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Allosteric properties of *Geobacillus* maltogenic amylase

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ABSTRACT

Maltogenic amylases (MAases, EC 3.2.1.133) have been gotten much attention due to their various applications in industry and commercial processes. MAases belong to subfamily 20 of glycoside hydrolase family 13 (NPase or CDases subfamily) and they have important differences with other members of the family. This enzyme consists of two subunits which form two active sites in the dimer form by binding of the central domain of each subunit to the N domain of the next one (domain-swapping dimeric structure). Allosterism is a possible way of regulating enzymatic activity and no evidence has been found regarding to the cooperativity and correlation between MAases subunits, therefore in this study the allosteric behavior of MAases from a native strain (*Geobacillus* sp. Gh6) was investigated. Unlike other members of α-amylase family, MAases showed positive cooperativity between their subunits and the enzyme exhibited sigmoidal nature towards all three cyclodextrin (CD) substrates with a Hill constant (*nH*) value equal to 2, 1.6 and 1.1 for α-CD, β-CD and γ-CD, respectively. On further analysis, the effect of glucose and maltose as MAases allosteric effectors in the presence of β-CD substrate showed that these two effectors had a biphasic effect; while they stimulated the enzyme activity at low concentrations (with a decrease in Hill constant), these metabolites acted as allosteric inhibitors at higher concentrations. Due to the key role of MAases in carbohydrate metabolization, an efficient regulating system for this enzyme is required. In this experiment, for the first time the allosteric properties of MAases were observed and investigated.

1. Introduction

Oligomerization is an important property of many natural enzymes which provides an additional level of complexity and plays an important role in numerous biological processes including increased catalytic efficiency, thermostability, and regulation of biological systems and also helps to minimize the size of a cell's genome [1]. Indeed, changes in enzymatic activity accompany with oligomer dissociation commonly are part of the allosteric regulation mechanisms in a number of enzymes [2]. One of the common mechanisms for protein oligomerization is three-dimensional domain swapping, in which a segment of the monomeric protein is replaced by an identical segment of a second monomer [3,4]. Only a few enzymes in the glycoside hydrolase family 13 (GH13), which belongs to α-amylase family, can form oligomers [3,5]. α −Amylase family is one of the most important members of clan H of glycosyl hydrolases and it includes many popular and important enzymes present in nature [6]. These enzymes are classified into three families (GH13, 70 and 77) in the sequence/structure-based glycoside hydrolase family system [7]. Glycoside hydrolase family 13 (GH13) includes the majority of the enzymes like α-amylase. Also, enzymes with a spectrum of other starch-modifying and hydrolyzing activities belong to the family, including neopullulanses (NPases; EC 3.2.1.135), maltogenic amylases (MAase; EC 3.2.1.133), cyclomaltodextrinases (CDases; EC 3.2.1.54) and *Thermoactinomyces vulgaris* α-amylase II (TVAII) [8,9]. Maltogenic amylase (MAase; EC 3.2.1.133) from *Thermus* (ThMA), which is classified under subfamily 20 of GH13, have 98% homology with our under study enzyme [10]. These enzymes have an extra N-terminal domain (N-domain) which mediates their domain-swapping dimeric structure Fig. 1 [11]. The crystal structure of ThMA shows that this enzyme is a homodimer and the unique N-terminal domain of one monomer is critically involved in dimerization by partly covering the top of the active-site cleft of the other monomer. The dimerization of the enzyme modifies the wide and highly accessible active-site cleft which forms a narrow and deep groove-shaped substrate-binding site [12–14].

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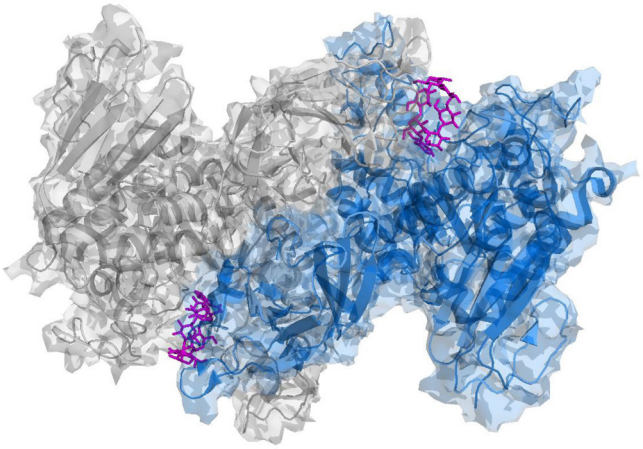

Fig. 1. Ribbon and surface representation of the domain-swapped homodimeric structure of *Thermus* sp. maltogenic amylase (PDB ID: 1GVI) with β-cyclodextrin (shown in purpl) in the active sites.

MAases are very unique because of their multi-substrate specificity toward starch, pullulan and CDs. This specificity is modulated by enzyme dimerization, which is distinctly different from the substrate specificity of α-amylases [15]. Dimeric MAases differ from typical α-amylases by: (1) being intracellular; (2) preferring CDs to starch or pullulan as substrate; and (3) exhibiting both high transglycosylation and hydrolytic activity on various substrates [12]. An aromatic platform in the active site was identified which is responsible in substrate recognition especially in determining the enzyme's preference toward β-CD using docking, molecular dynamics simulation and subsite structure analysis of a maltogenic amylase from *Bacillus lehensis* G1 [16].

Recently, Maases have been gained so much attention due to their industrial applications, including baking industry and starch conversion applications as anti-stalling agent for increasing shelf life, the synthesis of noncarcinogenic sweeteners, synthesis of novel carbohydrates and the development of new drugs for treatment of obesity, hyperlipidemia, dental caries and diabetes [13,17,18]. Several attempts have recently been done to improve the catalytic properties of maltogenic amylase in order to use in industry such as enhancing maltose production through mutagenesis of acceptor binding subsite +2 in maltogenic amylase [19]. There are many literatures about the purification, structural and functional peropreties of *Thermus*, *Bacillus stearothermophilus* and *Geobacillus* MAase, but there isn't much information on the allosteric properties of MAases and their domained relationship [9,11,20,21]. This information has major role in enzymatic characteristics and it can lead us to unique properties of MAase. For this reason, the allosteric characterization of the intracellular MAase from a thermophilic *Geobacillus* spp. was investigated for the first time in this study. In addition, the effects of some effectors on the allosteric behaviors of this enzyme were studied. The under study *Geobacillus* MAase is previously isolated from Gheynarge hot spring in Iran and it is more thermostable than that of dimeric MAases reported earlier in other species [22].

2. Materials and methods

2.1. Chemicals

3, 5-Dinitrosalicylic acid (DNS), phenylmethylsulfonyl fluoride (PMSF) and sodium dodecyl sulfate (SDS) were provided from Bioneer (Landon, UK). Isopropyl-D-thiogalactopyranoside (IPTG) and Kanamycin were obtained from Vivantis (Selangor Darul Ehsan, Malaysia) and Bio Basic INC (Markham, Canada), respectively. The Ni-NTA agarose, agarose and imidazole were purchased from Qiagen (Hilden, Germany), Acros (New Jersey, USA) and Applichem (Darmstadt, Germany), orderly. Glucose, Maltose, NaCl, NaH_2PO_4, and all other chemicals (reagent grade) were obtained from Merck (Darmstadt, Germany).

2.2. Expression and purification of maltogenic amylase

Expression and purification of His-tagged maltogenic amylase was carried out using pET28a expression vector containing maltogenic amylase gene from *Geobacillus* sp. Gh6 (GenBank accession No. GQ884176) in *Escherichia coli* BL21 (DE3) [22]. To produce protein, the *Escherichia coli* strain BL21(DE3) harboring the maltogenic amylase expression plasmid was cultivated in 5 ml of Luria-Bertani broth (LB) medium with 50 μg/ml kanamycin at 37 °C with reciprocal shaking (250 rpm min^{-1}) for 12–14 h. By reaching the optical density (OD_{600}) to 0.6, the cultures were induced with 1 mM IPGT. After adding IPTG, the cultivation process was continued for 6 h at 27 °C. Finally, the medium was cooled down and the cells were harvested by centrifuging at 5000 × g for 20 min and then the supernatant was discarded and the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 30 mM NaCl and 1 mM phenyl methane sulfonyl fluoride (PMSF), pH 8.0), the process was followed by disrupting *via* sonication process in an ice bath and the obtained suspension was clarified by centrifugation (12000 × *g*, 20 min, 4 °C) and the obtained proteins were applied on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and/or stored at −70 °C. Purification of His-tagged fusion proteins was carried out using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column. Bound proteins were eluted with buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0.

SDS-PAGE was carried out using a 12% (w/v) polyacrylamide gel [23] and protein bands were detected by Coomassie Brilliant Blue R250 staining. The protein concentration was determined by Bradford method [24], with bovine serum albumin as a standard.

2.3. Maltogenic amylase assay

Maltogenic amylase activity was assayed using the dinitrosalicylic acid (DNS) *via* the method described by Miller [11,25]. A mixture of sodium-acetate buffer (50 mM, pH 6.0) and 50 μl 1% α-, β-CD and γ-CD in the same buffer was pre-warmed at 60 °C. Next, the enzyme solution was added and the solution was incubated at 60 °C for 10 min. Then the reaction was stopped and colorized by adding DNS solution. The mixture was boiled for 5 min in a water bath, then cooled to room temperature, and the absorbance was measured at 540 nm. A standard curve of absorbance against amount of different concentrations of maltose was plotted. All reactions were performed under the optimum conditions (60 °C and pH 6.0) [26,27].

2.4. Allosteric properties of maltogenic amylase

The catalytic activity of enzyme was investigated at the presence of different amounts of α, β, γ-CD substrates (0–1.5%) under the assay conditions which was described in Section 2.3. In addition, the effect of 0.02% glucose and maltose in the presence of β-cyclodextrin (β-CD appeared to be the most preferred substrate) were investigated. The data were analyzed by GraphPad Prism software, version 5.04 and $K_{0.5}$, nH and V_{max} parameters were calculated.

2.5. Statistical analysis

All experiments were performed in triplicate. The results were expressed as means ± SD and statistical differences were evaluated

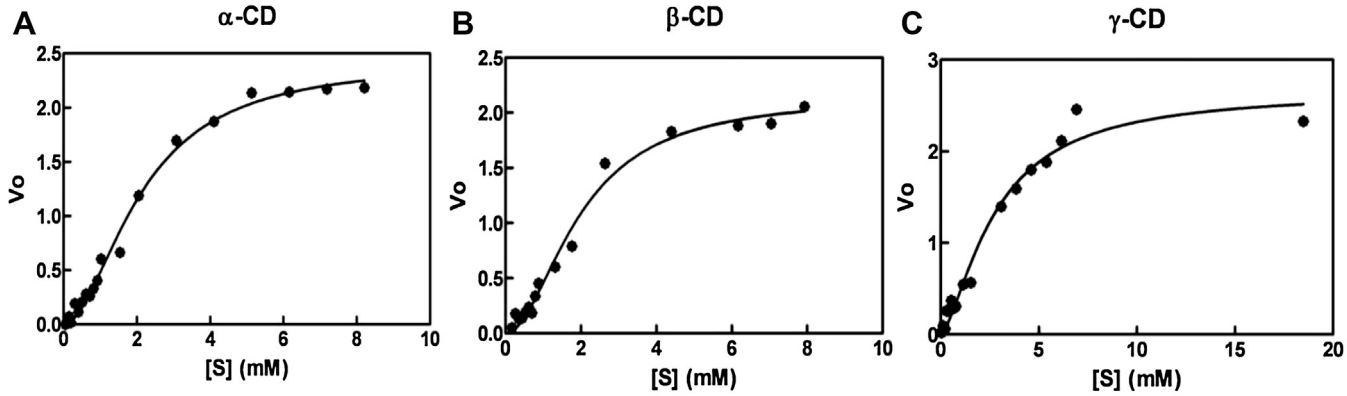

Fig. 2. Kinetic analysis of *Geobacillus* sp. Gh6 maltogenic amylase for hydrolysis of various substrates. The initial reaction rates were plotted against different concentrations of α-CD (A), β-CD (B) and (C) γ-CD substrates for maltogenic amylase. Data were fitted to sigmoidal curves using nonlinear regression with Graphpad Prism.

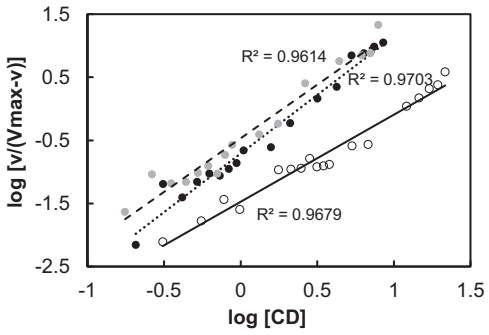

Fig. 3. Hill plots for substrate-saturation curves of *Geobacillus* sp. Gh6 maltogenic amylase for hydrolysis of various substrates including α-CD (●, dotted line), β-CD (●, dashed line) and γ-CD (○, solid line).

Table 1
Kinetic parameters of *Geobacillus* sp. Gh6 maltogenic amylase for hydrolysis of cyclodextrin substrates. The values were computed by non-linear regression of sigmoidal curves and Hill plots using Graphpad Prism. Measurements were repeated for three times. Different letters indicate that V_{max}, $K_{0.5}$ and nH values are significantly variable as determined by Tukey's test ($p < 0.05$).

Substrate	V_{max} (mM/min)	$K_{0.5}$ (mM)	nH
α-CD	$2.431 \pm 0.11^{a*}$	4.079 ± 0.22^{a}	2.0 ± 0.19^{a}
β-CD	2.152 ± 0.08^{b}	3.723 ± 0.13^{a}	1.6 ± 0.21^{a}
γ-CD	2.662 ± 0.04^{c}	4.579 ± 0.16^{b}	1.1 ± 0.11^{b}

* Different letters indicate that Vmax, K0.5 and nH of enzyme is significantly variable as determined by Tukey's test ($p < 0.05$).

by ANOVA, using SPSS. Tukey's test is used to determine significant difference between data (To determine which means amongst a set of means differ from the rest). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Investigation of allosteric properties of MAase

To investigate the allosteric behavior of the MAase, the substrate-activity curves and Hill plot for α-, β- and γ-cyclodextrin were depicted (Figs. 2 and 3). The maltogenic amylase was shown non-hyperbolic kinetics in the presence of all types of cyclodextrin as substrate, but instead had sigmoidal behavior. The sigmoidal nature of the velocity curve observable increased in the order of α-CD > β-CD > γ-CD. The $K_{0.5}$ and V_{max} values of the enzyme for each substrate were determined by non-linear regression of sigmoidal curves using Graphpad Prism. Results are summarized in Table 1.

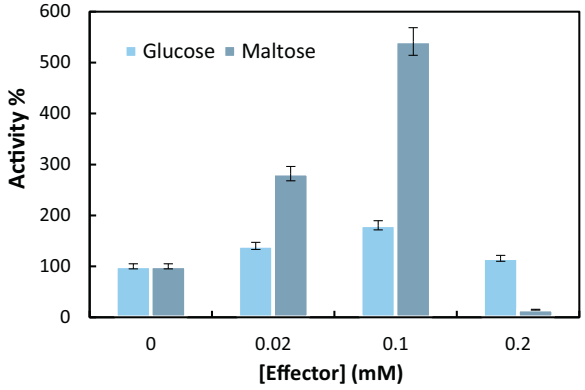

Fig. 4. Effect of glucose and maltose in various concentrations on the hydroltsis activity of the maltogenic amylase using β-CD substrate. The results represent mean ± SD of triplicate experiments.

The amount of $K_{0.5}$ for α-CD, β-CD and γ-CD were obtained 4.079, 3.723 and 4.579, respectively. In addition, calculation of Hill constant (nH) using the Hill equation revealed a decrease of nH for CD substrates in the order of α-CD > β-CD > γ-CD. The results revealed a significant increase in cooperativity with decrease in substrate size. MAase has nH of 2, 1.6 and 1.1 in the presence of α-CD β-CD and γ-CD, respectively.

3.2. Effect of glucose and maltose on the activity of MAase

The effect of glucose and maltose on MAase activity was studied in the presence of β-CD as substrate. Low concentrations of glucose and maltose (up to 0.1%) led to an increase in the activity of the enzyme, but by increasing their concentrations, a significant reduction was observed in the activity of the enzyme. The results showed that the concentration of maltose had more opposite effects than glucose (Fig. 4).

3.3. Effect of glucose and maltose as effectors on allosteric behavior of MAase

To investigate the effect of glucose and maltose as effectors on allosteric behavior of the MAase, the substrate-activity curves and Hill plot for β-CD were provided in the presence of 0.02% glucose and maltose as effectors (Figs. 5 and 6). Glucose and maltosein this concentration, as allosteric activators, and tend to increase the hyperbolic nature of the velocity curve. The $K_{0.5}$ and V_{max} values of the enzyme in the presence of these effectors were determined for β-CD substrate using Graphpad Prism and the Hill constants (nH)

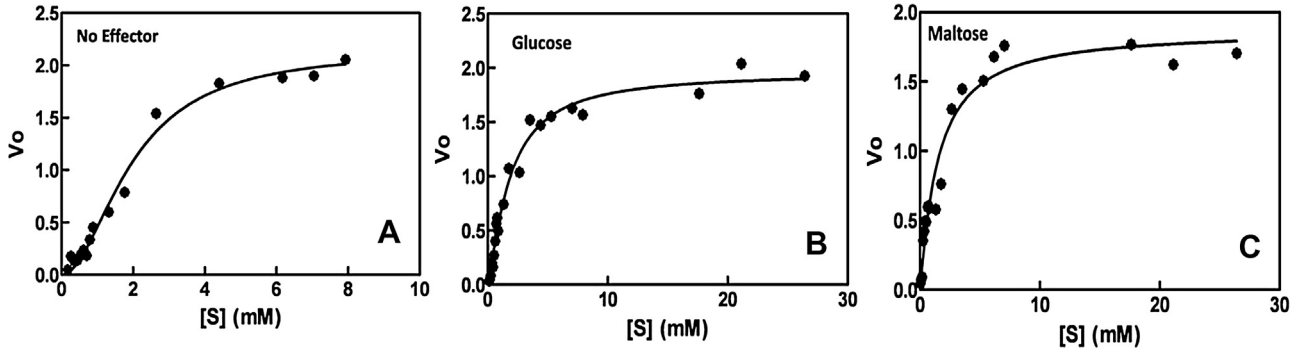

Fig. 5. Effect of glucose and maltose on substrate-activity curves of *Geobacillus* sp. Gh6 maltogenic amylase for hydrolysis of β-CD substrate. Final concentration of these additives was 0.02% (w/v). The initial reaction rates were plotted against different substrate concentrations using Graphpad Prism in the absence (A) and presence of glucose (B) and maltose (C) as effector.

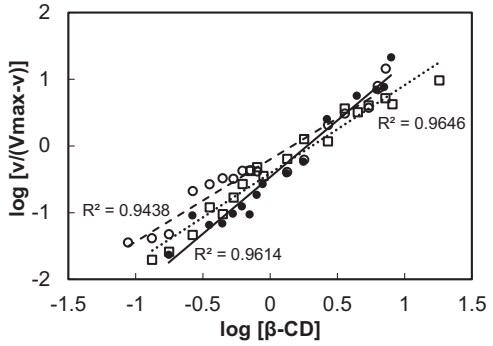

Fig. 6. Hill plots for substrate-saturation curves of *Geobacillus* sp. Gh6 maltogenic amylase for hydrolysis of β-CD in the absence (●, solid line) and presence of glucose (□, dotted line) and maltose (○, dashed line) as effectors. Final concentration of these additives was 0.02% (w/v).

Table 2
Effect of glucose and maltose as effectors on allosteric behavior of *Geobacillus* sp. Gh6 maltogenic amylase. The kinetic parameters of the enzyme in the presence of these effectors were determined for β-CD substrate using Graphpad Prism. Measurements were repeated for three times. Different letters indicate that V_{max}, $K_{0.5}$ and nH values in different conditions are significantly variable as determined by Tukey's test ($p < 0.05$).

Effector	V_{max} (mM/min)	$K_{0.5}$ (mM)	nH
No Effector	$2.152 \pm 0.06^{a*}$	3.723 ± 0.42^{a}	1.6 ± 0.08^{a}
Glucose	1.962 ± 0.05^{b}	2.176 ± 0.09^{b}	1.3 ± 0.07^{b}
Maltose	1.869 ± 0.11^{b}	1.556 ± 0.11^{c}	1.2 ± 0.12^{b}

* Different letters indicate that V_{max}, $K_{0.5}$ and nH of enzyme in different conditions is significantly variable as determined by Tukey's test ($p < 0.05$).

using the Hill equation were calculated for the enzyme in the presence of each effector. As shown in Table 2, a significant decrease in $K_{0.5}$ and nH of MAase was occurred in the presence of these effectors. all above results together indicate a reduction of MAase allosteric property by these two effectors.

4. Discussion

α-Amylases are known to act as a monomer, however MAases can be extracted from different strains, including *B. subtilis, B. stearothermophilus*, *Thermus* sp. IM6501, *Bacillus* sp. WPD616 have an extra N-terminal domains which can distinguish them from other amylolytic enzymes and it plays an important role in domain swapping, dimerization, stabilization, substrate specificity and shaping the active site cleft [1,11,13]. *Thermus* maltogenic amylase is a typical MAase, which can hydrolyze CDs much faster than it does for starch or pullulan and it exists as a homodimer in solution. Dimer formation of ThMA can change a wide substrate-binding site in a monomer to a pair of narrow and deep clefts in a dimer which prefere β-CD or maltooligosaccharides instead of bulky substrate such as starch or pullulan. Also, an extra suger- binding space at the bottom of the active site might be important for versatile transglycosylation activity of enzyme and it can accommodate disaccharides. Sugar molecules which binds to this site would compete with water molecules in attacking the enzyme-substrate intermediate. [12,28,29].

But in spite these properties, there is no report on the allosteric behavior of these enzymes and the correlation between the subunits. In this study, the allosteric behavior of the *Geobacillus* Gh6 MAase which has approximately 98% homology with ThMA was studied. In our previous study, substrate preference of this enzyme analyzed in details and it was shown that this enzyme preferentially hydrolyzed cyclodextrin (α, β-and γ-CD) substrates more than polymeric substrates, which could be due to the inhibiting space of polymeric substrates such as amylopectin, amylose and glycogen [19]. The active sites in oligomeric enzyme are usually provided by a subunit, however, in this enzyme, central domain of each subunit with N domain of the next one formed two active sites together in the dimer form. In the present study, for the first time, the allosteric behavior of this enzyme is reported with different CD substrates.

CDase activity was determined at different concentrations of α, β and γ-CD substrates and the initial velocity against substrate concentration was plotted. The results revealed that unlike other members of α-amylase family, this enzyme didn't exhibit the hyperbolic plot predicted using the Michaelis-Menten equation, but instead had sigmoidal behavior which increased in the order of α-CD > β-CD > γ-CD. In allosteric enzymes, binding of one substrate to catalytic site can alter the properties of other active sites located within the same enzyme [29]. This property of cooperativity accounts for the sigmoidal curve of velocity versus the substrate concentration, which was observed in this enzyme and the allosteric behavior was the highest one using α-CD substrate. This result suggests that the allosteric properties of this enzyme decreased by increasing substrate size. For more investigation of allosteric behavior, Hill curves were plotted for all substrates. The total number of substrate binding sites on the enzyme and the degree of cooperativity can be quantified by the Hill coefficient. The Hill coefficient is used to provide information about allosteric properties, measurement of cooperativity of ligand binding and the number of interacting sites. An nH value of greater than 1 indicates the existence of multiple (two or more) binding sites and describes the positive cooperativity effect. Also, there is no cooperativity properties when nH = 1. Finally, the Hill coefficient smaller than 1 describes the negative cooperativity [30].

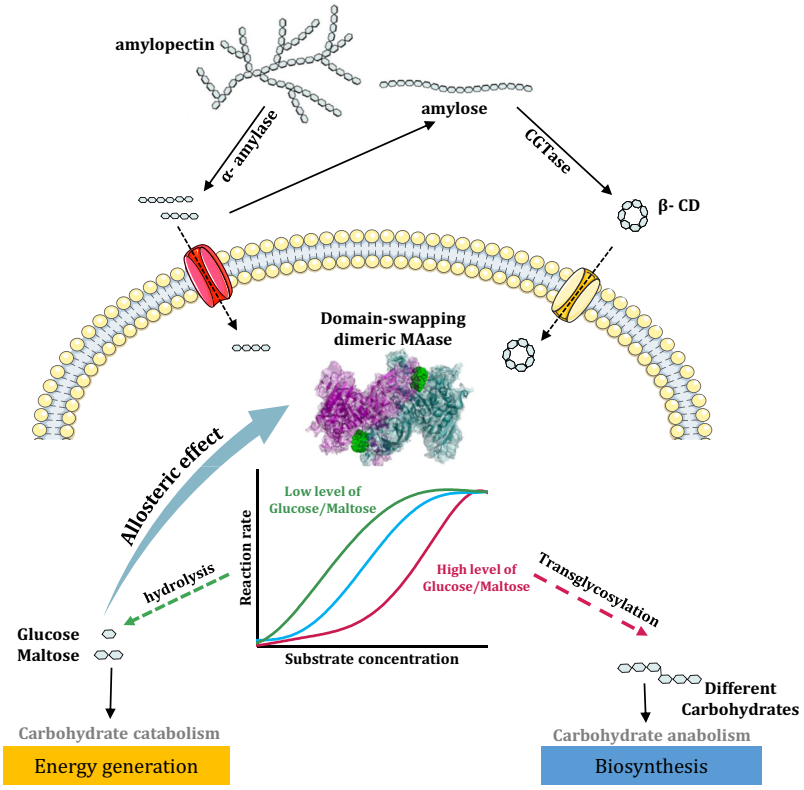

Fig. 7. A proposed the central role of MAases and the importance of its cooperative properties in utilizing carbohydrate in bacteria species including CGTase and CDase.

As shown in Table 1, the positive cooperativity between MAase subunits was observed by calculating Hill constant using all substrates. The highest constant was obtained with α-CD (nH = 2) compared to other substrates.

The central role of MAases in utilizing carbohydrate in bacterial species was well described by Park et al., 2000; Kim et al., 2001 and Shim et al., 2009 [15,28,29]. CGTase and CDase cooperatively play a critical role in carbohydrate metabolism in bacteria [28]. Starch is hydrolyzed to oligosaccharides by extracellular enzymes such as α-amylase and pullulanase. Next, these Maltooligosacarids are converted to α- or β- and γ-CD by extracellular CGTase and at the end, maltose and glucose is produced by CDase activity. Also, transglycosylation is occurred in the presence of high concentrations of oligosaccharides in cells. According to the participation of these enzymes in maltose and glucose production as a main fuel of the cells, in this study, the effect of these two compounds on allosteric behavior and MAase activity was investigated using β-CD as substrate. As it is illustrated in Fig. 4, the effects of maltose and glucose on enzyme activity at their various concentrations are different. The enzyme in the presence of 0.02% glucose and maltose as effectors approximately exhibits the hyperbolic behavior. According to Table 2, Hill constant was calculated to be 1.6 in the absence of effectors, and it decreased to 1.3 and 1.2 in the presence of glucose and maltose, respectively. Therefore, this enzyme showed decreased regulatory properties upon binding to these two metabolites. The determination of kinetic parameters indicated that $K_{0.5}$ value was reduced in the presence of both effectors especially maltose, while no significant difference was observed in the V_{max}. Due to the increased efficiency of the enzyme in the presence of the effectors, probably low concentrations of these compounds acts as allosteric activators (positive effectors) in cells. However, MAase activity was inhibited at high concentrations of these compounds. Cells coordinate metabolic flux through the allosteric regulation of enzymatic activity [31] and controling enzyme allosteric regulation is required to drive metabolic flux toward the desired levels [32]. Binding of allosteric regulators induces an interaction rearrangement of allosteric residues and regulates enzymatic activity [33]. When ThMA turns into a monomer through increased ionic strength or truncation of the N-terminal domain, the active site becomes wide and shallow, allowing access of the large substrates such as soluble starch, while small substrates such as CDs fit into the narrow and deep groove found in the dimer form of ThMA indicating that the dimeric enzyme hydrolyzes smaller substrates more efficiently [29]. Maltose and glucose are final metabolites in the extracellular (α-amylase and CGTase) and intracellular (MAases and CDases) environments. This process is related to hydrolysis of starch or other polysaccharides in the cell to supply cell energy. Thus, the inhibitory effect of higher concentrations of these metabolites on MAase activity is very considerable in the regulation of this process. Our proposed model for the central role of MAases and the importance of its cooperative properties in utilizing carbohydrate in bacteria species including CGTase and CDase is presented in Fig. 7.

Taken together, the data clearly showed that *Geobacillus* sp. Gh6 maltogenic amylase exhibited allosteric behavior that certainly has a major role in unique properties of this enzyme. As mentioned above, roles of such MAases in metabolism could be critical in bacterial cells because of these cooperative properties which require further study.

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