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Contributed Paper

α-Galactosidase from Ling-Zhi Mushroom (*Ganoderma lucidum*)

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ABSTRACT

Ling-Zhi mushroom (*Ganoderma lucidum*), a famous medicinal mushroom, was found to produce α-galactosidase. Fruiting bodies of *G. lucidum* strain L_6 were extracted with phosphate-buffered saline in the ratio of 1g :5 ml then the crude extract was assayed for α-galactosidase activity by hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside. During development of the fruiting body, the highest activity of the α-galactosidase was obtained in the second week in an amount of 402 mU/ml with specific activity of 212 mU/mg protein. Then the enzyme activity gradually decreased to half of this amount by the seventh week. The enzyme showed optimum activity at pH 6.0 and was stable between pH 4 and 8. The optimum temperature of the enzyme was 70°C and half of the enzyme activity remained after 1 hour at 78°C. The α-galactosidase activity was strongly inhibited by Ag^{+}, Hg^{++}, *p*-chloromercuribenzoate and galactose. When *p*-nitrophenyl-α-D-galactopyranoside was incubated with the enzyme, two oligosaccharides were detected by HPLC. This indicated the enzyme catalyzed a transgalactosylation reaction.

Keywords: α-galactosidase, Ling-Zhi mushroom, *Ganoderma lucidum*, transgalactosylation

1. INTRODUCTION

α-Galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) catalyze the hydrolysis of terminal α-1,6-linked galactosidic residues present in linear and branched oligosaccharides, polysaccharides and synthetic substrates such as *p*-nitrophenyl-α-D-galactopyranoside. The occurrence of this enzyme in nature is diverse, i.e. in plants, animals and microorganisms [1].

α-Galactosidases are generally involved in metabolic utilization of oligosaccharides such as melibiose, raffinose, stachyose and galactomannan [2,3], and have been implicated in the metabolism of galactolipids. In addition to the hydrolytic activity, α-galactosidases also catalyze transglycosylation reactions with broad acceptor specificity [4-6].

α-Galactosidases in microorganisms, especially, have been investigated from fungi and bacteria, having diverse forms and different properties [7-10]. Despite the wide distribution and diversity, the α-galactosidase seems to be less studied in mushroom compared with other fungi. However, the biochemical and/or physiological aspects in mushrooms differ from those in other fungi or bacteria, and hence other types of α-galactosidase may be present in the mushroom (*G. lucidum*). Characterization of α-galactosidase in the Ling-Zhi mushroom, *G. lucidum*, would be of benefit for understanding the physiolo-

gical role of the enzyme in mushrooms. In this paper, we present some of the biochemical characteristics of the crude enzyme, α-galactosidase from Ling-Zhi mushroom, and the ability of the enzyme on transgalactosylation activity in order to provide a new biocatalyst for the synthesis of new glycosides.

2. Materials and Methods

2.1 Source of the Enzyme

The Ling-Zhi mushroom, *Ganoderma lucidum* strain L_6, was cultivated at Department of Chemistry, Faculty of Science, Chiang Mai University. The fruiting bodies of the mushroom were used as a source of the α-galactosidase for this study.

2.2 Preparation of the Enzyme Crude Extract

The mushroom fruiting bodies were cut into small pieces, mixed with 10 mM phosphate buffer saline (PBS), pH 7.1, in the ratio of 1 g/5 ml, directly homogenized for 1 min then filtered through 4 layers of cotton cloth, and the filtrate was centrifuged at 7500x *g* for 15 min at 4°C. The supernatant obtained was used as starting crude enzyme preparation for measurement of glycosidase activities and determination of some properties.

2.3 Determination of the Enzyme Activity

The standard assay of the α-galactosidase activity was performed in a reaction mixture consisting of 0.2 ml of 4.0 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPGal), 0.2 ml of 0.2 M sodium phosphate buffer, pH 6.0, and 0.2 ml of the enzyme solution. After incubation for 10 min at 40°C, the reaction was stopped by addition of 2.7 ml of 0.2 M sodium borate buffer, pH 10.0. The *p*-nitrophenol released was measured by spectrophotometer at the absorbance of 400 nm.

One unit of the α-galactosidase was defined as the amount of the enzyme which released 1.0 mmole of *p*-nitrophenol from *p*-nitrophenyl-α-D-galactopyranoside per minute under the assayed condition. The specific activity was expressed in units per mg protein.

2.4 Determination of Protein Concentration

The total protein concentration was determined by the method of Bradford [11] using bovine serum albumin as a standard.

2.5 Effects of pH on the Enzyme Activity and Stability

The optimum pH for α-galactosidase activity was determined by incubating the enzyme preparation with *p*NPGal in 0.2 M KCl-HCl buffers (Clark and Lubs solutions) in the pH range 1.0-2.5 or 0.2 M McIlvaine buffers in the pH range 2.5-8.0 at 40°C for 10 min.

The pH stability of α-galactosidase was assessed by preincubation of the enzyme in McIlvaine buffers in the pH range 2.5-8.0 at 40°C for 1 hour and the residual enzymatic activity was measured under the standard assay.

2.6 Effects of Temperature on the Enzyme Activity and Stability

The optimum temperature for α-galactosidase activity was determined at pH 6.0 by performing the standard assay at temperatures ranging from 30-100°C. Thermal stability was measured by preincubation of the enzyme at different temperatures (30-100°C) for 1 hour at pH 6.0 and the residual enzymatic activity was determined by the standard assay.

2.7 Transgalactosylation Activity of the Enzyme

A 2.5 mM solution of *p*-nitrophenyl α-D-galactopyranoside (*p*NPGal) in 20 mM sodium phosphate buffer, pH 6.0 was incubated with 6.2 mU of the crude α-galactosidase at 25°C and the aliquots were taken at time intervals. The reaction was stopped by 5 min heating in boiling water bath. After cooling, the reaction products were analyzed by analytical HPLC on Nova Pak C18, 4mm (3.9x150 mm) column connected with Waters Tunable Absorbance Detector (Millipore

Corporation, USA) operating at wavelength 300 nm. The gradient elution profile with the system of 6% to 30% acetonitrile:water (v/v) over 30 min at a flow rate of 0.9 ml/min was used for product analysis. *p*-Nitrophenol and *p*NPGal were used as standards.

3. Results and Discussion

3.1 Glycosidase Activities from Mushroom Fruiting Bodies of Various Growth Stage

Figure 1 shows the variation in the enzyme activities of β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase, α-galactosidase, β-glucosidase and α-mannosidase from the crude extract of fruiting bodies at different stages of development. The α-galactosidase showed the highest activity in the second week and then gradually decreased to 0.5 fold by the seventh week of fruiting body development. In the crude extract of *G. lucidum*, the α-galactosidase activity was higher than other glycosidase activities, except for β-N-acetylhexosaminidase activity. It is common that α-galactosidase activity was found in mushroom fruiting body, and Ling-Zhi mushroom generally contained the α-galactosidase activity higher than the other glycosidases. On the other hand, the mushrooms in Genus *Pleurotus* spp. produced the greater amount of β-glucosidase activity during the fruiting body development [12,13].

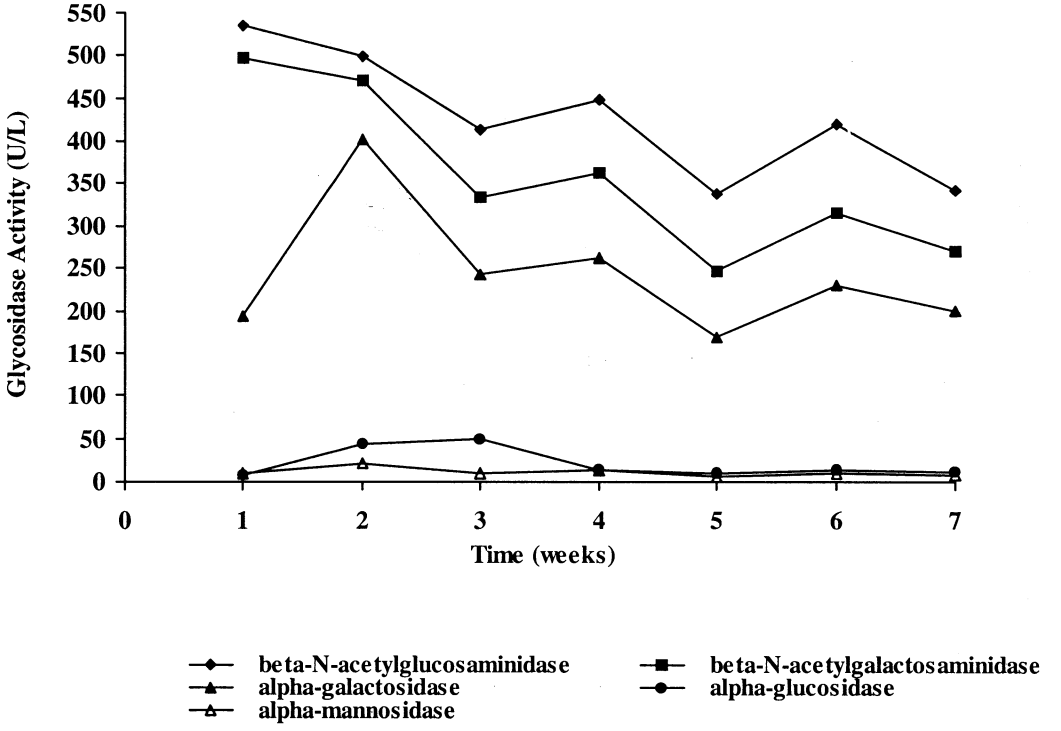

Figure 1. Glycosidase activities at various stages of fruiting body development.

3.2 Effects of pH and Temperature on the Enzyme Activity

The variation in activity of the α-galactosidase isolated from *G. lucidum* with pH is shown in Figure 2. The activity exhibited an optimum at pH 6.0. The enzyme maintained 80% of its maximum activity at pH 3.0, but rapidly decreased to 56% at pH 7.0. The pH optimal value is different from α-galactosidases isolated from other fungi such as the α-galactosidases from *Aspergillus niger* [7], *Calvatia cyathiformis* [14] and *Mortierella vinacea* [15] which have maximum activities at the extremely acidic pH range (2.7-4.0).

The effect of temperature on the rate of *p*NPGal hydrolysis catalyzed by the *G. lucidum* α-galactosidase is shown in Figure 3. The maximum activity was found at 70°C. Above 70°C, the enzyme activity declined very rapidly and was almost nil at and above 90°C. From this result, the *G. lucidum* α-galactosidase appears to be a thermostable enzyme.

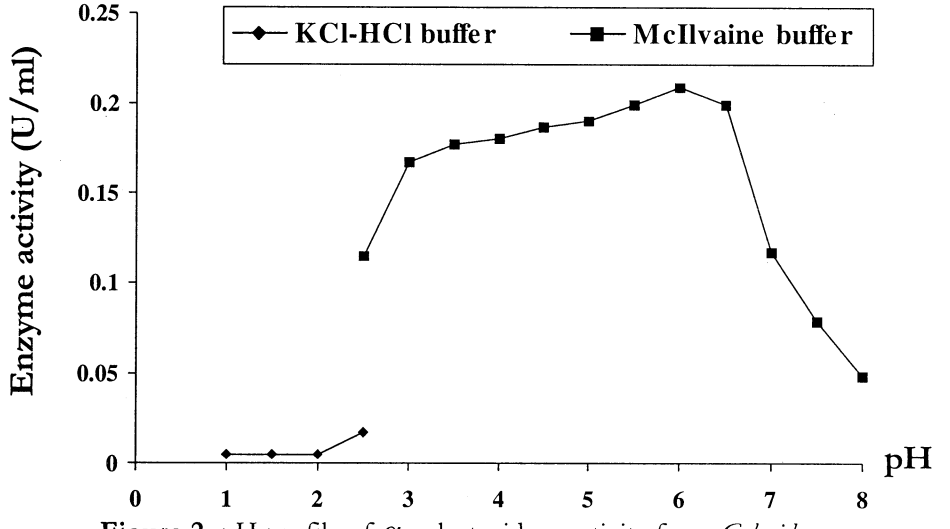

Figure 2. pH profile of α-galactosidase activity from *G.lucidum*.

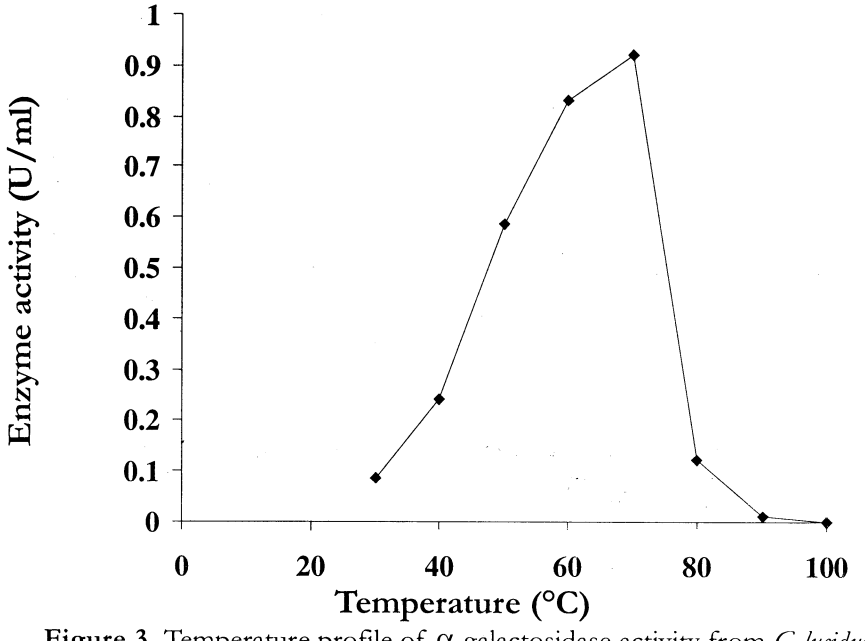

Figure 3. Temperature profile of α-galactosidase activity from *G.lucidum*.

3.3 Effects of pH and Temperature on Stability of the Enzyme

The stability of the α-galactosidase at various pH values was investigated by incubating the enzyme in buffer ranging from pH 2.5 to 8.0 for 1 hour at 40°C prior to assay at its optimal pH. An untreated enzyme sample (incubated in 0.2 M sodium phosphate buffer, pH 6.0) was used as control and assumed to have 100% activity. As shown in Figure 4, the enzyme was most stable at pH 6.5 and more than 75% of its activity between pH 3.5-8.0.

The effect of temperature on stability of the α-galactosidase was examined at pH 6.0 for 1 hour (Figure 5). The enzyme was thermostable up to 70°C, guickly inactivated at 80°C and completely lost its activity at 90°C. It is noteworthy that, the observed temperature stability and optimum temperature are higher by at least 10°C than the values reported for α-galactosidases from other sources [16-19].

On the basis of the properties of the α-galactosidase from *G. lucidum*, which can work well at nearly neutral pH and high temperature, the enzyme appears to have a potential for commercial and industrial applications.

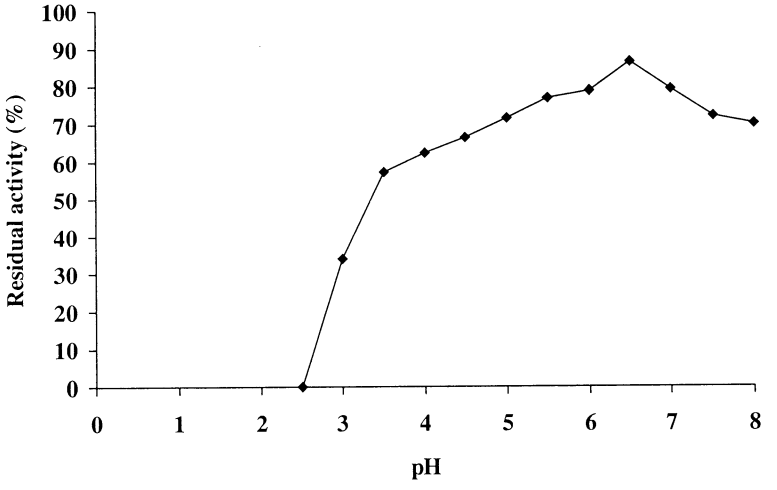

Figure 4. pH stability of α-galactosidase from *G. lucidum*.

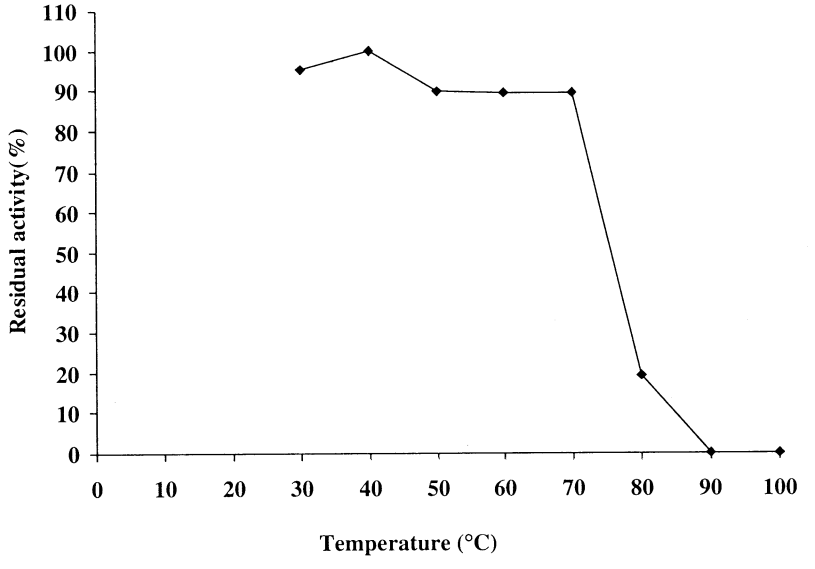

Figure 5. Temperature stability of α-galactosidase from *G. lucidum*.

3.4 Inhibition by Metal Ions and Some Group-Specific Reagents

As Table 1 shows, the enzyme was strongly inhibited by metal ions such as Ag^{+}, Hg^{2+} and Fe^{3+}. The enzyme activity was about 50% inhibited by 1 mM *p*-chloromercuribenzoate (PCMB) and more than 80% inhibited by 10 mM PCMB. Inhibition by the thiol specific inhibitor PCMB is comparatively strong indicated the presence of essential thiol group in the enzyme molecule. Similar result with the thiol-specific inhibitor is for that of the α-galactosidase from *Pycnoporus cinnabarinus* [20]. Ethylenediaminetetraacetate (EDTA) did not affect the enzyme activity, indicating that no divalent cation was required for the enzyme activation. Neither 2-mercaptoethanol nor dithiothreitol affected the enzyme activity indicating no disulfide bond involved in the enzyme catalysis.

Table 1. Effect of metal ions on the α-galactosidase activity from *G. lucidum*.

Compound	Final concentration (mM)	Relative activity (%)
None	-	100.0
$Pb(OAc)_3$	10	108.9
NaOAc	10	97.1
$BaCl_2$	10	97.9
$MnCl_2$	10	97.2
$CaCl_2$	10	96.2
$CoCl_2$	10	96.2
$MgCl_2$	10	96.0
$ZnCl_2$	10	95.7
$CdCl_2$	10	95.6
$CuCl_2$	10	92.3
$AlCl_3$	10	89.4
$FeCl_3$	10	16.7
$HgCl_2$	10	0
$HgCl_2$	1	12.8
$AgNO_3$	10	0
$AgNO_3$	1	13.3
Iodoacetic acid	10	117.6
2-mercaptoethanol	10	98.4
Dithiothreitol	10	91.9
EDTA	10	90.3
PCMB	10	18.2
PCMB	1	51.6

EDTA: Ethylenediaminetetraacetic Acid; PCMB: *p*-Chloromercuribenzoate.

3.5 Inhibition by Saccharides

The effect of saccharides on the *G. lucidum* α-galactosidase activity is summarized in Table 2. Among the saccharides tested, galactose was the most powerful inhibitor. Galactosamine, melibiose, stachyose and raffinose showed weak inhibition. In the presence of 10 Mm D-galactose, about 66% inhibition was found. Galactose inhibition has also been reported for α-galactosidases from *Trichoderma reesii* [17], *Aspergillus awamori* [21] and *A. tamarii* [22,23].

Table 2. Effect of saccharides on the α-galactosidase activity from *G. lucidum*.

Saccharides	Final concentration (mM)	Relative activity (%)
Control	-	100.0
Maltose	10	114.5
Sucrose	10	108.5
Trehalose	10	106.0
Mannose	10	104.3
L-Fucose	10	103.0
Glucose	10	100.4
D-Fucose	10	100.0
Glucosamine	10	91.3
Raffinose	10	91.3
Stachyose	10	88.2
Melibiose	10	87.2
Galactosamine	10	70.7
Galactose	10	34.2
Galactose	1	77.6

3.6 Transgalactosylation Activity of the Enzyme

The transferase activity of the *G. lucidum* α-galactosidase using *p*NPGal as substrate was determined. The conversion of *p*NPGal into *p*-nitrophenol and *p*-nitrophenyl galactooligosaccharides (OS) with time, as observed in an experiment starting from a 2.5 mM *p*NPGal concentration is given in Figures 6 and 7.

Following the transfer reaction over the time of 96 hours, the result showed that there were two types of oligosaccharide (OS-1 and OS-2) detected by HPLC after 24 –hour time (Figures 6 and 7). The ratio of OS-1: OS-2 was 6:1 and a total yield was 45.2% when 50% of *p*NPGal was utilized (Figure 7). These results suggested that the enzyme possibly catalyzed a galactotransfer reaction and new oligosaccharides were also produced in addition to the hydrolysis products. It is known that α-galactosides are interesting substrates for bifidobacteria which is claimed to be beneficial for human health [24]. The soybean oligosaccharides are reported to be bifidogenic substrates but another bifidogenic α-galactosides could be produced by enzymatic synthesis using α-galactosidase [25-27]. Therefore, the α-galactosidase from Ling-Zhi mushroom (*G. lucidum*) could be interesting for production of a potentially bifidogenic oligosaccharides.

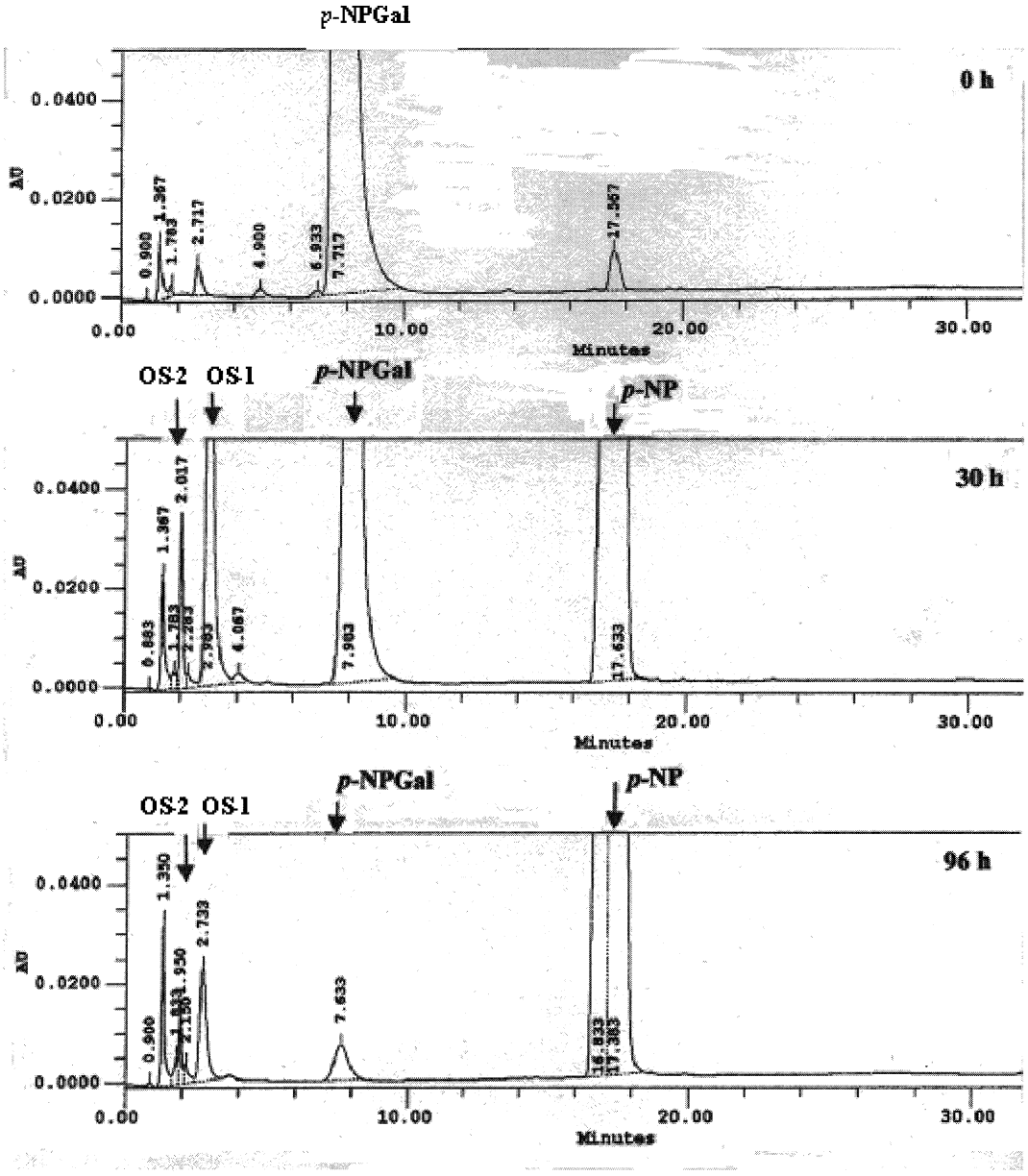

Figure 6. Transgalactosylation reactions catalyzed by the crude αgalactosidase with *p*NP-α-Dgalactopyranoside as substrate. Reactions were carried out at 2.5 mM substrate concentration in 20 mM sodium phosphate buffer (pH 6.0) at 25°C, and the products were analyzed by HPLC using spectrophotometric detector at 300 nm.

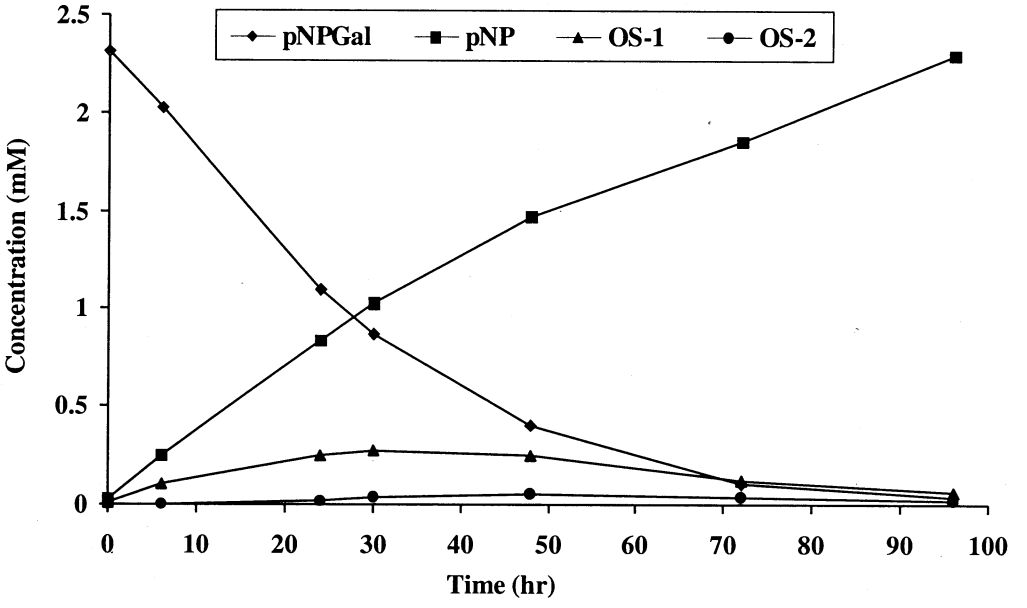

Figure 7. Time course of *p*NPGal conversion and oligosaccharide synthesis.

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