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Journal of Molecular Catalysis B: Enzymatic 16 (2001) 109–114

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Synthesis of galacto-oligosaccharides in AOT/isooctane reverse micelles by β-galactosidase

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Received 1 May 2001; received in revised form 6 July 2001; accepted 7 July 2001

Abstract

Synthesis of galacto-oligosaccharides (GOS) from lactose by β-galactosidase was investigated in AOT/isooctane reverse micelles where the enzyme was fairly stable. GOS yield increased with initial lactose concentration in both reverse micelles and aqueous system. Transgalactosylation reaction of the enzyme was strongly dependent on the molar ratio of water to surfactant (w_0) of reverse micelles. At pH 7.0 and 45°C, a maximum GOS concentration from 45% (w/v) lactose solution was 51.2% (w/w) in the reverse micelles with $w_0 = 15$, while a maximum of 31% in aqueous media. Effects of solvents on GOS formation were also examined.

Keywords: Galacto-oligosaccharides; β-galactosidase; AOT/isooctane; Reverse micelles

1. Introduction

Galactose-oligosaccharides (GOS) containing 3–10 molecules of galactose and glucose are also known as "Bifidus growth factor" and have been found to stimulate the growth of *Bifidobacteria* in the human body [1,2]. For this reason, GOS are now widely used as a foodstuff beneficial to human health. GOS are formed when lactose is subject to an enzymatic hydrolysis by β-galactosidase. The normal function of β-galactosidase is to hydrolyze lactose to glucose and galactose. However, under certain reaction conditions, the same enzyme also catalyzes transgalactosylation reaction and synthesizes GOS.

Although most of β-galactosidases belonging to the class of hydrolytic enzymes have the transgalactosylation capability, the main drawback of GOS synthesis by these enzymes is that the reaction equilibrium is shifted to favor hydrolysis over synthesis in aqueous systems, which leads to a low yield in GOS production. Therefore, some approaches to increase oligosaccharides formation from lactose have been proposed, including screening for microorganisms with high capabilities to GOS production [3], and enzyme reaction in organic media [4]. Synthesis of GOS in organic media has its special advantages because the thermodynamic equilibrium can be shifted to synthetic direction by reversing the normal hydrolysis due to the limitation of water activity in the reaction media. In a few recent studies, the glycosidation reaction in organic solvent showed enhanced oligosaccharides production [5–7]. Reverse micelles were also used as an effective low-water media for biotransformations [8]. The catalytic behaviors of enzymes entrapped in water pool of reverse micelles are quite different

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1381-1177/01/$ – see front matter
PII: S1381-1177(01)00051-0

from that in aqueous media. Syntheses of peptides and esters via enzymes in reverse micelles have been achieved [9,10]. To our knowledge, GOS production in reverse micelles has not been reported yet. In this article, GOS synthesis by β-galactosidase from *A. oryzae* in AOT/isooctane solution was investigated in order to improve the GOS yield and elucidate the characteristics of the hydrolytic enzyme in reverse micelles.

2. Experimental

2.1. Materials

β-galactosidase from *A. oryzae* was purchased from Solvay Enzymes Company (USA) and used without further purification. Standard GOS sample was purchased from Yakult Pharmaceutical Industry Co., Ltd. (Japan). AOT (dioctyl sodium sulfosuccinate) was a product of Aldrich. Other chemicals and solvents were of analytical grade and obtained from Shanghai Chemical Reagent Company (China).

2.2. Synthesis of oligosaccharides in reverse micelles

Reverse micelles were prepared by injecting of lactose solution containing the predetermined amounts of water (0.1 M phosphate buffer, pH 7.0) and β-galactosidase into a solution of 30 mM AOT in isooctane and mixed by a vortex for a few seconds to obtain a clear micellar solution. The concentrations of reactants in 20 ml of reverse micelles solutions were as follows: initial lactose 0.278–1.39 M, β-galactosidase 10 mg/ml. The GOS synthesis experiments were carried out in a water–jacket glass reactor under magnetic agitation at 45°C. At different time intervals, one milliliter of sample was taken from the reactor and heated to 100°C for 5 min. The reaction products in reverse micelles were extracted to an aqueous phase by addition of equal volume of deionized water to the sample and mixing vigorously. Top phase of the mixture was decanted after centrifuging (5000 rpm) for 20 min. Product in bottom phase was condensed and determined by HPLC. Yield of GOS is a weight percentage of oligosaccharides based on total saccharides in the reaction medium.

2.3. β-galactosidase stability studies

The enzyme-entrapped reverse micellar solution was prepared by directly injection appropriate amount of aqueous buffer (0.1 M phosphate buffer, pH 7.0) containing 5 mg of β-galactosidase to 20 ml of AOT/isooctane solution, and incubated in stopped glass tubes under magnetic agitation at 45°C. At different time intervals the mixture was taken for the measurement of enzyme activity as reported previously [11].

2.4. Determination of saccharides

Analysis of all saccharides produced from reaction was done on a SCL-10vp HPLC (Shimadzu) with a refractive index monitor (RID 10vp, Shimadzu), and a 250 mm × 4.6 mm column of KR100-5NH_2 (EKA Chemicals AB) eluted with 68:32 acetonitrile:water at 40°C at a flow rate of 1.0 ml/min.

2.5. Isolation and identification of the products

The carbohydrates in the reaction mixture (5 ml) were adsorbed on 80 g of charcoal and 80 g Celite on a column (30 mm × 300 mm) and desorbed by a discontinuous gradient of water and ethanol in water (5, 10, 15%, respectively). The fractions of trisaccharide eluted with 10% ethanol were concentrated and freeze-dried.

The ^{13}C-NMR spectra of the trisaccharides in D_2O at 25°C were recorded with a AVANCE-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of sodium 4,4-dimethyl-silapentanoate referred to external 1,4-dioxane (67.40 ppm).

3. Results and discussion

3.1. Stability of β-galactosidase in AOT/isooctane solution

In order to use the AOT/isooctane reverse micelles as reaction media for GOS synthesis, it is necessary to investigate the stability of β-galactosidase in the solution. As shown in Fig. 1, the enzyme activity decreased rapidly within early 1 h in the reverse micelles, and then followed by a slower decay during incubation at 45°C. The enzyme in the reverse micelles

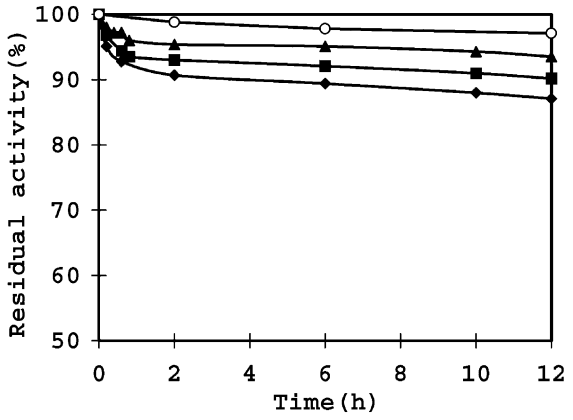

Fig. 1. The stability of β-galactosidase from *A. oryzae* in AOT/isooctane reverse micelles at 45°C: $w_0 = 30$ (▲); $w_0 = 20$ (■); $w_0 = 10$ (◆); aqueous system (○).

was less stable. The deactivation might be a result of the enzyme conformation change caused by electrostatic interactions between polar headgroups of the AOT and the β-galactosidase. It can be seen clearly the stability of β-galactosidase entrapped in the reverse micelles depends on the water content. The higher water content, the more stable the enzyme. The half-life of β-galactosidase was approximately 46 and 90 h at $w_0 = 10$ and 30 in the AOT/isooctane solution, respectively. This tendency could be due to the fact that the properties of water in reverse micelles at higher w_0 values approach to those of bulk water.

3.2. Reaction profile

HPLC was used to analyze the amount of GOS and other saccharides produced in the reverse micellar solution. The components in each peak had the same retention time as the standard GOS (Fig. 2). Fig. 3 shows time course of lactose hydrolysis and GOS formation in the AOT/isooctane solution at $w_0 = 30$ and initial lactose concentration of 45% (w/v). The general form of these curves is typical reaction process at the various experimental conditions studied. As the reaction proceeded, the hydrolysis of lactose resulted in monosaccharides, trisaccharide and a small amount of tetrasacchharide, while pentasaccharide and above were scarcely produced. After incubation for 2 h, the maximum yield of GOS was attained. Prolonging the reaction time to 2.5 h, GOS decreased slowly as they

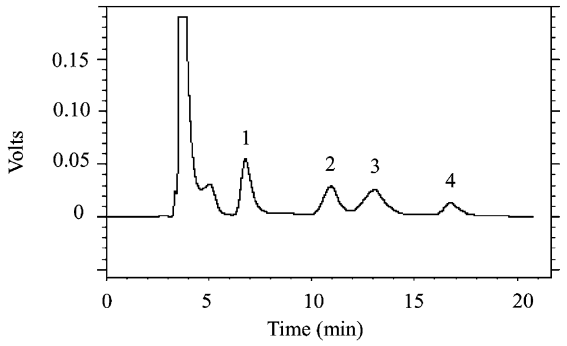

Fig. 2. HPLC chromatogram of a typical product synthesized in reverse micelles: 1, Monosaccharides; 2, Lactose; 3, Trisaccharide; 4, Tetrasaccharide.

were converted to monoses. The structure of trisacchairde, the major product isolated, was identified by ^{13}C-NMR, and the ^{13}C-NMR data shown in Table 1 were compared with the chemical shifts of GOS reported [12]. All the signals were identical with those of the β-D-Gal-(1-4)-β-D-Gal-(1-6)-D-Glc. According to these results, the main transgalactosylation product was identified as allolactose-type (β-1-6).

3.3. Effect of lactose concentration on GOS synthesis

The effect of lactose concentration on GOS synthesis was examined in a 30 mM AOT/isooctane

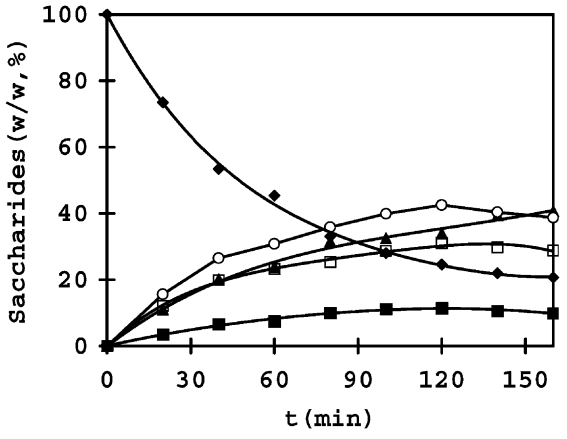

Fig. 3. The time course for lactose conversion, GOS synthesis with β-galactosidase from *A. Oryzae* in AOT/isooctane reverse micelles at 45°C. Initial lactose concentration is 45% (w/v) and $w_0 = 30$: (◆), Lactose; (▲), Monosaccharides; (□), Trisaccharide; (■), Tetrasaccharide; (○), Total of GOS.

Table 1
^{13}C chemical shift and assignment of signals[a]

Chemical shift (ppm)	Assignment	Chemical shift (ppm)	Assignment
105.02	C″-1	73.53	C″-3
104.06	C′-1	72.20	C-2α
96.68	C-1β	72.20	C″-2
92.91	C-1α	71.98	C′-2
77.82	C′-4	70.3	C-4β
76.39	C-5β	69.59	C-6α
75.91	C″-5	69.62	C-6β
75.70	C-3β	69.50	C-5α
75.13	C′-5	69.42	C-4α
74.79	C-2β	69.42	C″-4
73.92	C′-3	61.83	C″-6
73.53	C-3α	61.30	C′-6

[a] C, glucose residue; C′, internal galactose group; C″, nonreducing group.

($w_0 = 30$) solution and an aqueous media, respectively. As shown in Fig. 4, the yield of GOS increased with initial lactose concentration in both systems, but the yield was higher in the reverse micelles than in the aqueous system at the same initial lactose concentration. The maximum GOS yield of 42.5% (w/w) was obtained in the AOT/isooctane solution, and the maximum of 31% (w/w) in the aqueous system at 45% (w/v) lactose. The result suggests that the use of reverse micelles can shift thermodynamic equilibrium toward GOS synthetic direction.

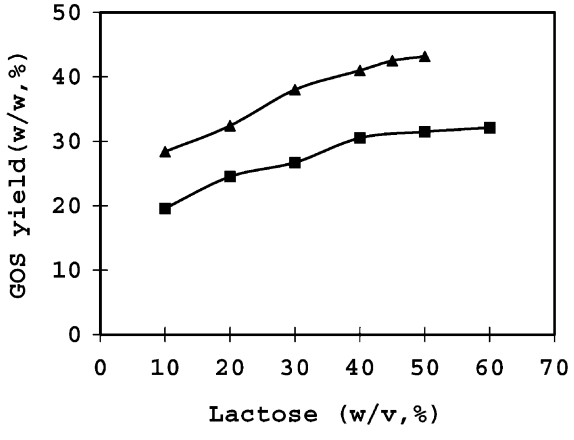

Fig. 4. Effect of lactose concentration on GOS synthesis in AOT/isooctane reverse micelles ($w_0 = 30$). The reaction mixture containing indicated concentration of lactose and the β-galactosidase in a total volume of 20 ml was incubated at 45°C and pH 7.0: (▲), Reverse micelles; (■), Aqueous system.

It is known that β-galactosidase catalyzes both hydrolysis and transgalactosylation reaction, that is, the enzyme transfers the galactose moiety of a β-galactosidase to an acceptor containing a hydroxyl group [13]. Lactose is hydrolyzed to glucose and galactose when water acts as acceptor. However, other sugars present in the solution can also serve as acceptor, GOS are formed by this way. From the viewpoint of transgalactosylation, water can be considered as a major factor unfavorable to the GOS synthesis when a large amount of water existing in the reaction system. In aqueous system, this disadvantage can be improved by increasing lactose concentration. In reverse micelles, the hydrophilic interactions between water and the surfactant decrease the water activity, which causes two important effects on GOS synthesis. First, the hydrolytic activity of the β-galactosidase is effectively inhibited. Second, lactose concentration is increased in micro-aqueous core of the reverse micelles. Precipitation of lactose was found when its concentration was above 52% (w/v) in the AOT/isooctane solution with $w_0 = 30$, while the saturation of lactose solution is 65% in the aqueous buffer at 45°C, which implies that lactose solution in the reverse micelles was condensed. Consequently, GOS formation was enhanced by increasing lactose concentration since more lactose competed with water as an acceptor for galactoly residue. Fig. 5 depicting the relationship between galactose and lactose when GOS concentration reached a maximum value, reflects that less free galactose was present in

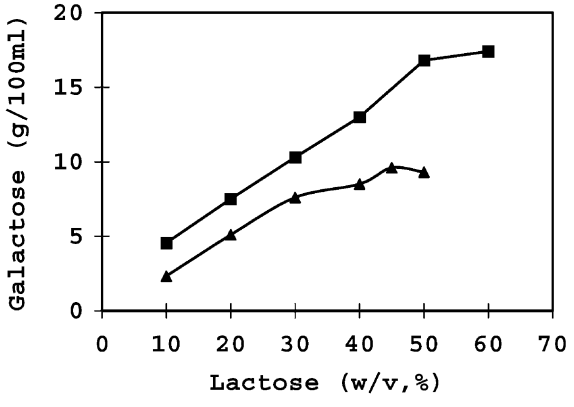

Fig. 5. Concentration of galactose at time GOS yield reached maximum yield in AOT/isooctane reverse micelles ($w_0 = 30$). Reaction conditions were the same as in Fig. 4. (▲), Reverse micelles; (■), Aqueous system.

the reverse micelles due to more galactose incorporated with lactose by transgalactosylation reaction.

3.4. Effect of water content on GOS synthesis

Water plays an important role in many enzymatic reactions in low-water media. The physical characteristics of water in reverse micelles depend on the w_0 value. In order to evaluate the effect of water concentration on GOS synthesis in the AOT/isooctane solution, the reactions were carried out in a range of water content (5–50). The dependence of GOS yield and w_0 was a bell-shaped curve (Fig. 6), indicating that GOS synthesis was unfavorable at either lower or higher water content in the reverse micelles.

In reverse micelles system, water added to form reverse micelles includes bound water and free water [14]. The bound water does not participate in enzymatic reaction because of the hydrophilic interactions with the polar headgroups of AOT. However, the free water can significantly influence the enzymatic behavior. A little amount of free water bound to the β-galactosidase is necessary to maintain the catalytic activity [11,15,16]. At w_0 value below 5, the enzyme could not function well. The product GOS, therefore, was difficult to be synthesized. The GOS yield increased with increasing of the water content until it reached to 15, so this w_0 value might be the basic need of the enzyme for the free water in order to reach maximal activity. A maximum of 51.2% (w/w) of GOS was obtained, including 174 mg/ml of trisaccharide and 56.4 mg/ml of tetrasaccharide. The free water whose properties and structure became closer to those of bulk water as w_0 increased further. The increased water did not affect the enzyme activity, but acted as a reactant participating in hydrolysis, resulting in decrease in GOS formation and increase in lactose hydrolysis (Fig. 6). GOS yield obtained in the reverse micelles, especially, verged on that in the aqueous media when the w_0 up to 50. In addition, the newly formed GOS hydrolyzed faster in the reverse micelles with higher w_0 was another reason that was responsible for decrease in GOS yield.

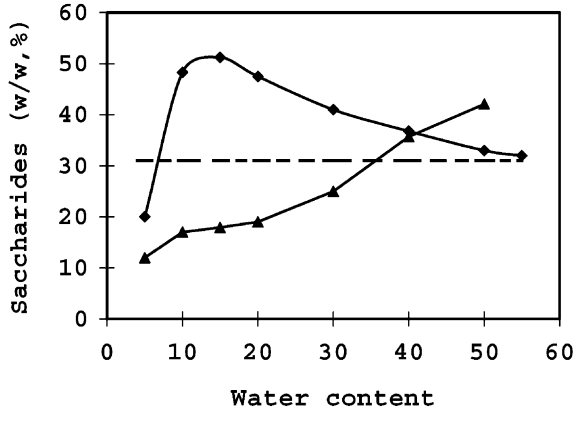

Fig. 6. Effect of water content on GOS synthesis in AOT/isooctane solution. The reaction mixture containing 45% (w/v) lactose and the β-galactosidase in a total volume of 20 ml was incubated at 45°C and pH 7.0. (◆), Yield of GOS in reverse micelles; (▲), Yield of monosaccharides in reverse micelles; (---), Yield of GOS in aqueous system.

3.5. Effect of solvents on GOS synthesis

GOS synthesis was carried out in the reverse micelles under $w_0 = 30$, 40% lactose and 10% (v/v) solvents. Table 2 shows the effect of the solvents on the GOS synthesis and lactose hydrolysis. The highest yield of GOS was attained in the reverse micelles containing propyl acetate and the lowest in the media with ethyl acetate. The effect of solvents on GOS synthesis may be elucidated on the basis of their log *P* values. It is indicated that solvents with higher log *P* values cause less inactivation of enzyme as compared to solvents with lower value [18]. The ethyl acetate is more polar than the other solvents, suggesting that it caused more unfavorable condition for enzymatic reaction including hydrolysis and transfer action. The β-galactosidase exhibited the higher hydrolytic

Table 2
Effect of solvents on GOS synthesis by β-galactosidase in reverse micelles

Solvent	Log *P* value[a]	GOS yield (%)	Lactose conversion (%)
Ethyl acetate	0.68	35	65
Propyl acetate	1.19	45	70
Chloroform	2.0	40	69
Toluene	2.6	42	72
o-Xylene	3.1	41	74
Hexane	3.5	43	76
Isooctane	4.5	41	72
Without solvent	–	40	72

[a] Data from Valerie Laroute and Mare Willemot [17].

activity in xylene and hexane, however, the transgalactosylation reaction was improved in the media containing propyl acetate due to a little of the solvent dissolved into the water pool in reverse micelles. This could have modified the reaction environment in favor of GOS formation. Hence, propyl acetate can be a co-solvent for improvement of GOS synthesis in the reverse micelles with high w_0 value.

In conclusion, GOS synthesis with β-galactosidase was enhanced by carrying out the reaction in the reverse micelles. This approach has several advantages over the aqueous media because it can inhibit the hydrolysis and increase the substrate concentration. So, the reverse micelles can hopefully be a novel reaction media for GOS and other oligosaccharides synthesis.

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