 1 min at 60 °C, followed by final elongation at 72 °C for 12 min. The PCR products were digested with *Dpn* I endonuclease. *Dpn* I was used to digest the parental DNA template to select for the mutation-containing synthesized DNA and the PCR product was then transformed into *E. coli* BL21 (DE3) as host cells. All mutations were confirmed by DNA sequencing analysis.

2.3. Bacterial cultivation and enzyme production

A single colony of *E. coli* BL21 (DE3) recombinants harboring the wild-type (WT) [16], W330A, W425A or W673A genes was inoculated and cultured in LB medium (0.5% NaCl, 0.5% yeast extract and 1% tryptone, w/v) containing 100 μg ml^{-1} ampicillin. Incubation at 37 °C with 250 rpm rotary shaking was performed as previously described [16]. Enzyme expression was induced by adding 0.4 mM of IPTG. The cells were collected by centrifugation at 12,000 × *g*, 4 °C for 30 min, broken by sonication, and the cell debris was removed. The supernatant which contained crude *Cg*AM was collected.

2.4. Purification of CgAMs

The crude WT or mutated *Cg*AMs (W330A, W425A or W673A) was purified by Histrap affinity column chromatography (GE Healthcare, UK, Histrap FF™, 1 ml column). Unbound proteins were washed off by 50 mM phosphate buffer containing 20 mM imidazole and 0.5 M NaCl, pH 7.4. The purified enzyme was eluted in the same buffer but with 500 mM imidazole [16].

2.5. Assay of CgAM activity

The activities of WT and mutated enzymes were measured by the following assays. The mean values of activities were determined from three independent experiments.

2.5.1. Starch transglucosylation activity

The enzyme solution was incubated with 0.2% (w/v) soluble potato starch and 1% (w/v) maltose in 50 mM phosphate buffer, pH 6.0 in a total volume of 1.0 ml at 30 °C for 10 min, and terminated by heating the solution at 100 °C. The absorbance at 600 nm was measured after adding iodine solution as previously described [16,17]. One unit was defined as the amount of enzyme that produced 1% decrease in the color of starch–iodine complex per min under the described conditions.

2.5.2. Disproportionation activity

The 50 μl reaction mixture containing 100 mM maltotriose and enzyme in 50 mM phosphate buffer pH 6.0 was incubated at 30 °C for 10 min. After adding 30 μl of 1 N HCl to stop the reaction, the product was reacted with 0.92 ml of glucose oxidase reagent for 10 min, and the absorbance was measured at 505 nm [18]. One unit was defined as the amount of enzyme which produced 1 μmol of glucose per min under the described conditions.

2.5.3. Cyclization activity

The 1.5 ml reaction mixture containing 2% (w/w) pea starch and enzyme in 50 mM phosphate buffer, pH 6.0 was incubated at 30 °C for 90 min and stopped by boiling. Eight unit of glucoamylase was added and incubated at 40 °C for 30 min to hydrolyze linear maltodextrin, then boiled to stop the reaction. The cycloamylose (CA) product formed was measured by high performance anion exchange chromatography with pulsed amperometric detection technique (HPAEC-PAD) [16]. One unit of enzyme was defined as the amount of enzyme which produced 1 nC of CD34 per min under the described conditions.

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