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Immobilization of β-fructofuranosidase from *Aureobasidium* sp. ATCC 20524 on porous silica

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SUMMARY

β-Fructofuranosidase P-1 from *Aureobasidium* sp. ATCC 20524, which produces a fructo-oligosaccharide (1-kestose) from sucrose, was immobilized covalently onto alkylamine porous silica with glutaraldehyde at high efficiency (44.4%). Optimum pore diameter of porous silica for immobilization of the enzyme was 91.7 nm. The enzymatic profiles of immobilized enzyme were almost identical to the native one except its stabilities to temperature and metal ions were improved. 1-Kestose was produced continuously and selectively from 40% (w/v) sucrose at fast flow rates by a column packed with the immobilized enzyme for up to 26 days, and the effluent concentration of 1-kestose remained in the range 113–135 mg ml^{-1}.

INTRODUCTION

In previous papers, we reported about the production and purification of β-fructofuranosidase (EC 3.2.1.26) from *Aureobasidium* sp. [5,6] which produces a fructo-oligosaccharide (1-kestose) from sucrose [2]. We have investigated the immobilization of β-fructofuranosidase to construct a bioreactor system for industrial production of 1-kestose. Inorganic supports are easier to use in continuous systems because of their physical and chemical stabilities [11] and immobilization by covalent bonds is favorable for long-term reactions. We described the efficiency of immobilization of β-fructofuranosidase onto *shirasu* porous glass and the effect of flow rate on production of 1-kestose from sucrose previously [3,4]. In the present paper, we describe the immobilization of β-fructofuranosidase from *Aureobasidium* sp. ATCC 20524 onto porous silica, the superior properties of the preparation and the long-term continuous selective production of 1-kestose by this column system. Porous silica is an inexpensive inorganic support and has been used for the immobilization of glucoamylase [8,9] and dextransucrase [7]. It is considered to be useful for industrial scale operations. While previous work described the utilization of porous silica as a support of β-galactosidase for the production of galacto-oligosaccharides [10], there is no published report concerning the immobilization of β-fructofuranosidase onto porous silica for the production of fructo-oligosaccharide from concentrated sucrose.

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MATERIALS AND METHODS

Cultivation and preparation of enzyme. Aureobasidium sp. ATCC 20524 was cultivated for β-fructofuranosidase production in liquid culture (sucrose 20%, yeast extract 2%, $NaNO_3$ 1%, K_2HPO_4 0.75%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 6.5–7) with the same conditions as described previously [5]. β-Fructofuranosidase P-1 was solubilized by Kitalase and partially purified by fractionations involving ethanol, calcium acetate and ammonium sulfate and DEAE-Cellulofine and Sephadex G-200 chromatography (once) before the immobilization as described in a previous paper [6].

Preparation of immobilized enzyme. Preparation of supports and immobilization of enzyme were carried out by the procedure proposed by Weetall [11] with slight modifications. Porous silica (CARiACT; Fuji-Davison Chemical Ltd., Kasugai, Japan) was used as support for immobilization of the enzyme.

Silanization of porous silica was carried out as follows. Fifteen ml of γ-aminopropyltriethoxysilane and 35 ml of toluene were added to 1 g of porous silica and incubated at 110 °C for 12 h. It was washed with toluene and then

dried. The number of amino groups on the surface of porous silica was measured by an amino-picric acid titration method [1].

Immobilization of enzyme was carried out as follows. The alkylamine porous silica (0.3–1 g) was activated by the addition of 2.5% (v/v) glutaraldehyde (3–10 ml) with stirring for 1 h, and the excess glutaraldehyde was washed off with water. Enzyme solution (ca. 10–500 U) was added to 0.1–1 g of activated support and stirred at room temperature for 2 h. The excess enzyme was then washed off with water. The preparation was used for further experiments.

Enzyme activity assay. The immobilized enzyme (ca. 25 mg) was employed in the reaction mixture. The enzyme was assayed using 30% (w/v) sucrose as substrate in a total volume of 1 ml of 75 mM McIlvain buffer, pH 5.5. The reaction was carried out at 50 °C for 20 min and stopped by boiling for 10 min.

Released glucose in the reaction mixture was measured by the glucose oxidase method (Glucose test B; Wako pure chemical industries Ltd., Osaka, Japan). 1-Kestose and other products were measured by HPLC with μ-Bondapack CH (3.9 × 30 mm, Waters) under conditions as described previously [6]. One unit of enzyme was defined as the quantity of enzyme responsible for the transfer of 1 μmol of fructose in 1 min.

Continuous reaction of immobilized enzyme. The immobilized enzyme (0.96 g support, ca. 260 U) was packed in a glass column (6 mm i.d.) with a bed volume of ca. 0.85 cm^3. The enzyme column was continuously operated at 30 °C using 40% (w/v) sucrose as substrate in 75 mM McIlvain buffer (pH 5.5) at a flow rate of 20 ml h^{-1}.

RESULTS AND DISCUSSION

Silanization of porous silica and immobilization of enzyme

The relationships between the amount (μmol) of amino groups per g of support and the degree of enzyme immobilization is shown in Table 1. There was a tendency to increase the amount of amino groups as the pore diameter of silica decreased. The enzymatic activity increased with increasing silica gel pore size. The maximum activity of immobilized enzyme, 222 U g^{-1} support (44.4% of immobilization), was found with CARiACT-100. It is estimated ca. 0.74 U of enzyme were immobilized on 1 μmol of amino groups of CARiACT-100. CARiACT-100 was selected as the support for further experiments.

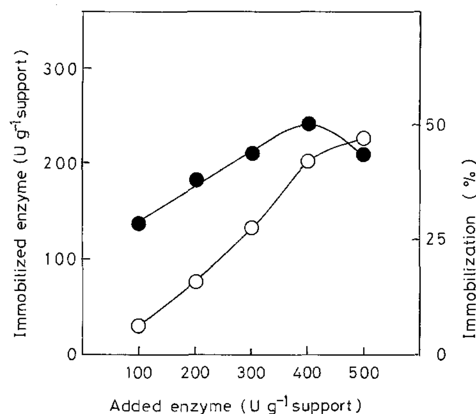

Fig. 1. Effect of the amount of added enzyme on the immobilization onto CARiACT-100. Symbols: immobilized enzyme, —o—; immobilization, —•—.

The effect of enzyme concentration on the efficiency of immobilization is shown in Fig. 1. While there was a tendency to increase the amount of immobilized enzyme as the increase of enzyme concentration, the maximum efficiency of immobilization, 50.3%, was found with 400 U g^{-1} support of added enzyme. The optimum pore size for immobilization of the enzyme was similar to that for glucoamylase (80 nm) [9].

Properties of the immobilized enzyme

The effects of reaction pH on the activity of immobilized and native enzyme are shown in Fig. 2. The opti-

TABLE 1

Loading of amino groups and immobilization of the enzyme on various pore sizes of silica

Support		Loading of amino groups (μmol g^{-1} support)	Immobilization	
No.	Pore diameter (nm)		Activity (U g^{-1} support)	%
CARiACT-15	15.0	711.4	137	27.4
CARiACT-30	30.0	552.4	156	31.2
CARiACT-50	50.0	403.2	181	36.2
CARiACT-100	91.7	301.6	222	44.4

50 U of enzyme were added to 0.1 g of support.

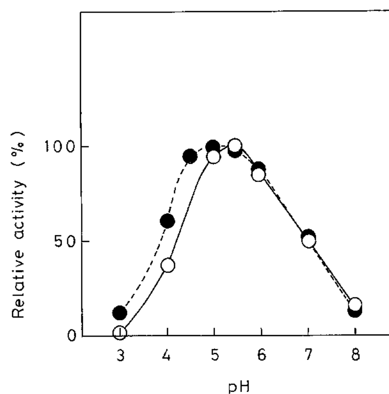

Fig. 2. Effect of pH on the activity of immobilized and native enzymes. Symbols: immobilized enzyme, —o—; native enzyme, ---●---.

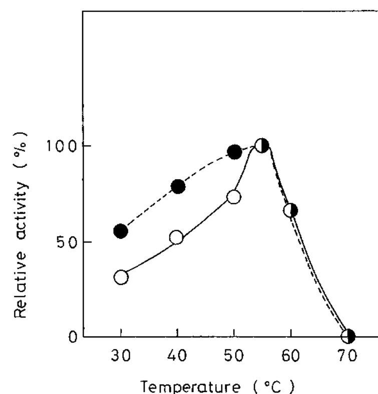

Fig. 4. Effect of temperature on the activities of immobilized and native enzymes. Symbols: immobilized enzyme, —o—; native enzyme, ---●---.

mum reaction pH of immobilized enzyme, 5–5.5, was not changed by immobilization as reported for dextransucrase [7].

The stabilities of enzyme at various pH are shown in Fig. 3. The immobilized enzyme was stable within the wide range of pH 5–10 and retained more than 90% of its maximum activity after 3 h. The stability of native enzyme at pH 3 and 11 was increased by immobilization.

The effect of reaction temperature on the activity of immobilized and native enzyme is shown in Fig. 4. The optimum temperature of immobilized enzyme, 55 °C, was identical to that of the native enzyme.

The stability of immobilized enzyme and native enzyme at various temperatures is shown in Fig. 5. The immobilized enzyme was stable at 55 °C but inactivated at 70 °C after 15 min. At temperatures higher than 50 °C, the immobilized enzyme activity decreased more slowly than the native enzyme. At 55 °C the immobilized enzyme still exhibited more than 95% of its maximum activity.

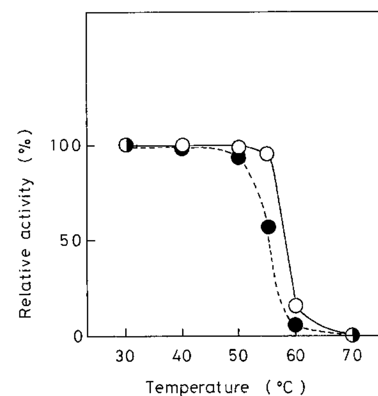

Fig. 5. Effect of temperature on the stabilities of immobilized and native enzymes. Symbols: immobilized enzyme, —o—; native enzyme, ---●---. The activities were measured after 15 min incubation at each temperature.

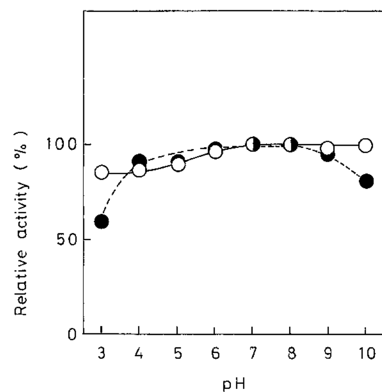

Fig. 3. Effect of pH on the stabilities of immobilized and native enzymes (pH 3–8, McIlvain buffer; pH 8–11, Michaelis buffer). Symbols: immobilized enzyme, —o—; native enzyme, ---●---. The activities were measured after 3 h incubation at each pH.

The K_m values for the immobilized and native enzymes for sucrose by a Lineweaver-Burk plot of the enzyme reaction were 0.59 and 0.43 M, respectively. The affinity to substrate of the enzyme was not significantly changed by immobilization.

The effect of metal ions on the activity of immobilized and native enzymes is shown in Table 2. The inhibition of metal ions, especially Ag^{+}, Hg^{2+}, etc., to immobilized enzyme was less than to the native enzyme. Other metal ions tested did not inhibit either enzyme.

Continuous reaction of immobilized enzyme column

Continuous production of 1-kestose was carried out at a flow rate of 20 ml h^{-1} and 30 °C. The result is shown in Fig. 6. The effluent concentration of 1-kestose remained

TABLE 2

Effect of metal ions on the activities of immobilized and native enzymes

Metal ion (1 mM)	Relative activity (%)	
	Native	Immobilized
$AgNO_3$	26	93
$CuSO_4$	6	26
$ZnSO_4$	44	63
$Pb(OAc)_2$	12	33
$HgCl_2$	3	20
Control	100	100

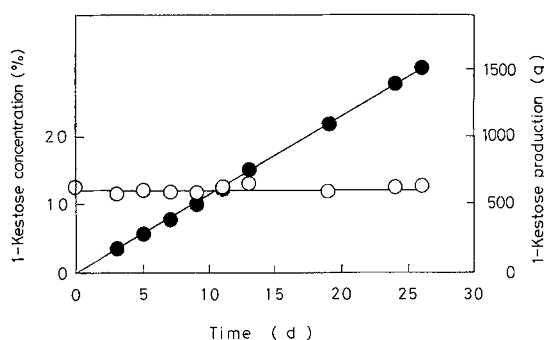

Fig. 6. Continuous reaction by a column packed with the immobilized enzyme. Symbols: 1-kestose concentration, —○—; 1-kestose production, —●—.

in the range 113–115 mg ml^{-1} for up to 26 days during which time a total of 1512 g of 1-kestose was produced. At these operating conditions, nystose was not produced. While the enzymatic reaction mixture comprised various fructo-oligosaccharides such as 1-kestose, nystose, fructosylnystose, etc. in batch reaction, selective production of 1-kestose was obtained in the continuous system because of the fast flow rate of concentrated sucrose due to the physical strength of porous silica.

β-Fructofuranosidase was successfully and covalently immobilized onto porous silica at high efficiency. The enzymatic properties of the immobilized enzyme were superior to the native enzyme. The immobilized enzyme was stable during continuous operation for an extended period of time (26 days) for the production of 1-kestose at high flow rates of concentrated sucrose. Porous silica is suitable as a support matrix for the enzyme in large scale reactors due to its physical strength and relatively low price (ca. $50 per kg). For the above reasons, the system using porous silica is considered to be useful for the construction of industrial scale bioreactors for the selective production of 1-kestose from concentrated sucrose.

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