Appl Biochem Biotechnol (2010) 160:1955–1966
DOI 10.1007/s12010-009-8704-y

Preliminary Investigation on the Action Modes of an Oligosaccharide-Producing Multifunctional Amylase

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Received: 4 January 2009 / Accepted: 28 June 2009 /
Published online: 7 August 2009

Abstract The action modes of an oligosaccharide-producing multifunctional amylase (OPMA) were investigated using glucose and some oligosaccharides as its substrates. OPMA did not cause the hydrolysis of maltose or isomaltose, but it catalyzed the α-1,6-transglycosylation of maltose to produce isomaltose or did the self-condensation of isomaltose to form isomaltotetraose and 4-O-α-isomaltosyl isomaltose. OPMA exhibited strong α-1,6-transglycosylation activity in addition to its α-1,4-hydrolytic activity on higher oligosaccharides substrates rather than bisaccharides. OPMA displayed high acceptor specificity in its transglycosylation or condensation reaction. OPMA seemed to only take glucose or isomaltose as the acceptor molecule in its transglycosylation or condensation reaction, which made glucose or isomaltose form higher products, and as a result, glucose or isomaltose were absent in the final products. In view of the simultaneously formation of several transglycosylation or condensation products, it was predicted that there might be separate donor and acceptor sites in OPMA's active center and the fact that the catalytically active form of this enzyme included its homodimer or homotrimer supported this prediction. Accordingly, a special pathway, isomaltose pathway, for OPMA catalysis was proposed to emphasize the central or important signification of isomaltose in OPMA catalysis.

Keywords Amylase · Action mode · Condensation · Hydrolysis · Transglycosylation

Introduction

We have reported that the oligosaccharide-producing multifunctional amylase (OPMA) is a novel starch-degrading enzyme secreted from *Bacillus sp.* ZW2531-1 [1, 2]. *Bacillus sp.* ZW2531-1 is a newly isolated mesophilic spore-forming bacteria from Chinse soil and its 16S rDNA sequence accession number in GenBank is EF567395 [1, 2]. OPMA is an extracellular

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enzyme and has a molecular weight of 66 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and has an isoelectric point (pI) at pH 5.3 [1]. This enzyme is a new member of α-amylase family due to its unique catalytic features and activities. OPMA exhibits an unusual catalytic versatility besides its high substrate specificity compared with other amylases such as α-amylase (EC 3.2.1.1) and cyclomaltodextrin glucanotransferase (EC 2.4.1.19). OPMA only digests starch rather than other glucan oligo- or polysaccharides such as β-cyclomaltodextrin and pullulan. OPMA catalyzed starch degradation into maltose, maltotriose, and isomaltotriose as well as small amounts of isomaltotetraose, but does not produce glucose. Apparently, OPMA is distinguished from other typical amylases, such as α-amylase or other popular multifunctional amylases such as maltogenic amylase (MAase) [3, 4], neopullulanase (NPase) [5, 6], or cyclodextrin-hydrolyzing enzymes (CDase) [7, 8], not only by its multiple activities, but also by its product composition though they all have the catalytic versatility [9–11].

The unique catalytic properties of OPMA make OPMA have more potential than other starch-degrading enzymes in the production of maltooligosaccharides or isomaltooligosaccharides because the elimination of glucose from the products of starch degradation with OPMA can be canceled.

Though it has been confirmed that OPMA has both abilities to catalyze the hydrolysis and transglycosylation reactions on starch and some oligosaccharides [1], the action modes of OPMA and the reaction rules displayed by OPMA are still unclear. To better utilize OPMA in starch sugar processing, we explore the action modes and reaction rules of OPMA through using glucose and some oligosaccharides containing one or two α-1,4- and/or α-1,6-glucosidic linkages such as maltose, isomaltose, maltotriose, or their mixture as the substrates or substrate mixture in this study.

Materials and Methods

Enzyme OPMA was purified from the culture broth of *Bacillus sp.* ZW2531-1 after the fermentation of strain *sp.* ZW2531-1 overnight by 30∼60% saturated ammonium sulfate precipitation, followed by twice Sephadex gel filtration chromatography as described previously [1]. The purified OPMA had a specific activity of 13.75 U/mg and a molecular weight of 66 kDa on SDS-PAGE [1].

Standard Sugar and Substrates All sugar including glucose, maltose, isomaltose, maltotriose, isomaltotriose, and isomaltotetraose, either as standard materials or as substrates, were purchased from Sigma Co., Ltd.

Catalytic Reaction of Substrate(s) by OPMA A reaction system (1 ml) containing glucose or oligosaccharide alone or their mixture (1–5% *w/v* for each) 500 μl, purified OPMA 400 μl (approximately 27.5 U) and 0.2 M NaOAc-HAc buffer (pH 6.0) 100 μl was incubated at 50°C for 24 h. The reaction was stopped by heat treatment at 100°C for 5 min. After centrifugation, 5 μl or 10 μl reaction mixture was applied to thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Each catalytic reaction was repeated at various substrate concentration(1–5% *w/v*).

Analysis and Isolation of Reaction Products The sugar composition of products was analyzed by thin layer chromatography and high-performance liquid chromatography. In TLC analysis, the ascending mode was used on silica gel GF254 with a solvent mixture of

1-butanol/acetic acid/water (8:1:1, *v*/*v*), the sugar was detected by spraying with the solution of 1 g diphenylamine, 0.5 ml aniline, 25 ml acetone, and 2.5 ml phosphoric acid. In the HPLC analysis, Sweden Kromasil NH_2 column, 5 μm, 150×4.6 mm, was applied and maintained at 25°C, the elution of sugars was carried out with 60% acetonitrile, 1 ml/min, with a differential refractometer,W40. To quantify the molar ratio of products, the *r*ation of each *p*eak area to its *m*olecular weight (RPM) is automatically calculated and the molar ratio of products is the ratio of their RPM.

Determination of Enzyme Activity Amylase activity was determined by measuring the amount of reducing sugars generated from starch by the catalysis of the amylase at 50°C for 30 min as described previously [7]. The reaction system contained 1 mL of 5% (*w*/*v*) soluble starch in 0.2 M sodium acetate buffer (pH=6.0) and 0.5 mL of the enzyme liquid. The reaction was stopped by adding 1.0 mL of DNS and heated in boiling water for 5 min. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of reducing ends per half hour under the catalysis conditions described above.

Measurement of Protein Concentration The protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.

Substrate-PAGE PAGE in the presence of substrate (isomaltose or maltotriose; substrate-PAGE) is the electrophoresis under native conditions. The polyacrylamide gel (PAG) was prepared in the same way as SDS-PAG, except that SDS was replaced by substrate (0.2% *w*/*v*). Substrate-PAGE was carried out in the same way as SDS-PAGE, but the sample buffer contained neither SDS nor β-mercaptoethanol, and the samples were not boiled.

Results

Action of OPMA on Glucose Since glucose (G1) has been proved absent in the degradation products of soluble starch by OPMA as described previously [1, 2], here it was first examined whether any product could be produced from glucose by transglycosylation or condensation with OPMA. After incubation of OPMA with glucose alone at 50°C for 24 h, in addition to glucose itself, none of other product was detected either by TLC or HPLC at any time. This result indicated that OPMA did not act on the free glucose, i.e. neither transglycosylation nor condensation reaction occurred when glucose alone was provided as the substrate. However, the result above did not demonstrate whether or not this enzyme acts on the enzyme-bound intermediate glucose.

Action of OPMA on Maltose or its Mixture with Glucose When maltose (G2) alone was used as the substrate, only isomaltose (IG2) was detected in addition to maltose either by TLC or HPLC (Fig. 1a and b) . The molar ratio of maltose to isomaltose in the reaction systems always maintained 1:1 (Fig. 1b). These results clearly indicated that transglycosylation reaction, rather than hydrolysis reaction, of maltose took place since glucose could not be detected at all in the reaction system, and there was an equilibrium between α-1,4- and 1,6-transglucosylation of OPMA-glucosyl intermediate (OGI) resulted from maltose in OPMA's active center.

When maltose plus glucose was used as the substrate, the new product was still only isomaltose, and the balance of the equilibrium between maltose and isomaltose did not be disrupted by the free glucose molecule (Fig. 1a, lanes 3 and 6). The results in Fig. 1a

Fig. 1 **a** TLC analysis of the products from maltose or maltose plus glucose by OPMA. Lanes *1* and *2* ▶ standard mixture of glucose and oligosaccharides: glucose (*G1*), isomaltose (*IG2*), pantose (*PG3*), and isomaltotetraose (*IG4*) or maltose (*G2*), maltotriose(*G3*), and isomaltotriose (*IG3*). Lanes *3–5* reaction product(s) from maltose with active OPMA (*3*), inactive OPMA (*4*), without OPMA (*5*). Lanes *6–8* reaction product(s) from maltose plus glucose with active OPMA (*6*), inactive OPMA (*7*), or without OPMA (*8*). **b** HPLC quantitative analysis of products obtained from maltose or maltose plus glucose by OPMA. *Std* the elution profiles for the standard oligosaccharide mixture. The retention times of these standards are *marked*. The abbreviations of glucose and oligosaccharides are as described in Fig. 1a

showed that only the enzyme-bound intermediate glucose rather than free glucose could act as the acceptor molecule for transglycosylation reaction though plenty of maltose or free glucose molecules (substrates) were present in the reaction system. Furthermore, no condensation reaction between any two substrate molecules occurred.

According to the results mentioned above, it could be concluded definitively that OPMA had the strong ability to catalyze α-1,6 transglycosylation of maltose to form isomaltose, but not to catalyze the hydrolysis of maltose to produce glucose. Figure 2 showed the scheme of the action mode of OPMA on maltose.

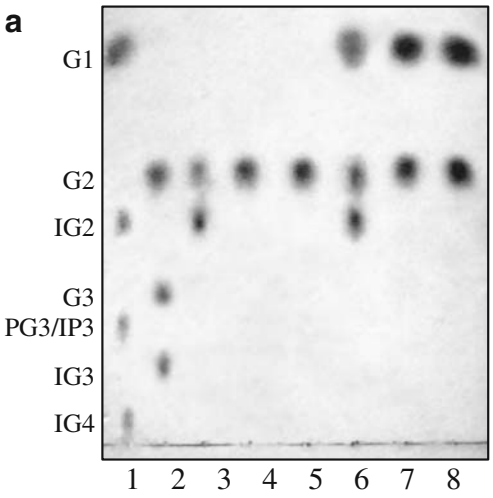
Action of OPMA on Isomaltose or its Mixture with Glucose When isomaltose (IG2) was utilized as the substrate, the composition of the reaction products seemed more complex (Fig. 3). In addition to isomaltose, several higher oligosaccharides such as maltotriose (G3), isomaltotriose (IG3), isomaltotetraose (IG4), and a indefinite tetrasaccharide as well as a small amount of panose (PG3) or isopanose (IP3) were detected in the final products, but neither glucose nor maltose were produced (Fig. 3b). The indefinite tetrasaccharide was guessed to be 4-O-α-isomaltosyl isomaltose (G4') because its position on the chromatographic matrix or its retention time on HPLC lay between isomaltotriose and isomaltotetraose (Fig. 3a and b). These results demonstrated that the self-condensation rather than the hydrolysis or transglycosylation of isomaltose in the early stage was the dominant reaction, and the hydrolysis of G4' or transglycosylation of PG3 or IP3 were proceeded immediately after their formation to produce main final products, maltotriose (G3), and isomaltotriose (IG3). This showed that G4', PG3, or IP3 seemed to be the unstable intermediate products.

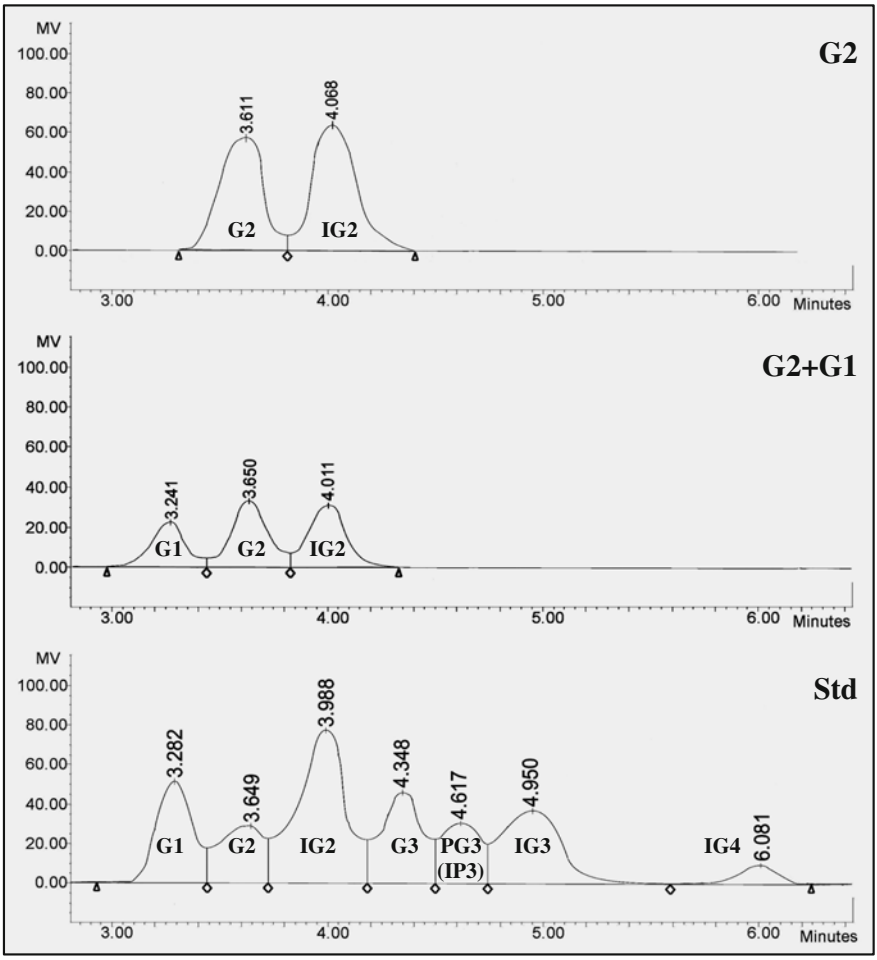
The less formation of maltotriose than isomaltotriose from the intermediates panose and isopanose suggested that OPMA had the higher activity for α-1,4 glucosidic linkage cleavage than for α-1,6 glucosidic linkage cleavage, and consequently, OPMA had higher activity for α-1,6 glucosidic linkage formation than for α-1,4 glucosidic linkage formation.

When isomaltose plus glucose was used as the substrate, the condensation between glucose and isomaltose in the early stage was the dominant reaction, and the products were maltotriose and isomaltotriose as well as a small amount of PG3 or IP3 (Fig. 3). Obviously, the reaction rules in isomaltose plus glucose system was the same as that in single isomaltose system.

The action modes of OPMA on isomaltose or isomaltose plus glucose were proposed in Fig. 4 based on the results described above.

The proposed modes in Fig. 4 explained the composition of the final products. Obviously, tetrasaccharides existed in the final products only when isomaltose alone was used as the substrate, the addition of glucose to the isomaltose reaction system made several trisaccharides be the dominant products (Fig. 4), isomaltotriose and maltotriose were resulted from the intermediate panose or isopanose and were accumulated as the dominant products. The action modes of Fig. 4 indicated that isomaltotriose and maltotriose seemed to be stabler products than panose or isopanose, which made less panose or isopanose exist in the final products.


a
G1
G2
IG2
G3
PG3/IP3
IG3
IG4
1 2 3 4 5 6 7 8


b
MV
G2
3.611
4.068
G2
IG2
3.00
4.00
5.00
6.00
Minutes
G2+G1
3.241
3.650
4.011
G1
G2
IG2
Std
3.282
3.649
3.988
4.348
4.617
4.950
6.081
G1
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(IP3)
IG3
IG4

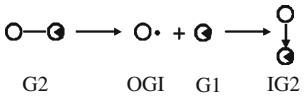

Fig. 2 Action mode of OPMA on maltose. *Black line* α-1,4 glucosidic linkage; *downwards arrow* α-1,6 glucosidic linkage; ◔, glucose with reducing end; ○, glucose without reducing end; ○•, OPMA-glucosyl intermediate (*OGI*). Other abbreviations of glucose and oligosaccharides are as described in Fig. 1a

The ratio of maltotriose (G3), isomaltotriose (IG3), and isomaltotetraose (IG4) from isomaltose was about G3:IG3:IG4=1:2:3 and the ratio of G3:IG3 from isomaltose plus glucose was about G3:IG3=1:2 by their HPLC quantitative analysis. These facts indicated that the formation of α-1,6-glucosidic linkage was more than that of α-1,4-glucosidic linkage in transglycosylation or condensation in both isomaltose reactions system, and besides the enzyme-bound intermediate glucose, isomaltose could also act as an acceptor molecule (Fig. 4). These conclusions corroborated, and supplemented, those deductions made from maltose.

Based on the scheme in Fig. 4, it could be presumed that there was more than one binding site, at least one donor, and one acceptor binding sites, in the active OPMA, and they were responsible for binding glycosyl donor (substrate) molecule and acceptor (intermediate product or another substrate) molecule, respectively, so as to simultaneously realize the association of substrate(s) and product(s) or another substrate with special amino acid residue(s) in OPMA.

Compared the results above with that derived from maltose or maltose plus glucose system, the reaction activity of glucose and isomaltose substrates in Fig. 3 was distinguished from that of glucose and maltose substrates in Fig. 1, so was the reaction activity of glucose and isomaltose substrates in Fig. 3 from that of glucose substrate and isomaltose product in Fig. 1. According to the deductions above, it could be suggested that there was a structural difference between OPMA's donor and acceptor binding sites, which made isomaltose in a distinct state in OPMA's donor or acceptor binding site.

Action of OPMA on Maltotriose or its Mixture with Glucose or Maltose To confirm the conclusions drawn from the catalytic reactions of glucose, maltose, and isomaltose above, an attempt was made to predict the main products resulted from maltotriose or its mixture with glucose or maltose with OPMA. Since enzyme-bound intermediate glucose or isomaltose molecule was perhaps the acceptor molecule, the probable schemes of the reaction process of maltotriose or its mixture with glucose or maltose by OPMA were proposed in Fig. 5a and b.

When maltotriose was used as the single substrate, plenty of indefinite tetrasaccharides as well as small amounts of isopanose and isomaltotriose were detected in addition to maltotriose (Fig. 6a (lane 3) and b). The indefinite tetrasaccharides were likely 6-O-α-maltotriosylglucose (G4'') and 4-O-α-isomaltosyl isomaltose (G4') according to its position on the chromatographic matrix (Fig. 6a lane 3) or its retention time on HPLC (Fig. 6b). The ratio of these final products were IP3:IG3:(G4'+G4'')=1:1:3 by HPLC. When maltotriose plus glucose was used as the substrates, the final products were isopanose and isomaltotriose with the ratio of IP3:IG3=1:1 by HPLC (Fig. 6a (lane 6) and b). As to the case of maltotriose plus maltose as the substrates, it seemed to be the simple mixture of both final products from maltotriose and maltose (Fig. 6a lane 11). The experimental results of Fig. 6 verified the predicted action mode showed in Fig. 5.

The results above also proved that the conclusions about the action modes of OPMA drawn from the catalytic reactions of glucose, maltose, and isomaltose were reliable and practical.

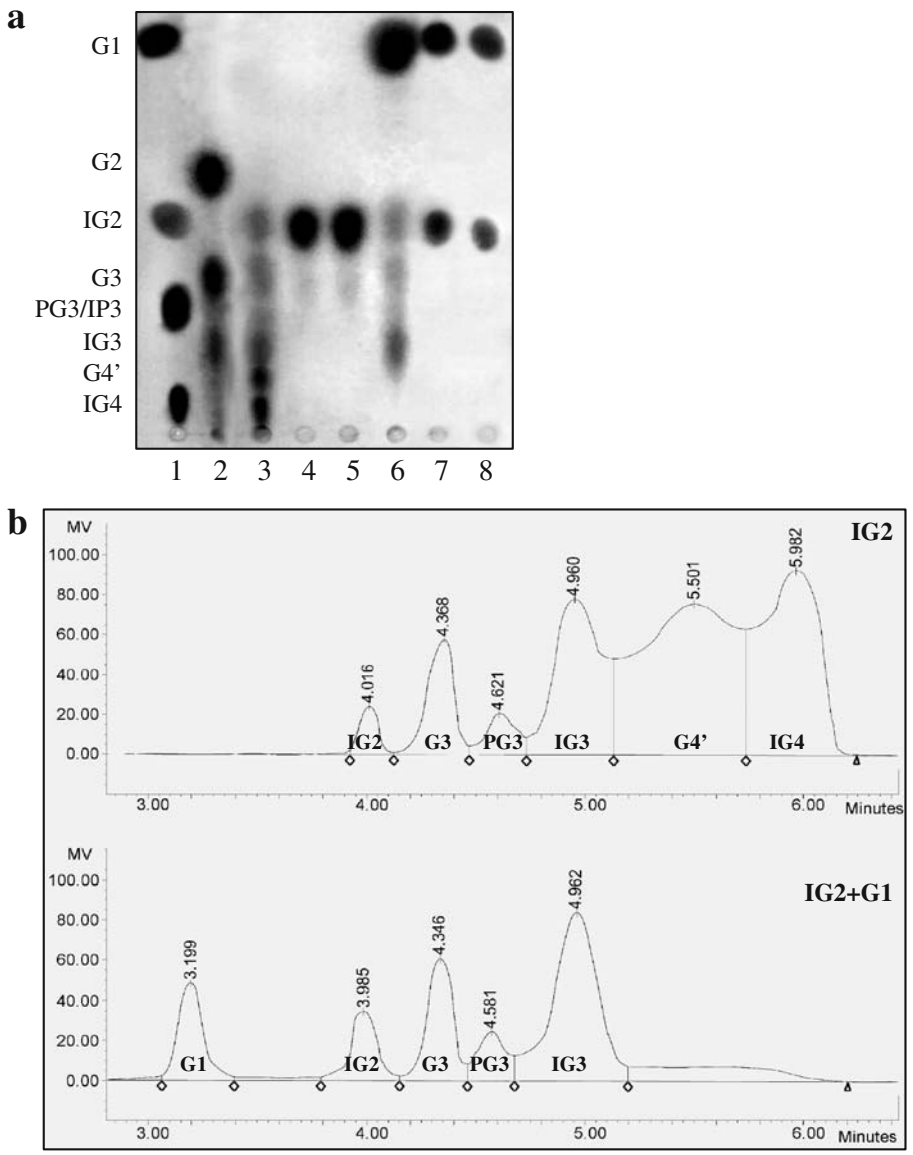

Fig. 3 **a** TLC analysis of the products from isomaltose or isomaltose plus glucose by OPMA. Lane *1* standard mixture of G1, IG2, PG3, and IG4. Lane *2* standard mixture of G2, G3, and IG3. Lane *3–5* reaction products from isomaltose with active OPMA (*3*), inactive OPMA (*4*), without OPMA (*5*). Lane *6–8* reaction products from isomaltose plus glucose with active OPMA (*6*), inactive OPMA (*7*), without OPMA (*8*). G4' represents 4-O-α-isomaltosyl isomaltose (pointed by an *arrow*). Other abbreviations of glucose and oligosaccharides are as described in Fig. 1a. **b** HPLC quantitative analysis of products obtained from isomaltose or isomaltose plus glucose by OPMA. The elution profiles for the standard oligosaccharide mixture were shown as in Fig. 1b. The abbreviations of glucose and oligosaccharides are as described in Figs. 1a and 3a

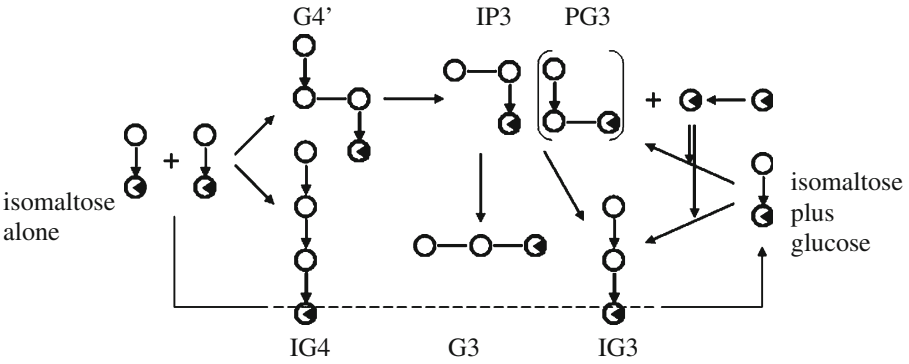

Fig. 4 Action modes of OPMA on isomaltose or isomaltose plus glucose. All symbols and abbreviations of glucose and oligosaccharides are as described in Figs. 1, 2, and 3

Oligimerization of OPMA Figures 3 and 6 showed that more than one product, including transglycosylation or condensation products, was formed at the same time in the reaction system of isomaltose or maltotriose. According to the characteristics of these products, it was consequently suggested that the active form of OPMA might include its homodimer or homooligomer. To clarify whether or not OPMA could exist in the form of a dimer or a oligomer, substrate-PAGE of purified OPMA in the presence of substrate (isomaltose or maltotriose) was carried out and the result in Fig. 7 showed that the prufied OPMA showed all three forms with the molecular weights of 66 kDa, 130 kDa, and 200 kDa at the same time, though it is a monomer with a molecular weight of 66 kDa on SDS-PAGE [1]. The result indicated that the purified OPMA could form a dimer and a trimer simultaneously besides its monomer (Fig. 7) in the presence of substrate.

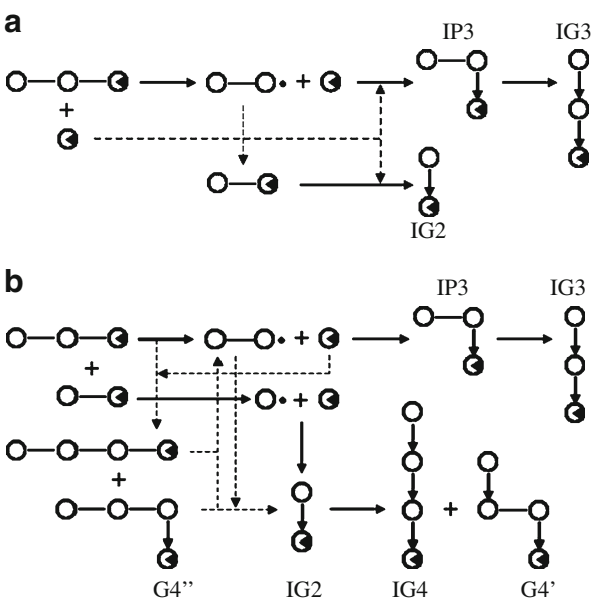

Fig. 5 Action modes of OPMA on maltotriose plus glucose (**a**) and on maltotriose or its mixture with maltose (**b**). *G4"* represents 6-O-α-maltotriosylglucose. Other symbols and abbreviations of glucose and oligosaccharides are as described in Figs. 1, 2, and 3

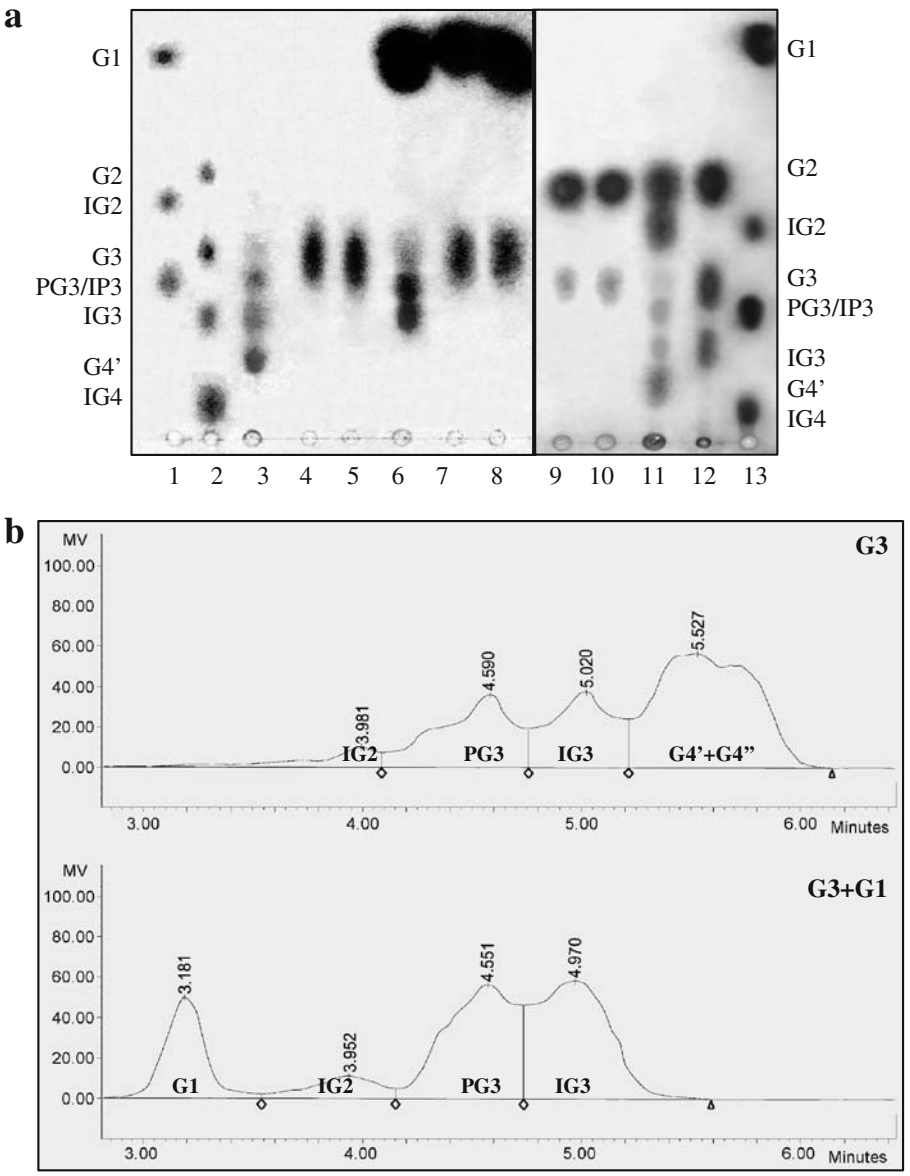

Fig. 6 **a** TLC analysis of the products from maltotriose or maltotriose plus glucose or maltose by OPMA. Lanes *1*, *2*, *12*, and *13* standard mixture of glucose and oligosaccharides. Lanes *3–5* reaction product(s) from maltotriose with active OPMA (*3*), inactive OPMA (*4*), without OPMA (*5*). Lanes 6–8 reaction product(s) from maltotriose plus glucose with active OPMA (*6*), inactive OPMA (*7*), or without OPMA (*8*). Lanes *9–11* reaction product(s) from maltotriose plus maltose without OPMA (*9*), with inactive OPMA (*10*), or active OPMA (*11*). All abbreviations are as described in Figs. 1 and 3. **b** HPLC quantitative analysis of products obtained from maltotriose or maltotriose plus glucose by OPMA. The elution profiles for the standard oligosaccharide mixture were shown as in Fig. 1. All abbreviations are as described for Figs. 1 to 5

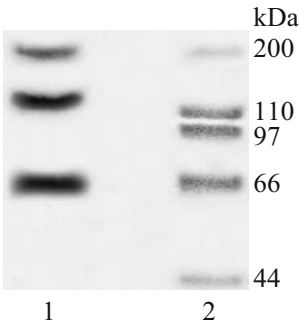

Fig. 7 Substrate-PAGE of purified OPMA in the presence of substrate (isomaltose or maltotriose). *1* purified OPMA which had a molecular weight of 66 kDa on SDS-PAGE. *2* protein ladder (200 kDa, 110 kDa, 97 kDa, 66 kDa, 44 kDa)

Discussion

We previously reported that OPMA, oligosaccharide-producing multifunctional amylase, was a novel starch-degenerating enzyme, it not only hydrolyzed α-1-4 glucosidic linkage but also formed α-1,6 glucosidic linkage [1]. In this report, its action modes and reaction rules were investigated based on the products derived from some standard oligosaccharides or their mixture with glucose or maltose. OPMA did not cause the hydrolysis of bisaccharide, maltose, or isomaltose, but it catalyzed the α-1,6-transglycosylation of maltose to produce isomaltose and the self-condensation of isomaltose to form tetrasaccharides, isomaltotetraose, or 4-O-α-isomaltosyl isomaltose. OPMA showed stronger transglycosylation activity to form α-1,6-glucosidic linkage than to form α-1,4-glucosidic linkage in addition to its α-1,4-hydrolytic activity on tri- or higher oligosaccharide and starch, which, therefore, led to the accumulation of some oligosaccharides having α-1,6 glucosidic linkage such as isomaltotriose.

Taken together, a special pathway for OPMA catalysis, named isomaltose pathway was proposed as showed in Fig. 8 in this study in order to emphasize the central or important signification of isomaltose in OPMA catalysis (Fig. 8). In this pathway, the main final

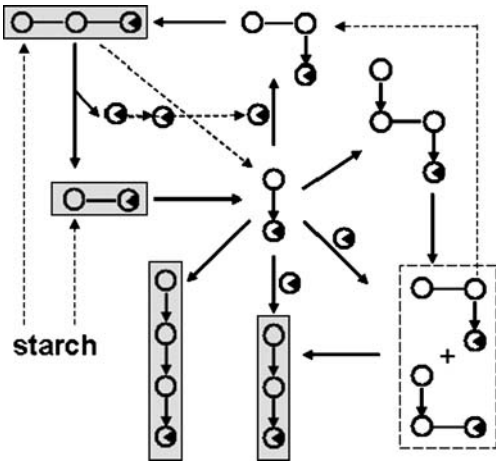

Fig. 8 Isomaltose pathway of OPMA. Symbols and abbreviations are as described for Figs. 1 and 2

oligosaccharide products from starch with OPMA could be supposed apparently to be maltose, maltotriose, isomaltotriose, and isomaltotetraose (shaded in dark gray) because maltose and maltotriose were the initial plenty products from starch, and isomaltotriose and isomaltotetraose were the terminal products. The predicted products obtained from starch based on this pattern were consistent with the actual ones that have been detected in starch degradation in previous report [1, 2]. Hence, the isomaltose pathway was proved to be reliable and effective.

OPMA displayed a high acceptor specificity. It only took enzyme-bound intermediate glucose or isomaltose, as the acceptor molecule, which caused the lack of glucose or isomaltose in the final products. Actually, this case has been seen in starch products by OPMA in our previous report [1, 2]. Thus, the several lower oligosaccharides such as di- or trisaccharides should appeared in OPMA's final products when glucose existed as one of the substrates, otherwise, higher oligosaccharides such as tri- or tetrasaccharides were formed.

Only a few enzymes in the α-amylase family are known to form dimers or oligomers [12]. OPMA could form homodimer or homotrimer like MAase [13], CDase [14], or NPase [15]. Dimerization or oligomerization could make them provide simultaneously more than one binding site for donor and acceptor molecules in enzyme active center for transglycosylation or condensation reaction [3, 4, 8, 15–17].

The great significance of investigating the action modes of OPMA in this study meant more than the action modes pattern themselves. The significance of this study is to provide helpful information for OPMA application. With the action modes and reaction rules, we could estimate what equilibrium product would be formed when any α-glucosyl saccharide or oligosaccharide was supplied as the substrate, or reversal, determine which α-glucosyl saccharide or oligosaccharide substrate should be provided when a final α-glucosyl oligosaccharide product was expected.

Among the glucosyl oligosaccharides, isomaltooligosaccharides are all-purpose, and functional oligosaccharides. Obviously, the absence of glucose in OPMA's products not only advanced the value of OPMA's products, but also simplified the purification process of OPMA's products from starch, one of the most abundant and low-cost biomaterial in the world, since the glucose in oligosaccharide products often prevents their use in food industry [18, 19]. Therefore, OPMA was expected as a more excellent candidate enzyme for the industrial production of isomaltooligosaccharides from starch than the traditional ones [18, 20].

Acknowledgement This work was supported by the National High Technology Research and Development Program of China (No. 3F0086071465) and the National Natural Science Foundation of China (No. 30870518).

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