Immobilization on Chitosan of a Thermophilic Trehalose Synthase from *Thermus thermophilus* HJ6

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A thermostable trehalose synthase (TtTSase) from *Thermus thermophilus* HJ6 was immobilized on chitosan activated with glutaraldehyde. The yield of immobilization was evaluated as 39.68%. The optimum pH of the immobilized enzyme was similar to that of the free enzyme. However, the optimal temperature ranges were shifted by about 4°C owing to better thermal stability after immobilization. The half-life of heat inactivation for free and immobilized enzymes was 5.7 and 6.3 days at 70°C, respectively, thus showing a lager thermostability of the immobilized enzyme. When tested in batch reaction, the immobilized enzyme retained its relative activity of 53% after 30 reuses of reaction within 12 days, and still retained 82% of its initial activity even after 150 days at 4°C. A packed-bed bioreactor with immobilized enzyme showed a maximum yield of 56% trehalose from 100 mM maltose in a continuous recycling system (bed volume: 10 ml) under conditions of pH 7.0 and 70°C.

Keywords: Chitosan, immobilization, trehalose synthase, *Thermus thermophilus*, thermostability, bioreactor

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a nonreducing disaccharide that is widely present in various organisms, including bacteria, yeast, fungi, insects, invertebrates, and plants [7, 21]. The nonreducing disaccharide serves as a carbohydrate reserve and a protecting agent against a variety of environmental stresses such as heat, cold, desiccation, dehydration, and osmotic pressure [1]. These functions have been applied to the development of additives, stabilizers, and sweeteners, which are useful in the food, cosmetic, and pharmaceutical industries [15, 18, 20]. Trehalose synthase (TSase; E.C. 5.4.99.16) converts maltose into trehalose by an intramolecular rearrangement of the α-1,4-linkage of maltose to the α,α,-1,1-linkage of trehalose in the absence of coenzyme [23]. This enzymatic process has the advantages of simple reaction, high substrate specificity, high conversion yield, and low cost. Therefore, it is one of the most efficient processes for the production of trehalose in the point of industrial application.

The immobilization of enzyme has been often regarded preparative for commercial applications owing to low stability [6]. Advantages of immobilized enzymes are that the generally expensive biocatalysts can be used repeatedly in batch or continuous reactors. Enzymes can be immobilized on various supports and by different methods. Recently, immobilization of recombinant TSase for trehalose production has been reported [4]. The recombinant TSase was immobilized with a high yield onto commercially available Eupergit C250L, an epoxy-activated acrylic bead. In our studies, chitosan was selected as the support for enzyme immobilization. Chitosan is a natural polyaminosaccharide obtained by *N*-deacetylation of chitin, and is non-toxic, biocompatible, and biodegradable [2, 19]. Its reactive amino and hydroxyl groups after chemical modifications make possible the coupling of enzymes [10]. Therefore, chitosan has been used as a support material for immobilization of enzymes [3, 22].

In a previous study, we have cloned and analyzed the expression of the gene encoding a hyperthermostable trehalose synthase from *Thermus thermophilus* HJ6 and studied its molecular and enzymatic properties [9]. In this paper, we report the optimization of TSases immobilization on porous chitosans, the properties of the immobilized preparation, and the performance of a bioreactor for trehalose production at high temperature.

MATERIALS AND METHODS

Chemicals, Strains, and Plasmids

Chitosan (C3646, from crab shells), Tween 80, sodium tripolyphosphate, Triton X-100, and glutaraldehyde were obtained from Sigma Co.

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(St. Louis, MO, U.S.A.). All chemicals were of analytical purity and used as purchased. The column (XK16/20) for the packed bed bioreactor was purchased from GE Healthcare. *E. coli* DH5α and BL21 (DE3) codon plus (Novagen, Inc., San Diego, CA, U.S.A.) were used as the cloning and expression host cells, respectively. The plasmid pJLA503 was preserved in our laboratory as the expression vector for the *TtTS* gene.

Chitosan Particle Preparation

Chitosan particles were prepared by a precipitation method [3]. Chitosan was solubilized in 2% acetic acid to a final concentration of 2% (w/v). The solution was filtered through Whatman GF/A filters and added dropwise through a capillary into a gently stirred coagulation liquid [1 N sodium hydroxide and 26% (v/v) ethanol]. The obtained particles were centrifuged and washed with distilled water until neutrality. The mean size of the particles was determined from optical micrographs taken with an Eclipse 50i optical microscope (Nikon, Japan).

Enzyme Immobilization

Trehalose synthase (TtTSase) from *Thermus thermophilus* HJ6 cloned in *E. coli* was produced as previously described [8, 9]. Immobilization on chitosan particles was carried out adding 1 g (wet weight) particles to a mixture of 3.5 ml of 0.02 mol/l potassium phosphate buffer (pH 7.0) and 1.5 ml of TtTSase (10 mg/ml). The mixtures were gently shaken at 100 rpm for 2 h, and after a further 16 h at 25°C in the presence of 2.5% (v/v) glutaraldehyde, the mixture was centrifuged at 12,000 rpm for 2 min. The immobilized enzymes were washed with 0.02 mol/l potassium phosphate buffer (pH 7.0) until protein was no longer detected in the washing solution. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as the standard. The immobilized protein amount was calculated by subtracting the protein recovered in the supernatant from the protein subjected to immobilization. The immobilization yield was calculated as the ratio of immobilized protein to protein subjected to immobilization.

Assay of Enzyme Activity

Enzyme activity was assayed by measuring trehalose produced from 50 mM maltose solution. Maltose solution in 50 mM potassium phosphate buffer (pH 7.0) was incubated with free or immobilized enzyme at 70°C for 30 min in a water bath. The reaction mixture was boiled for 10 min to stop the reaction. The trehalose produced was analyzed by HPLC (Waters Alliance 2690). Chromatography was performed using a carbohydrate analysis column (Waters, 300 mm×3.9 mm) on a Waters Alliance 2690 system and a Waters model 410 detector. Separation of the samples was accomplished with a linear gradient using eluant (acetonitrile:water, 75:25). The column temperature was set at 25°C. A Pierce standard amino acid mixture (hydrolysis standard) was used to calibrate the analyses. The authentic glucose, maltose, and trehalose were used as controls.
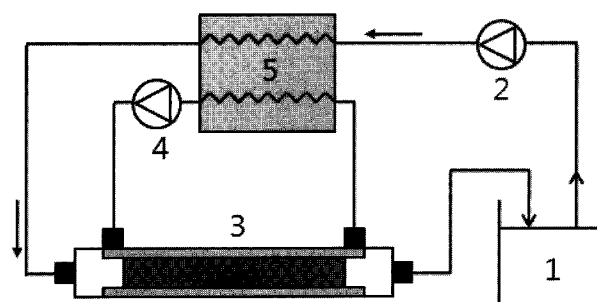
Fig. 1. Scheme of the packed-bed immobilized enzyme reactor. 1, Substrate reservoir; 2, piston pump; 3, thermostat column reactor; 4, water circular; 5, hot waterbath.

One unit of TtTSase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmole of trehalose per minute under the reaction conditions.

Continuous Conversions in a Packed-Bed Reactor

The chitosan particles with TtTSase bound were packed into a XK 16/20 glass column (16×200 mm) (GE Healthcare, Uppsala, Sweden) equipped with a jacket for constant temperature operation. The reaction temperature was maintained at 70°C using a water circular (Jeio Tech CW-05G, Korea). The continuous production was carried out using a packed-bed bioreactor (16×100 mm) containing 8.6 g (dry weight) of immobilized enzyme preparation. The reactor was filled with 10 ml of immobilized enzyme resin and the working volume (Vw) was evaluated as 10 ml. The column was continuously fed from the bottom with 100 mM maltose solution in 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min using a piston pump (Fig. 1). The samples at the outlet of the reactor were taken and used for further analyses. Two duplicates were made for each determination.

RESULTS AND DISCUSSION

Immobilization of TtTSase

Chitosan is a suitable biocompatible and inexpensive matrix for a bioreactor in which the main features of the free enzyme are preserved. Furthermore, it has been used as a support material for immobilization of thermostable enzymes [5, 16]. In view of the thermostable and practicable industrial applications, the thermostable trehalose synthase (TtTSase) was immobilized on chitosan. Chitosan supports were prepared by a precipitation method, and the mean diameter of the prepared particles was 2.0 mm. The amount of

Table 1. Immobilization yield of support and specific activity of immobilized enzyme.

Support	Immobilized protein (mg/mg support)	Immobilization yield (%)	Immobilized enzyme activity[a]	Specific activity of immobilized enzyme[b]
Chitosan	0.16	39.68	0.072	0.45

[a]Unit of activity: μmol maltose/min/mg dry support.
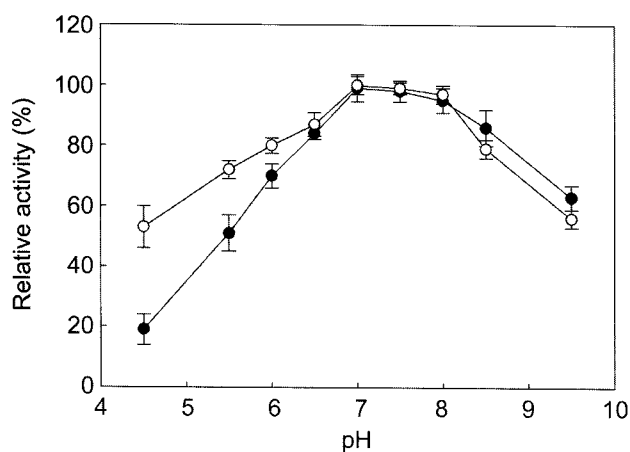[b]Unit of specific activity: μmol maltose/min/mg protein.

immobilized protein on chitosan particle, the immobilization yield, the activity of TtTSase related to the weight of dry supports as well as the specific activity of immobilized enzyme are summarized in Table 1. The immobilization yield reached 39.68% when the conditions were as follows: coupling time of 18 h at 25°C in 20 mM potassium phosphate buffer (pH 7.0) and enzyme loading of 0.01 g protein/g-supports. The immobilization capacity of chitosan was much lower than that of Eupergit C250L (92% of immobilization yield) that has been previously reported [4]. The specific activities of the free and the immobilized TtTSase were 0.42 and 0.45 U/mg protein, respectively, when measured at pH 7.0 and 70°C. As proven by the presented data, the prepared chitosan particles were applicable for immobilization of thermophilic TtTSase.
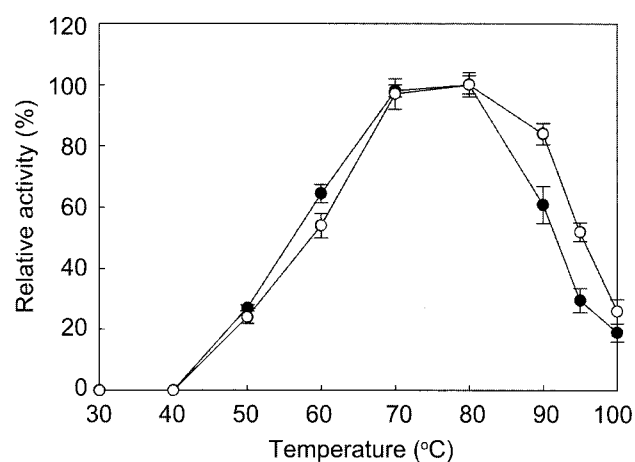
Properties of the Immobilized Enzymes

The effect of pH on the activity of the free and the immobilized TtTSase was investigated within pH 5.0 and 10.0 at 70°C (Fig. 2). The optimum pHs of free and immobilized enzymes were 7.0. The optimal pH ranges of immobilized TtTSase were broader than the corresponding ranges of the free enzymes, and expand the range of useful conditions for enzyme toward the acidic region (Fig. 2). This change was probably due to multi-point ionic interaction between the enzyme and the chitosan matrix, which has a polycationic character, containing cationic NH_3^+ and polar -OH groups. Similar observations for immobilization of enzyme on chitosan have been reported in the literature [16].

The effect of temperature on activity was investigated from 20 to 100°C (Fig. 3). As shown in Fig. 3, the optimum temperature was found to be 70-80°C for both free and immobilized enzymes. However, the whole curve was shifted by about 4°C for the immobilized enzymes, which
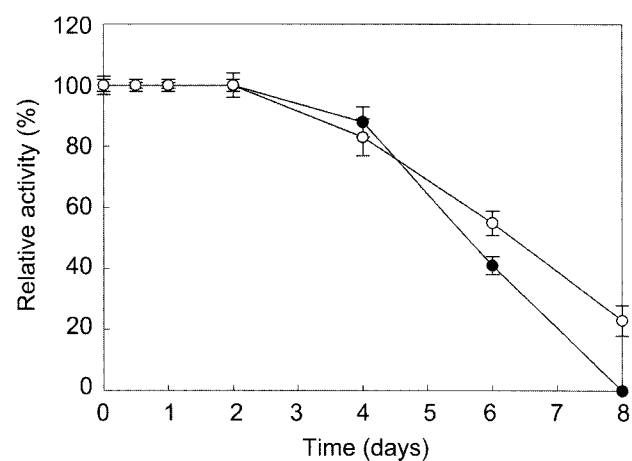

Fig. 3. Effect of temperature on the activities of the free (closed circles) and immobilized (open circles) trehalose synthases.
Error bars denote the variations from three independent experiments.

is often found after immobilization [11]. This shift could be explained as the stability of TtTSase caused by the covalent binding. In general, the activity of the immobilized enzyme, especially in a covalently bound system, is more resistant than that of the soluble form against heat and denaturing agents. The thermal stability of the enzyme was examined at pH 7.0 and 70°C. The free and immobilized enzymes showed high thermostability, and the half-life was 5.7 and 6.3 days, respectively (Fig. 4). Thus, the heat stability of immobilized enzyme was higher than that of free enzyme.

The reusability of the immobilized enzyme was examined because of its importance for repeated applications in a bioreactor. The reactions with immobilized enzyme were carried out at 70°C for 1 h in 1.0 ml of the mixture containing the immobilized enzyme (100 mg support) and

Fig. 2. Effect of pH on the activities of the free (closed circles) and immobilized (open circles) trehalose synthases.
Error bars denote the variations from three independent experiments.

Fig. 4. Thermostability of free (closed circles) and immobilized (open circles) trehalose synthases at 80°C.
Error bars denote the variations from three independent experiments.
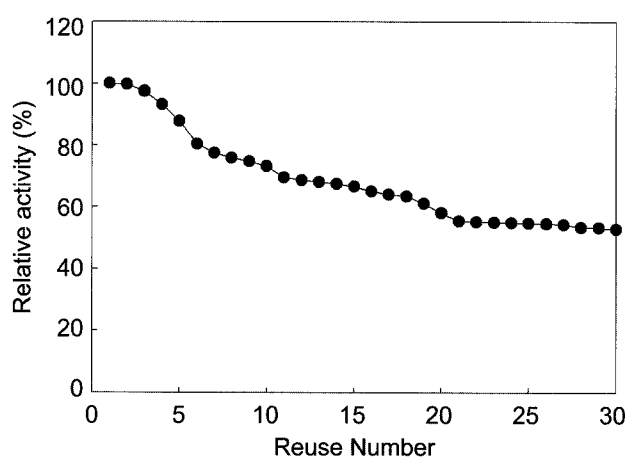
Fig. 5. Effect of reuse number on the activity of immobilized trehalose synthase.

100 mM maltose. After the reaction, the support was separated from the reaction solutions through centrifugation and subsequently washed with distilled water. Immobilized TtTSase by using chitosan was used repeatedly, 30 times within 12 days. After 10 reuses, the immobilized TtTSase retained 73% residual activity. This recycling of the immobilized enzyme was successfully performed 30 times in batch reactions with 47% loss of activity (Fig. 5). Storage stability is an important factor for the commercial application of enzymatic bioconversion. Storage stability of immobilized enzyme was investigated in 50 mM sodium phosphate buffer (pH 7.0) at 4°C for 150 days. Upon 150 days of storage, the residual activity of the immobilized enzyme still retained 82% of the initial activity (data not shown). Thus, the high reusability and storage stability make the immobilized TtTSase very attractive for long-term continuous conversion of maltose solution.

Packed-Bed Enzyme Reactor for Continuous Trehalose Conversion

The TtTSase immobilized onto chitosan was used to prepare a packed-bed bioreactor whose configuration is shown in Fig. 1. A packed-bed enzyme reactor was employed for the continuous conversion of maltose (100 mM) in 50 mM sodium phosphate buffer (pH 7.0) at 70°C, and then the trehalose synthesized was analyzed by HPLC. Table 2 shows the conversion rate obtained in the feeding substrate. The conversion rate was calculated at the steady state as the reaction proceeded. The maximum yield of trehalose from maltose reached 56% when the retention time was 200 min. Although the immobilization yield of the present system was lower than that of the previously reported system [4], the trehalose production was enhanced more than that (42%) of the former. As the retention time increased, the conversion yield into glucose increased and

Table 2. The composition of continuous trehalose production by packed-bed bioreactor[a].

Retention time (min)	Product composition[b]		
	Trehalose (%)	Glucose (%)	Maltose (%)
200	56	14	30
150	51	11	38
100	39	7	54
75	24	5	71
50	20	4	76
25	19	3	78
10	16	3	81

[a]Packed-bed bioreactor: working volumn (immobilized enzyme pellets; 10 ml).
[b]Product compositions were measured in the steady state of the reaction.

reached 14% at a retention time of 200 min. Trehalose synthases from *Pimelobacter* sp. R48 [13] and *T. aquaticus* [14] catalyzed the conversion of maltose into trehalose and trehalose into maltose and released a small amount of glucose from both of these substrates. In the batch reactions of the free and immobilized enzymes, under identical conditions, we found that both product compositions were almost the same at 120 min as having maximized trehalose production yield. The product was composed of trehalose (48%), glucose (20%), maltose (32%) (data not shown). Thus, the conversion yield of glucose in the continuous packed-bed reactor was lower than that in the batch reaction, and the trehalose production was more enhanced in the continuous reaction using a packed-bed bioreactor than in the batch reaction. This result might be explained in terms of product inhibition due to by-products formed during the batch reaction [12].

In an attempt to develop a thermostable and inexpensive matrix, a trehalose synthase (TtTSase) from *Thermus thermophilus* HJ6 was immobilized on chitosan. The immobilized TtTSase on chitosan support was more enhanced in the thermostability and storage stability than free enzyme. Using the continuous packed-bed reactor, a maximum yield of 56% trehalose was reached, with a good operational stability at 70°C. The high temperature processing has several valuable advantages in industrial applications: (i) longer half-life of enzyme activity, (ii) increased substrate solubility and higher product concentrations, (iii) reduced risk of microbial growth, and (iv) reduced product inhibition [17]. Therefore, the immobilized TtTSase system on chitosan may be an industrially feasible trehalose production method by a biological process at high temperature.

Acknowledgment

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