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Synthesis of isomalto-oligosaccharides by using recombinant dextransucrase and *Hypocrea lixii* dextranase

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ABSTRACT

Due to their abundant nutrition and potential prebiotic functions, the potential development of oligosaccharide synthesis is very important. In this context, Synergistic catalytic manner of recombinant dextransucrase and Hypocrea lixii dextranase were elucidated according to carbohydrate analysis by carbohydrate gel electrophoresis and high performance liquid chromatography. The molecular weight of dextrans in the dynamic process of double enzymatic system with low concentrations sucrose showed an increasing firstly and then decreasing trend. High concentrations sucrose in dextransucrase and dextranase system led to accumulation of oligodextrans with molecular weight below 15 kDa. Comparative study of isomalto-oligosaccharides synthesis through both sucrose conversion and dextran hydrolysis was performed. Dextran hydrolysis through Hypocrea lixii dextranase gave rise to IMOs with degree of polymerization (DP) 2-15. Combined usage of recombinant dextransucrase and Hypocrea lixii dextranase produced isomalto-oligosaccharides with a DP range of 2-10, which showed great advantage and potential industrial value.

Keywords: dextransucrase; dextranase; double enzymatic catalysis; oligosaccharides;

INTRODUCTION

Oligosaccharides have been widely used for functional food and pharmaceutical industries, due to their abundant nutrition and potential prebiotic functions [1, 2]. Especially, isomalto-oligosaccharides (IMOs) have been used to treat chronic constipation, improve serum lipid profiles and lower cholesterol levels [3], which exhibit excellent effects on promoting food absorption and digestion and maintain normal intestinal microbial balance [4, 5]. Therefore, the synthesis and application of oligosaccharides have become a great interest of both researchers and industries.

Generally, isomalto-oligosaccharides are prepared by three different approaches, 1) by using glucoamylase to catalyze high concentration glucose to form maltose and isomaltose; 2) by using transglycosylation to synthesize isomaltose and panose from maltose [4, 6]; 3) by using α-amylase to hydrolyze starch to produce a mixture of maltose, panose and IMOs [7]. These tranditional techniques are not friendly to the environment with high cost, and the yield of IMOs need to be further improved. Acceptor reactions of free and immobilized dextransucrase also can synthesize isomalto-oligosaccharides [8, 9, 10], and the hydrolysis of dextran using acid or soluble and immobilized dextranase can give rise to isomalto-oligosaccharides [11, 12]. There were also reports about the combined use of dextransucrase and dextranase to synthesize oligodextrans and isomalto-oligosaccharides [13, 14].

Along with these research, we conducted a comparative study of isomalto-oligosaccharides synthesis through both sucrose conversion and dextran hydrolysis in seek of the most appropriate industrial IMOs (DP 2-10) preparation method,, and the dynamic process was monitored by using carbohydrate analysis by carbohydrate gel electrophoresis (PACE), and the synergistic catalytic manner of dextransucrase and dextranase has been elucidated. The results showed that dextran hydrolysis through *Hypocrea lixii* dextranase gave rise to IMOs with DP 2-15, combined usage of recombinant dextransucrase and *Hypocrea lixii* dextranase produced isomalto-oligosaccharides with a DP range of 2-10, which showed great advantage and potential industrial value.

EXPERIMENTAL SECTION

Chemicals

Dextran standards (dextran 1400, 670, 410, 270, 150, 50, 25, 12, and 5 kDa) for the calibration curves of molecular weight were purchased from Sigma. 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was purchased from Tokyo Chemical Industry. Other chemicals were of analytic grade.

Enzymes

Preparation of dextransucrase and its activity

Dextransucrase used in this study was a recombinant enzyme constructed in our laboratory using *Escherichia coli* BL21 (DE3)/pET28-dexYG as the host [16]. In brief, the fermentation for enzyme production was carried out in a shaker. The culture conditions and medium were the same as the previous reports [17].

After fermentation, the culture broth was centrifuged at 12,000 ×*g* and 4 °C for 5 min in a 0.02 M acetic acid–sodium acetate buffer (pH 5.4) [19]. After centrifugation (12,000 ×*g* and 4 °C, 5 min), the supernatant (crude dextransucrase) was stored at 4 °C. One unit of dextransucrase activity was defined as 0.1 mg fructose liberated per hour [16, 17]. The fructose was determined using the 3,5-dinitrosalicyclic acid assay (DNS) according to the previous report [20].

Preparation of dextranase and its activity

Dextranase was aslo prepared in our lab [21]. The fermentation was carried out in a liquid medium composed of (w/v) 1.5% dextran (70 kDa), 0.5% peptone, 0.4% $K_2HPO_4 \cdot 3H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.02% KCl. The culture condition was 5 days at 28 °C in a 200 rad rotary shaker.

After fermentation, the culture broth was centrifuged at 8000 ×*g* and 4 °C for 15 min. The obtained supernatant was crude dextranase. The crude enzyme solution was treated with ammonium sulfate precipitation. Furthermore, the concentrated dextranase was stored in 0.02 M acetic acid–sodium acetate buffer (pH 5.4).

The reaction condition for activity analysis was performed according to our previous studies [21]. One unit of dextranase activity was defined as the amount of enzyme that hydrolyzed dextran to yield reducing sugars equivalent to 1 μM glucose per min under the optimal conditions, and the reducing sugars were determined by Miller's method [22].

Synergistic synthesis of oligodextran using dextransucrase and dextranase

All of the enzymatic reactions were carried out in 0.02 M acetic acid–sodium acetate buffer (pH 5.4). The catalytic condition was 25 °C and 150 rad with a total volume of 100 mL. The reaction systems were selected with various concentrations of sucrose, dextransucrase and dextranase (Table 1). In all cases, dextransucrase and dextranase were added simultaneously into sucrose solution after the solution was equilibrated to 25 °C. Samples were took out at intervals and boiled for at least 6 min, then diluted to 5 mg/mL.

The molecular weight of dextran in each samples were determined by high-performance liquid chromatography (HPLC, Waters, Milford, USA) using a TSK gel G4000XL (7.8 mm ×30 cm) (TOSOH, HOSOL, Japan) column connected to a differential refractive index detector (Knauer, Berlin, Germany) [21]. Chromatography was operated at 60 °C with a flow rate of 0.6 mL/min. Calibration curves of the retention time and molecular weight were prepared for dextran standards (Sigma): 1400, 670, 410, 270, 150, 50, 25, 12, and 5 kDa dextran.

Table 1 The experimental parameters in the dextransucrase-dextranase synthesis system

Sucrose concentration (mg/mL)	Dextransucrase concentration (U/mL)	Dextranase concentration (U/mL)	Sampling time (h)
Group I			
20	2.0	2.5	1-8, 24
40	2.0	2.5	1-8, 24
60	2.0	2.5	1-8, 24
80	2.0	2.5	1-8, 24
Group II			
200	3.0	3.0	2-10, 24-32
300	3.0	3.0	2-10, 24-32
400	3.0	3.0	2-10, 24-32
500	3.0	3.0	2-10, 24-32
600	3.0	3.0	2-10, 24-32
Group III			
60	2.0	2.5	1-8, 24
60	4.0	2.5	1-8, 24
60	7.0	2.5	1-8, 24

Synthesis and analysis of isomalto-oligosaccharides

Synthesis of IMOs by using double enzyme system

Constant concentrations of dextransucrase (5.0 U/mL) and dextranase (12.0 U/mL) with two different concentrations of sucrose were performed in this experiment. The reactions were carried out in the same conditions described in section 2.3.1. Samples were took out at 4 h, 8 h, 12 h, 24 h, 28 h and 32 h and boiled for at least 6 min, and then diluted to 5 mg/mL for PACE analysis, respectively.

Preparation of IMOs by the hydrolysis of dextran

Industrial dextran 20 kDa was used as the hydrolysis substrate. Two different concentration of dextranase (6 U/mL and 12 U/mL, respectively) were reacted with 50 mg/mL dextran 20kDa at 25 °C with a mechanical stirring device. Samples were took out at an interval of 1 h and boiled for at least 6 min and then diluted to 5 mg/mL for PACE analysis, respectively. In addition, 200 μL of 5 mg/mL dextran 20 kDa was added to trifluoroacetic acid (TFA) at a final concentration of 0.5 mol/L. The partial acid hydrolysis was performed at 80 °C for 3 h, then the reaction solution was treated with methanol and dried in a 40 °C nitrogen evaporator for three times to remove TFA. The dried sample was stored at 4 °C.

Analysis of IMOs using PACE

All of the samples produced in above section 2.3.2.1 and 2.3.2.2 were treated with 8-amino-naphthalene-1,3,6-trisulfonic acid (ANTS) for derivatization [15, 23]. The derivatized sugars (3 μL) were separated on a vertical slab gel electrophoresis apparatus, Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA) [15]. 30% (w/v) polyacrylamide and 8% (w/v) polyacrylamide were used as resolving gel and stacking gel respectively. Both resolving and stacking gels were prepared in pH 8.2 0.1 mol/L Tris-boric acid and were cast and cooled at least 2 h before use. Samples were electrophoresed at 200 V for the first 20 min and then at 700 V for the last 40 min. The gel imaging was performed in an InGenius LHR CCD camera system (Syngene, Cambridge, UK) under UV 365 nm.

RESULTS AND DISCUSSION

The effect of different concentrations sucrose on Synergistic synthesis of oligodextran indouble enzyme system

Dextransucrase and dextranase were added simultaneously into sucrose solution after the solution was equilibrated to 25 °C, and the effect of different concentrations sucrose on the molecular weight change of oligodextrans in the dynamic double enzyme reaction process was studied. The results were showed in Fig. 1.

Four curves in Fig. 1A showed the similar trend of increasing firstly and then decreasing, which due to the activity of dextransucrase towards dextran synthesis in the initial reaction stage. The dextranase began to degrade reaction only the dextran reached a certain size, which attributed to the *Hypocrea lixii* F1002 dextranase showing high hydrolytic affinity towards high molecular weight dextrans. Therefore, the molecular weight of dextran products increased firstly and then decreased gradually. The curves also showed that the molecular weight of dextran products was inverse proportional to the sucrose concentration at initial stage but direct proportional at later period, which

assumed to be caused by the processive polymerization mechanism of dextransucrase [24] that there was less time need for dextransucrase reacting with lower concentration sucrose to reach a high molecular weight dextran; the reached dextran was hydrolyzed firstly by *Hypocrea lixii* dextranase and was certainly lower than dextrans produced in higher concentration sucrose systems at each same time period.

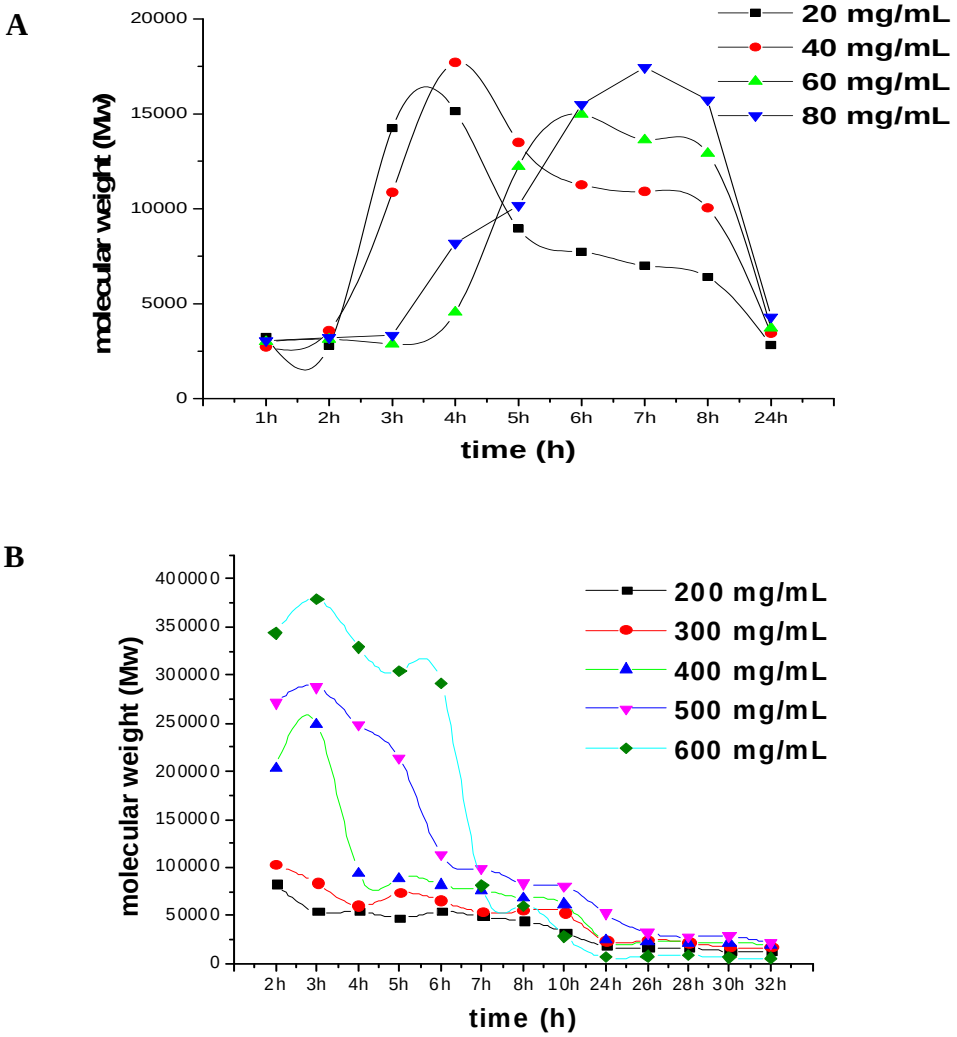


Fig. 1. Effect of the concentrations of sucrose on the molecular weight change of dextrans

A, The HPLC results of dextransucrase (2.0 U/mL) and dextranase (2.5 U/mL) system with low concentration of sucrose solutions range from 20 mg/mL to 80 mg/mL. B, The HPLC results of dextransucrase (3.0 U/mL) and dextranase (3.0 U/mL) system with high concentrations of sucrose range from 200 mg/mL to 600 mg/mL

It was reported that dextransucrase reacting with increasing concentration of sucrose gave rise to low molecular weight dextrans [25, 26]. Daniel J. Falconer [27] reported that the molecular weights of dextrans products were direct proportional to the concentration of sucrose. In the current study, molecular change of dextran with the passage of time in double enzyme system with series high concentrations of sucrose (200 mg/mL, 300 mg/mL, 400 mg/mL, 500 mg/mL and 600 mg/mL) was given in Fig. 1B. For every high concentration of sucrose, the molecular weight change of dextran showed an overall downward trend. Unlike the low concentration sucrose systems, the increasing firstly and then decreasing trend happened much earlier and was not so obvious. This was due to the sucrose substrate inhibition which changed the allosteric conformation of the dextransucrase [28, 29, 30]. In addition, the molecular weight of dextran products increased with the increasing sucrose concentration (200 mg/mL to 500 mg/mL) for every time point during the reaction process. However, when sucrose solution reached a certain high

concentration, increasing the sucrose content made molecular weight of dextran decreased (600 mg/mL in this study), which suggested that the dextran molecular weight showed a restrictive relationship with sucrose concentration.

Effect of different ratios of dextransucrase to dextranase on the molecular weight change of dextran in double enzyme system

The effect of different ratios of dextransucrase to dextranase on the molecular weight change of dextrans in the dynamic double enzyme was studied. The results were showed in Fig. 2.

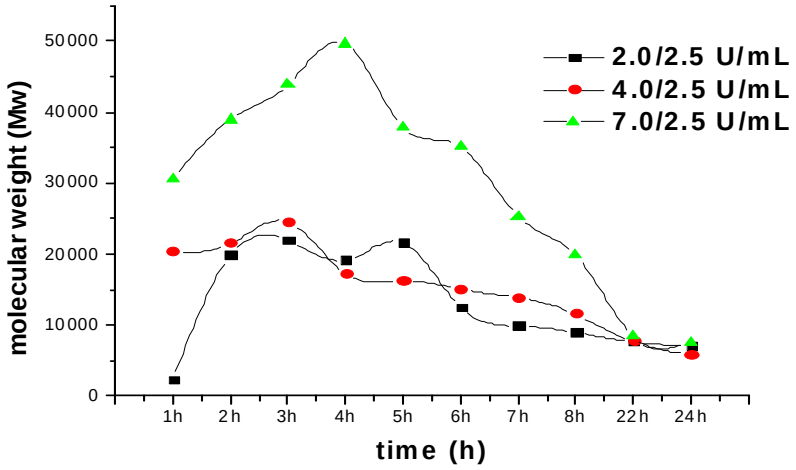


Fig. 2. Effect of different ratios of dextransucrase to dextranase on the molecular weight change of dextrans

The double enzyme system was composed of sucrose (60 mg/mL) and dextranase (2.5 U/mL) with different concentrations of dextransucrase (2.0, 4.0 and 7.0 U/mL, respectively)

The molecular weight of dextran products increased faster with the increase of dextransucrase concentration, which showed a direct proportion between dextran molecular weight and dextransucrase concentration in the beginning reaction stage. When the ratio of dextransucrase and dextranase was greater than one (4.0/2.5 U/mL, 7.0/2.5 U/mL in this study), the polymerization (reaction of dextransucrase) rate of dextran chains was faster than its cleavage (reaction of dextranase) rate. However, when the concentration of dextransucrase and dextranase was similar (2.0/2.5 U/mL in this study), the molecular weight change of dextran products had a small magnitude fluctuation period, which showed a coordinate state of both polymerization and degradation in the double enzyme system.

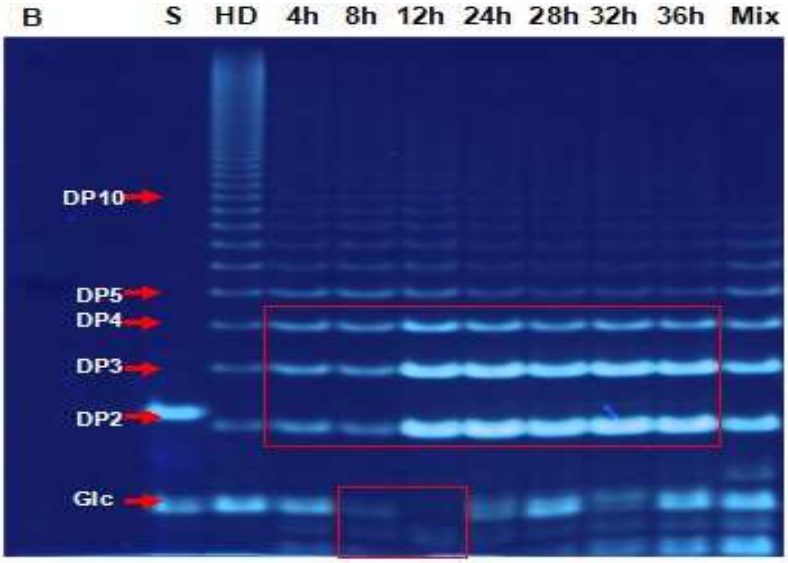
Synthesis of IMOs by the combined using of dextransucrase and dextranase

A comparative study of IMOs synthesis through both dextran hydrolysis and sucrose conversion was performed in this study. The results of analysis of sucrose conversion in double enzyme system comprised of dextransucrase and dextranase was showed in Fig. 3A and Fig. 3B. IMOs synthesized in this system were composed of saccharides with DP 2-10. The products with DP 2-3 maintained almost unchanged in the long period of 24-36 h and the major DP of IMOs was between 2 and 4, and few IMOs were DP 5-10, which suggested that the acceptor reaction of dextransucrase was not dominant when dextranase concentration was increased [13, 28, 31]. Different concentration of sucrose was also compared (Fig. 3A and Fig. 3B). The intensity of IMOs bands in lane (4 h to 12 h) in Fig. 3A were much higher than those of Fig. 3B, which suggested that IMOs accumulated faster with the increasing concentration of sucrose.

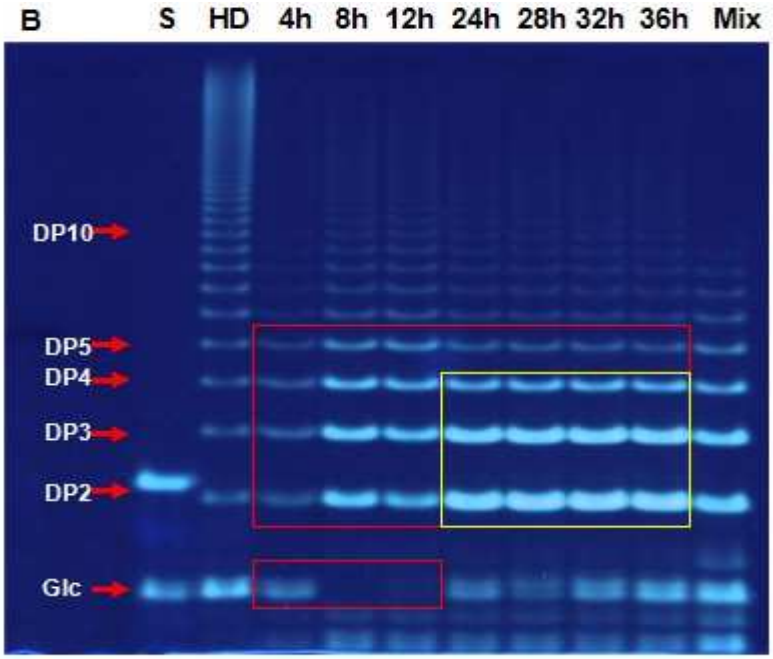
Products prepared by dextran hydrolysis were detected clearly by PACE (Fig. 3C and Fig. 3D). The intensity of IMOs bands in Fig. 3C were much higher than those of Fig. 3D in every time point, which suggested that the IMOs production was direct proportional to the concentration of dextranase. Partial acid hydrolysis produced oligosaccharides with DP 2-15 containing oligodextrans and a significant amount of glucose. By contrast, the hydrolysates with dextranase were mainly composed of DP 2-15 without glucose in the same reaction time (3 h). Therefore, enzymatic preparation of isomalto-oligosaccharides showed great advantage, since functional IMOs were more effective with less digestible sugars like glucose and maltose [32]. However, the double enzymatic synthesis of

IMOs (DP 2-10) was much better than the dextranase hydrolysates (DP 2-15), as IMOs with larger DP (DP>10) were absorbed slowly with less amount and thus showed less effective prebiotic functions [33].

A



B



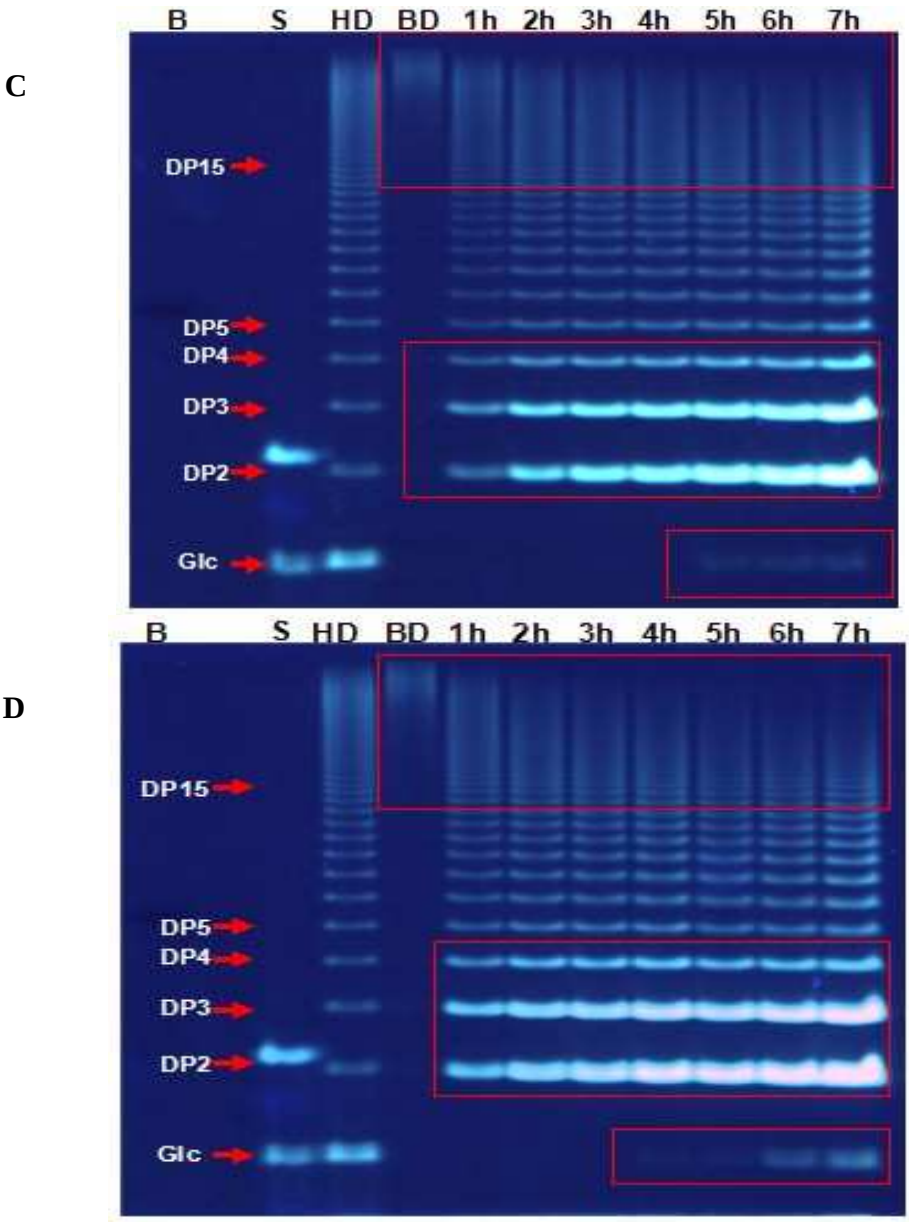


Fig. 3. PACE analysis of the isomalto-oligosaccharides produced by sucrose conversion (A and B) and dextran hydrolysis (C and D)
Lane B, Bromophenol blue; Lane S, Glucose and laminaribiose; Lane HD, partial acid hydrolysates of dextran 20 kDa used as markers; Lane 4 h to 32 h, IMOs products synthesized in the dextransucrase (5.0 U/mL) and dextranase (12.0 U/mL) system with 200 mg/mL (A) and 300 mg/mL (B) sucrose; Lane Mix, mixture of IMOs separated from oligodextrans after ethanol precipitation; Lane 1 h to 7 h, dextranase (C 6 U/mL, D 12 U/mL respectively) hydrolysates for every time point

There were clear bands of glucose in the earlier double enzymatic reaction stage (lane 4 h in Fig. 3A and Fig. 3B) but gradually faded (lane 8 h) and then disappeared (lane 12 h), which suggested that dextran synthesis was the main reaction happened in this period. In the initial catalytic process of dextransucrase, the enzyme attacked C-1 of D-glucosyl moieties of sucrose [24]. Therefore, there should be a large mount of glucose to be transferred in double enzyme systems with high concentrations sucrose. With the reaction proceeded, more and more D-glucosyl moieties were linked to form dextran chains. Accordingly, the glucose bands in the carbohydrate gel electrophoresis decreased gradually and eventually disappeared. Previous study showed that Hypocrea lixii dextranase was a typical endodextranase which produced isomaltotriose and isomaltose [21]. Isomaltotriose decreased and isomaltose was the major product after excessive hydrolysis [21]. Thus, glucose bands appeared again in the later period of IMOs synthesis (lane 24-36 h in Fig. 3A and Fig. 3B) as well as dextran hydrolysis (lane 5-7 h in Fig. 3C and lane 4-7 h in

Fig. 3D) by dextranase. The figure showed clearly how the reaction proceeded in the double enzyme system and the preparation of IMOs can be controlled without glucose and larger DP through adjusting the reaction time.

CONCLUSION

Double enzymatic catalysis of recombinant dextransucrase and *Hypocrea lixii* dextranase was researched in this study. Products in double enzymatic system with low concentrations sucrose (< 80 mg/mL) showed an increasing first and then decreasing trend of molecular weights. High concentrations sucrose (200 mg/mL to 600 mg/mL) in dextransucrase and dextranase system led to accumulation of oligodextrans with molecular weight below 10 kDa. Acceptor reaction of dextransucrase was partial inhibited by high concentration of sucrose and dextranase. Combined use of recombinant dextransucrase and *Hypocrea lixii* dextranase produced isomalto-oligosaccharides with the DP range from 2 to 10. The specific structural identification of synthesized IMOs was in progress. This study provided a simple method for the preparation of IMOs, which showed great potential industrial values.

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