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ORIGINAL RESEARCH PAPER

Characterization of a recombinant sucrose isomerase and its application to enzymatic production of isomaltulose

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Abstract

Objective To characterize a recombinant isomerase that can catalyze the isomerization of sucrose into isomaltulose and investigate its application for the enzymatic production of isomaltulose.

Results A sucrose isomerase gene from *Erwinia sp.* Ejp617 was synthesized and expressed in *Escherichia coli* BL21(DE3). The enzymatic characterization revealed that the optimal pH and temperature of the purified sucrose isomerase were 6.0 and 40 °C, respectively. The enzyme activity was slightly activated by $Mn^{2+}$and Mg^{2+}, but partially inhibited by Ca^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+} and EDTA. The kinetic parameters of K_m and V_{max} for sucrose were 69.28 mM and 118.87 U/mg, respectively. The time course showed that 240.9 g/L of isomaltulose was produced from 300 g/L of sucrose, and the yield reached 80.3% after bioreaction for 180 min.

Conclusions This recombinant enzyme showed excellent capability for biotransforming sucrose to isomaltulose at the substrate concentration of 300 g/L. Further investigations should be carried out focusing on selection of suitable heterologous expression system with the aim to improve its expression level.

Keywords Sucrose isomerase · Isomaltulose · Biotransformation · Purification · Characterization

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Introduction

Isomaltulose (α-D-glucopyranosyl-1,6-fructose) is a natural isomer of sucrose (α-D-glucopyranosyl-1,2-fructose), which has a similar appearance and taster to sucrose (Zhan et al. 2019). It is commonly used as a functional carbohydrate with superior properties, including lower glycemic index, slower digestion, lower insulin reaction, less cariogenicity and prolonged energy release (Zheng et al. 2019). Recently, isomaltulose has attracted wide attention of researchers because of its industrial applications either as an ideal sucrose substitute in food processing or as a starting material for producing surfactants and polymers (Mu et al. 2014; Watzlawick and Mattes 2009).

Sucrose isomerase (EC 5.4.99.11, SIase) catalyzes the isomerization of sucrose into isomaltulose and trehalulose. In this bioconversion process, isomaltulose is the main product, and a little amount of

glucose and fructose are by-products (Duan et al. 2016; Kim et al. 2015; Wu et al. 2016; Zhang et al. 2002). SIase has been isolated and characterized from several microbial strains, such as *Ervinia rhapontici*, *Serratia plymuthica*, *Klebsiella pneumonia*, *Pseudomonas mesoacidophila* and *Pantoea dispersa*. Mattes et al. firstly reported the heterologous expression of the SIase from *Protaminobacter rubrum* CBS 547.77 in *E. coli* (Mattes et al. 1995). After which, other SIases from *P. dispersa* UQ68J (Wu and Birch 2004), *Klebsiella* sp. LX3 (Zhang et al. 2002), *E. rhapontici* NX-5 (Li et al. 2011) and *Enterobacter* sp. FMB-1 (Cho et al. 2007) were heterologously expressed in *E. coli*.

In this study, to generate new biocatalyst for the biotransformation process to produce isomaltulose, genome mining method was used to identify new SIase. After codon optimization, the synthesized SIase gene was cloned and successfully expressed in *E. coli* BL21 (DE3). The detailed properties of this recombinant SIase were investigated and sucrose isomerization reaction was conducted as well. This work revealed that the recombinant SIase might be a candidate catalyst for industrial production of isomaltulose.

Materials and methods

Microorganisms, media, and plasmids

Escherichia coli BL21 (DE3) and plasmid pET28b (+) were obtained from Invitrogen (Karlsruhe, Germany) and Novagen (Darmstadt, Germany), respectively. Restriction endonucleases (*Nco* I and *Xho* I) were purchased from Takara (Dalian, China). Luria–Bertani (LB) culture medium (Tryptone 10 g/l, yeast extract 5 g/l, and NaCl 5 g/l) was employed for cultivation of strain and expression of recombinant enzyme. Kanamycin (Kan), isopropyl-β-D-thiogalactoside (IPTG) and sucrose were purchased from Sangon (Shanghai, China). Protein molecular weight marker was purchased from TaKaRa (Dalian, China). The Ni–NTA affinity chromatography column was from GE Healthcare (Uppsala, Sweden). Other chemicals employed were of analytical pure and commercially available.

Genome mining

Novel SIase genes were searched by genome mining method in GenBank using the known SIase sequence as a query sequence (Wang et al. 2019). Protein sequence alignment was performed through the online Blast program of National Centre of Biotechnology Information (NCBI).

Cloning, expression and purification of SIase

Herein, a 1847 bp *Erwinia sp*. Ejp617 SIase gene (GenBank: G37835) was optimized based on the codon preference against *E. coli*, and was chemically synthesized (Tsingke, Hangzhou, China). The synthesized gene was ligated into the pET28b between *Nco* I and *Xho* I sites to form the recombinant plasmid pET28b-SI. Then, pET28b-SI was transformed into *E. coli* BL21 (DE3) by heat shock method (Roychoudhury et al. 2009), the recombinant cells was cultivated in LB medium and was identified by Kan resistance screening. All DNA manipulations were performed by conventional protocol (Sambrook and Russell 2001).

The recombinant cells were cultured at 37 °C until the obstacle density at 600 nm (OD_{600}) reached 0.6–0.8, the induction process was started by shifting the fermentation temperature to 25 °C and addition of 0.1 mM IPTG. The induction process lasted for 10 h. Then, the cell pellet was collected by centrifugation at 10,000×*g* at 4 °C for 10 min, and washed twice with 50 mM Tris–HCl buffer (pH 7.0). Cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.0). The suspension was sonicated with an ultrasonic breaker (Branson Ultrasonic, Shanghai, China) at 300 W for 30 min on ice, and then centrifuged at 12,000×*g* at 4 °C for 20 min. The supernatant was collected as crude protein solution.

The crude SIase sample was isolated on an AKTA Purifier equipped (GE Healthcare, New Jersey, USA) with a Ni–NTA affinity chromatography column. The molecular mass of SIase was investigated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Tripathi et al. 2011). The protein concentration was quantified by the Bradford method (Bradford 1976). The final protein solution was used for further experiments after measuring the protein concentration and enzyme activity.

Determination of enzyme activity

The SIase activity was investigated in a 1 mL mixture: 100 mM potassium phosphate buffer (pH 6.0), 100 mg/ml sucrose and 10 μg enzyme solution. The obtained solution was incubated at 30 °C for 15 min. Then, the reaction solution was boiled for 7 min to terminate the reaction. Finally, the reaction mixture centrifuged at 12,000×*g*. One unit of sucrose isomerase activity was defined as the amount of enzyme producing 1 μmol isomaltulose per min under the above conditions.

Quantification of sugar composition

Quantification of sugar composition was carried out by High performance liquid chromatograph (HPLC) equipped with a refractive index detector (Waters 2414, Waters Ltd., Milford, MA, USA) using a ZORBAX carbohydrate analysis columns (Agilent Technologies Ltd., California, USA), mobile phase acetonitrile: water = 75:25 (v/v), flow rate 1.0 ml/min at 30 °C. The retention times of fructose, glucose, sucrose and isomaltulose were 8.947, 9.885, 12.716 and 13.535 min, respectively.

Enzyme characterizations

Effects of pH and temperature on SIase activities

Assay reactions were performed at different pH from 4.6 to 10.0 in 100 mM disodium hydrogen phosphate-citric acid buffer (pH 4.6–7.5), 100 mM Tris–HCl buffer (pH 7.5–8.6) and 100 mM Gly-NaOH buffer (pH 8.6–10.0). The pH stability was confirmed by detecting the residual activity of pre-incubated SIase at various pH ranging from 4.6 to 10.0. The optimum temperature of SIase was assayed by detecting enzyme activity at various temperatures (20–55 °C). Thermal stability was evaluated by determining residual enzyme activity of pre-incubated SIase at different temperatures ranging from 22 to 55 °C.

Effect of metal ion and reagents on SIase activity

SIase activity was measured by pre-incubating the enzyme with 1 mM various metal ions (Cu^{2+}, Al^{3+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Ag^{3+}, Fe^{3+} and Mn^{2+}) for 2 h. To evaluate the effect of reagents, the SIase was pre-incubated with various reagents (DMSO, EDTA, Tween-20, Tween-80, and Triton X-100) at a concentration of 5% for 2 h. The activity of untreated SIase was set as control.

Kinetic parameters

For kinetic analysis, SIase activity was measured at different sucrose concentrations (50–250 mM) at pH 6.0 and 30 °C. The maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) were determined from Michaelis–Menten plots.

Time course of isomaltulose production

The reaction was carried out in 10 ml mimic biotransformation system containing 100 mM potassium phosphate buffer (pH 6.0). The substrate concentration was set at 300, 400 and 500 g/L, respectively. The enzyme concentration was 0.1 mg/ml. The reaction was conducted at 30 °C for specified time and taken with a time interval of 30 min. The production of isomaltulose was calculated after the reaction was terminated by boiling for 7 min.

Docking analysis

The three-dimensional structure of sucrose molecule was drawn by Chem3D, and the program was run to minimize the energy and optimize the spatial structure of sucrose molecule. To generate the enzyme model, the X-ray crystallographic structures of SIase (PDB: 4HOX, a resolution of 2.0 Å) was used as template that shared 78.24% sequence identify with SIase. AutoDock Vina was used for docking to predict the binding energy and tune the ligand placement in the binding site under the default docking parameters, and point charges were initially assigned according to the AutoDock semi-empirical force field.

Results and discussion

Expression of sucrose isomerase

The SIase gene from *Erwinia* sp. Ejp617 was selected after database mining using a well-studied SIase from *Erwinia rhapontici* (GenBank: AAK28735.1) as probe, which showed a sequence similarity of 74.6%

(Börnke et al. 2001). Then, the *Erwinia* sp. Ejp617 SIase gene was synthesized after codon optimization. The recombinant *E. coli* harboring pET28b-SI was constructed and induced by 0.1 mM of IPTG at 25 °C for 10 h. SDS-PAGE showed that pET28b-SI had a partly soluble expression (Fig. 1a) and its molecular weight was estimated to be approximate 70 kDa (Fig. 1b), which was consistent with the predicted MW value. The purified SIase showed a single band demonstrating that the enzyme has been purified to homogeneity. The specific activity of purified SIase reached 18.86 U/mg, which was slightly higher than the recombinant *E. coli* with 14.5 U/mg (Li et al. 2013). According to reports by Zhang et al. (2019a), the common soluble expression level of SIase in *E. coli* is not high and most of recombinant protein will be folded incorrectly as inclusion body. For better industrial application, more hosts, such as *Yarrowia lipolytica* (Zhang et al. 2019a) can be considered to improve the expression of SIase.

Characterizations of the SIase

Effect of pH on SIase activity

The SIase activity was assayed at different pH values at 30 °C using sucrose as substrate. As shown in Fig. 2a, its optimum pH was 6.0. The results showed that the optimal pH of SIase was 6.5 (Fig. 2b). Furthermore, relative SIase activity was decreased rapidly when pH is higher than 9.0 and almost no activity when pH is 10, which was similar to SIase from *P. dispersa* (Wu and Birch 2005) and *Erwinia rhapontici* NX-5 (Ren et al. 2011). Previous studies have suggested that SIase showed narrow pH spectrum for activity, such as *Enterobacter* sp. SIase (Cha et al. 2009), *K. pneumonia* SIase (Aroonnual et al. 2007) and *Erwinia* sp. SIase (Kawaguti et al. 2010). Therefore, the stability of the SIase is unlikely to be affected when protein exposed to lower pH (Li et al. 2017). Meanwhile, the enzyme that can tolerate acidic environment would reduce the side-reaction between sucrose and protein (Li et al. 2011). Thus, this study indicated that pH had a significant effect on the activity of purified SIase.

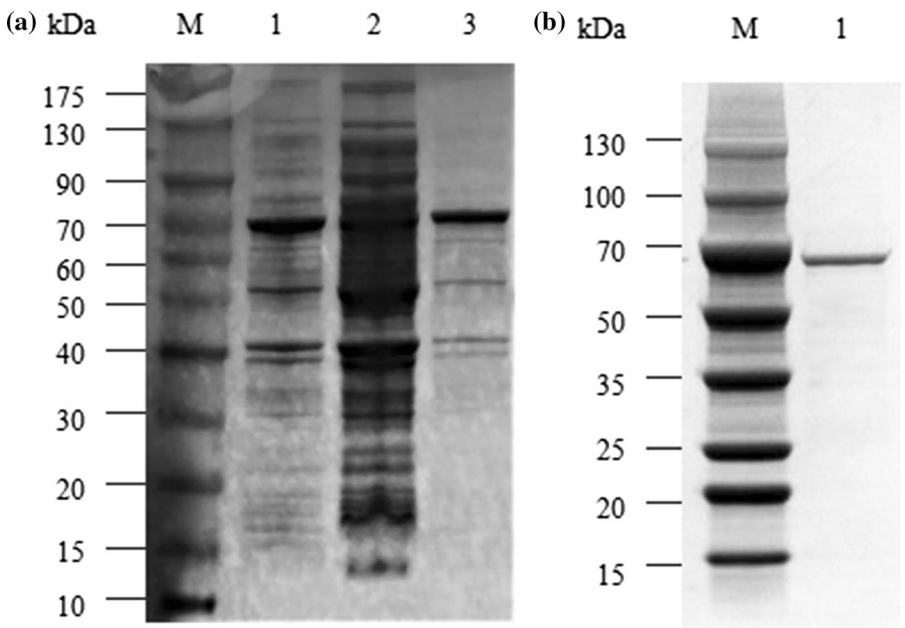

Fig. 1 **a** SDS-PAGE analysis of expression products and purified SIase. *Lane M* standard proteins marker of different molecular weights, *lane 1* supernatant of the cell extract, *lane 2* pET28b-SI induced by IPTG, *lane 3* precipitation of cell extract. **b** SDS-PAGE analysis of purified enzyme. *Lane M* standard proteins marker of different molecular weights, *lane 1* the purified enzyme

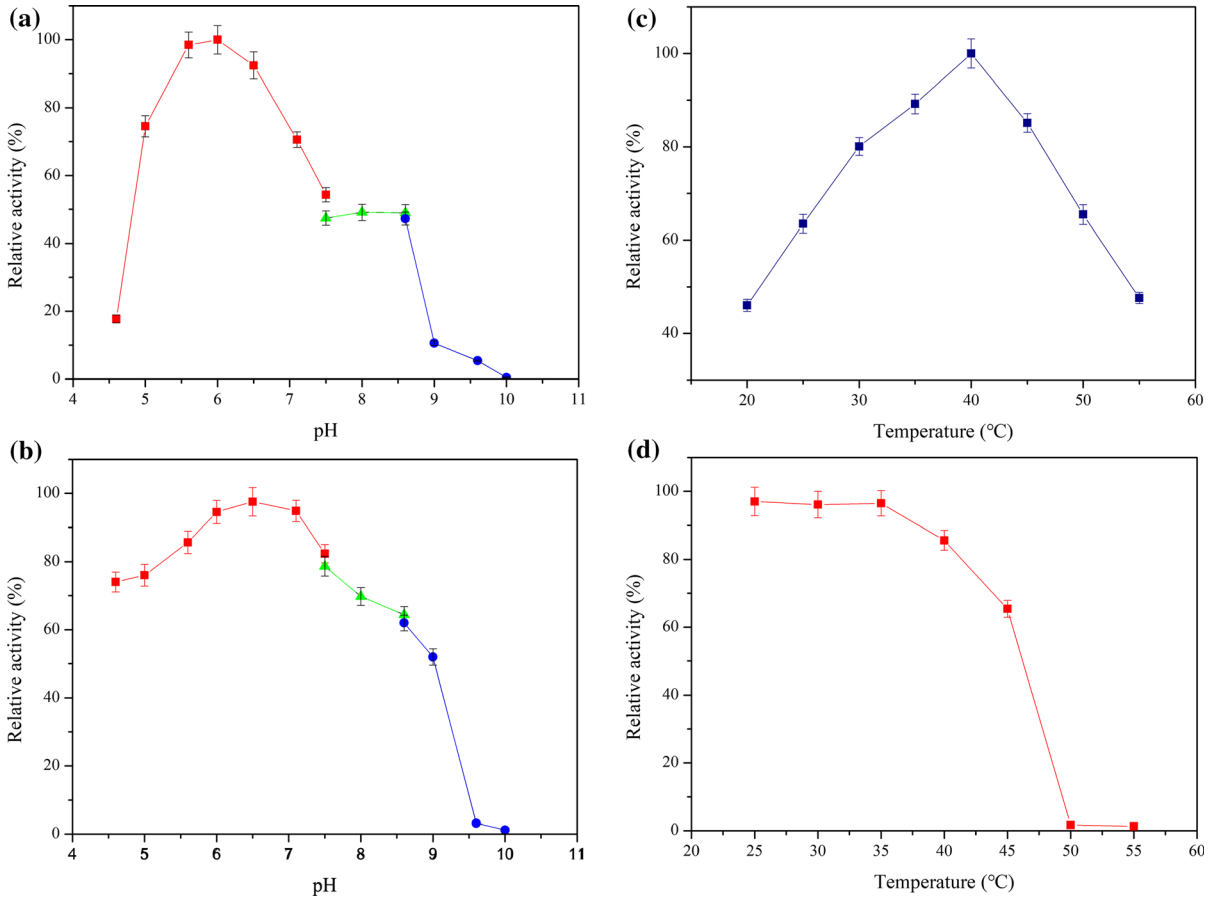

Fig. 2 Enzyme characterization. **a** Effect of pH on the SIase activity. The enzyme activity was measured at different pH values with 100 mM disodium hydrogen phosphate-citric acid buffer, pH 4.6–7.5 (■), Tris–HCl buffer, pH 7.5–8.6 (▲), Gly-NaOH buffer, pH 8.6–10.0 (●). **b** pH stability of the SIase. The purified sucrose isomerase was pretreated in different buffers with pH ranging from 4.6–10.0 at 4 °C for 24 h and the residual activity was tested. **c** Effect of temperature on the SIase activity. Reactions were performed at 100 g/L substrate for 15 min at various temperature (20–55 °C) with 100 mM disodium hydrogen phosphate-citric acid buffer (pH 6.0). **d** The thermo-stability of the SIase. The enzyme was incubated at different temperatures ranging from 22–55 °C in 100 mM disodium hydrogen phosphate-citric acid buffer (pH 6.0) for 1 h and the residual activity was tested

Effect of temperature on SIase activity and thermo-stability

The optimum temperature of SIase was assayed at temperatures ranging from 20 to 55 °C. As shown in Fig. 2c, purified SIase had maximal activity at around 40 °C, which was similar to that of *Pseudomonas mesoacidophila* SIase (Nagai et al. 1994). This optimum temperature was also higher than that of *Erwinia rhapontici*, *Pantoea dispersa* and *Erwinia* sp. strain (Contesini et al. 2013; Li et al. 2011, 2017). However, when the reaction temperature reached below or above 40 °C, the activity decreased gradually.

The thermo-stability results indicated that the activity of the purified SIase maintained about 94.7% of its original activity after incubation at 35 °C for 1 h. However, when the incubation temperature reached 50 °C and 55 °C, after incubation for 1 h, the residual activity was sharply reduced to 1.7% and 1.3%, respectively. These results showed that SIase obtained in this study was more stable below 50 °C, which is similar with SIase from *Enterobacter* sp. FMB-1 (Lee et al. 2011). Wu et al. also proved that the purified SIase from *Erwinia rhapontici* WAC2928

was unstable at high temperature (Wu and Birch 2005). During the industrial scale, SIase is required to have high thermal stability to maintain the enzymatic catalytic efficiency and reduce enzymatic consumption. Therefore, the thermal stability of the SIase can be improved through protein engineering strategies.

Effect of metal ions and reagents on SIase activity

The SIase activity was measured by pre-incubating the enzyme with various metal ions and reagents, and the activity of untreated SIase was set as control, because ions and reagents are reported may significantly affect the activity of SIase (Zhang et al. 2019b). As shown in Table 1, EDTA slightly reduced the SIase activity to 84.8%, suggesting that SIase may be a metal-dependent enzyme. Furthermore, it was observed that Mn^{2+} and Mg^{2+} possessed the positive effect on the SIase activity, and the metal ions including Ca^{2+}, Ba^{2+}, Cu^{2+} and Zn^{2+} partially inhibited the SIase activity. This phenomenon was analogous to most of isomerases, such as glucose isomerase (Liu et al. 2015) and arabinose isomerase (Oh 2007), which required metal ions as cofactor and the enzymatic activity was significantly enhanced after incubation with metal ions.

Table 1 Effect of metal ions and other reagents on sucrose isomerase activity

Reagent	Relative activity (%)
Control	100 ± 1.65
Fe^{2+}	101.25 ± 2.02
Ni^{2+}	96.79 ± 1.56
Ca^{2+}	77.51 ± 1.74
Ba^{2+}	78.60 ± 2.13
Cu^{2+}	82.68 ± 2.41
Mn^{2+}	121.98 ± 1.68
Al^{3+}	94.59 ± 1.96
Zn^{2+}	85.92 ± 2.87
Mg^{2+}	113.12 ± 1.69
Fe^{3+}	88.25 ± 2.31
EDTA	84.79 ± 2.14
Tween-20	94.82 ± 3.14
Tween-80	106.28 ± 1.87

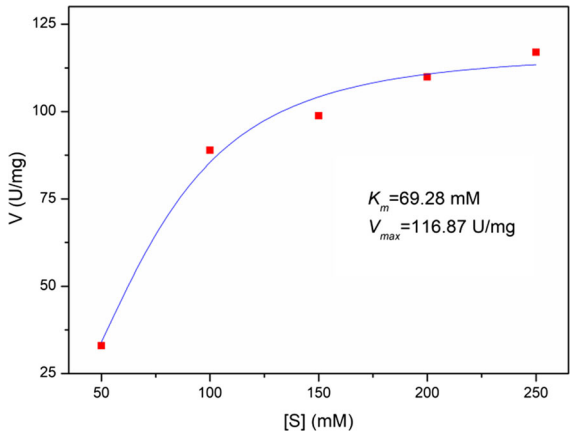

Fig. 3 Michaelis–Menten plot of purified SIase using sucrose as a substrate

Determination of kinetic parameters

Michaelis–Menten constant K_m and maximum reaction rate V_{max} were calculated from Michaelis–Menten plots using sucrose as substrate. From Fig. 3, the values of K_m and V_{max} were calculated to be 69.28 mM and 118.87 U/mg, respectively. To be noted, the obtained K_m value was lower than that of SIases from *Klebsiella planticola* UQ14S (Wu and Birch 2004) and *Klebsiella* sp. strain LX3 (Zhang et al. 2002). It was worth mentioning that low K_m and high V_{max} values of SIase was advantageous for practical application. Unlike previously reported SIases (Pilak et al. 2020; Ren et al. 2011; Salvucci 2003), the SIase in this study showed no obvious reverse reaction producing glucose, fructose, trehalulose from

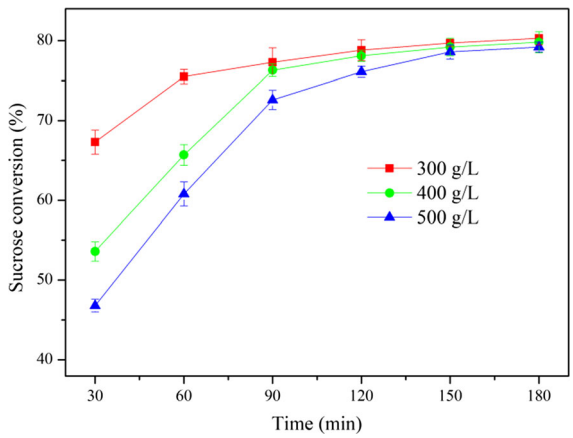

Fig. 4 Time course of isomaltulose synthesis by the purified SIase

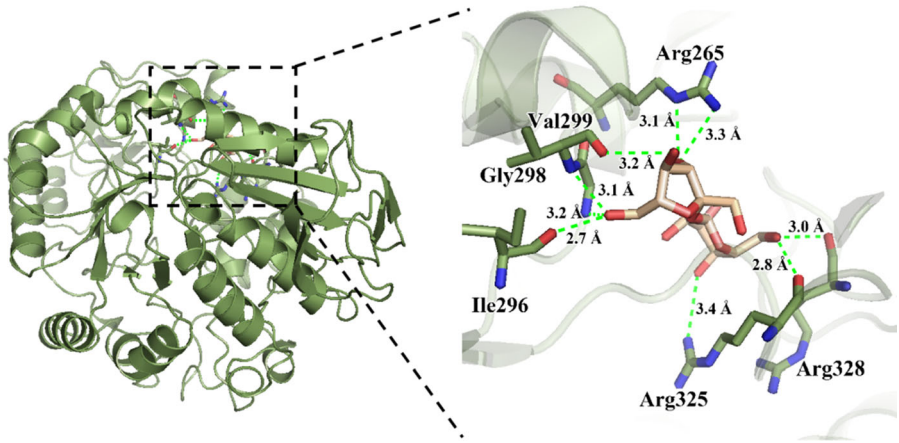

Fig. 5 The overall structure of SIase model and the zoom view of molecular docking of SIase with the substrate

isomaltulose. These properties were very important, indicating that there may be differences in enzyme active sites. Therefore, the SIase in this study is a competitive biocatalyst candidate for the production of isomaltulose on a large scale, and this method is considered to be very practical and is likely to be used in industrial applications.

Time course of isomaltulose production

The isomerization process from sucrose to isomaltulose was further investigated in a mimic reaction system using different sucrose concentration. As shown in Fig. 4, at the sucrose concentration of 300 g/L, the yield of isomaltulose reached 75.5% within 60 min, and finally reached up to 80.5% while the reaction time extending to 180 min. However, once the substrate concentration was further increased to 400 and 500 g/L, the catalytic efficiency of SIase was decreased, the corresponding yields were 79.6% and 78.4%, respectively. The yield is higher than that obtained from the immobilized cell biotransformation by *Erwinia* sp. D12 and the enzymatic reaction by *Erwinia* cells, which showed 75% and 63% yield of isomaltulose, respectively (Contesini et al. 2013; Kawaguti and Sato 2010). Higher substrate concentration resulting in lower product yields was probably because the increased liquid viscosity introduced by high substrate concentration may affect mass-transfer efficiency and the kinetics performance of SIase (Li et al. 2017).

Docking and in silico *analysis*

The SIase model was built based on the crystal structure of SIase (PDB: 4hox, a resolution of 2.0 Å) (Fig. 5). The primary amino acid sequence of SIase shows 78.2% identity and 56% similarity to protein 4hox. And the Q-Mean values of this model are 0.52, which indicated that the quality is satisfied. Sucrose molecule was docked into the substrate-binding pocket of SIase by docking under semi-empirical force field, which yielded 20 possible results. They showed stronger binding with negative values towards energy. The active site is surrounded by a loop (Arg265, Ile296, Gly298, Val299, Ard325, and Arg328), forming a pocket large enough to hold a sucrose molecule. The hydrogen bonds were formed between SIase and sucrose molecule. In fact, all of the known SIases had predicted secondary structures containing an *N*-terminal TIM barrel $(\beta/\alpha)_8$ A domain with a B subdomain inserted between β sheet 3 (AS3) and α helix 3 (AH3). In addition, there was a *C*-terminal domain containing 7 to 10 β sheets (Wu and Birch 2004).

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