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Production of dTDP-4-keto-6-deoxy-D-glucose by Immobilization of dTDP-D-glucose 4,6-dehydratase

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Abstract The dTDP-D-glucose 4,6-dehydratase from *Salmonella enterica* was immobilized using covalent binding to cyanogen bromide activated sepharose. The immobilized enzyme was used to produce dTDP-4-keto-6-deoxy-D-glucose, a key sugar intermediate that can be used economically to produce diverse classes of unusual sugars appended in various antibiotics. The enzyme was immobilized on the sepharose after activation with cyanogen bromide. The maximum immobilization (80.03%) was achieved after 14 h of coupling. The covalently immobilized enzyme was stable, and an average of 78.4 % conversion was achieved until 120 h of immobilization when it was repeatedly used. Similar conversion was noticed for the first batch using the enzyme entrapped-hydrogel but activity was gradually decreased in the following batches. The production of dTDP-4-keto-6-deoxy-D-glucose by using an immobilized enzyme has high potential for commercial application.

Key words: Immobilization, dTDP-D-glucose 4,6-dehydtarase, chitosan-xanthan hydrogel, cyanogen bromide

Several categories of natural products contain deoxysugar moieties that are essential for their biological activities. Within these natural product categories are the glycosylated macrolactones of erythromycin and tylosin families, vancomycin family of glycopeptides antibiotics as well as aminoglycoside antibiotics [12]. dTDP-4-keto-6-deoxy-D-glucose has enormous synthetic potential that is reflected by the number of biosynthetic branches proceeding through it to give the final unusual deoxysugars such as dTDP-β-L-rhamnose, epivancosamine, vancosamine, rubranitrose, daunosamine etc. [1, 5, 23]. The dTDP-D-glucose 4,6-dehydratase catalyzes a fascinating oxidation-reduction reaction converting dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose in the presence of NAD^+ [11, 15] (Fig. 1). Although this enzyme is characterized from various organisms, no effort has been carried out toward its application for the industrial production of dTDP-4-keto-6-deoxy-D-glucose. The industrial application of this enzyme would benefit from the availability of its immobilized form, in particular in the prospect of repeated use and recovery of active enzymes. The dTDP-glucose 4,6-dehydratase (RfbB) from *Salmonella enterica* has been proven as an effective means for the formation of this compound. The active sites of RfbB have been previously characterized in the laboratory by site-directed mutagenesis experiment [18–20]. The protein is a homodimer of 43 kDa subunit and is highly specific for dTDP-D-glucose.

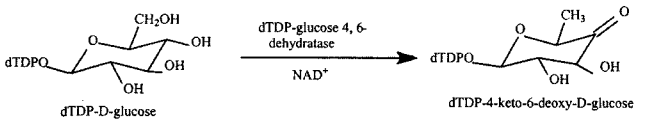

Fig. 1. Dehydration reaction triggered by dTDP-D- glucose 4,6-dehydratase.

Although there have been several reports on immobilization of enzymes, but the most of them rely on either covalant linkage between the enzyme and the support or its entrapment by the application of natural or synthetic polymers [6, 10, 13, 22, 24]. Cyanogen bromide activated sepharose has previously been extensively used for the immobilization of antibody [2]. The activation results in the formation of reactive cyclic imido-carbamate functionality on the sepharose, which reacts with the enzyme to form a covalent bond during the coupling process (Fig. 2). Kim and coworkers also have reported the high production of D-tagatose from immobilized L-arabinose isomerase on cyanogen bromide activated agarose [13]. Similarly, it has been reported that hydrogel formed by using chitosan and xanthan has fibrous structure with good

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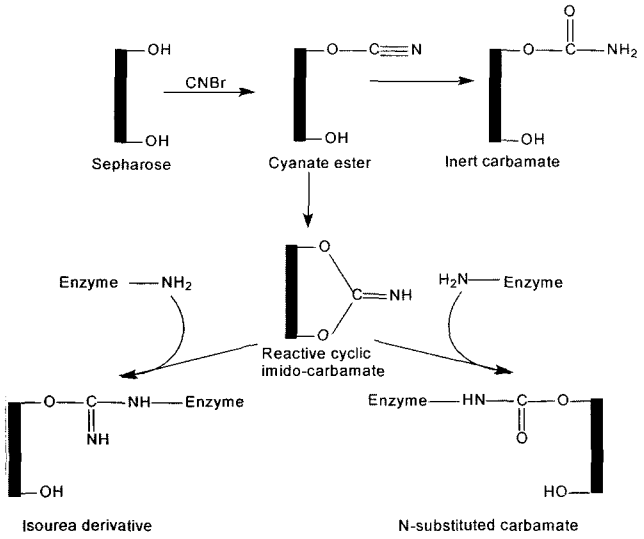

Fig. 2. Schematic diagram for the activation of sepharose by cyanogen bromide and immobilization of enzyme by covalent linkage [16].

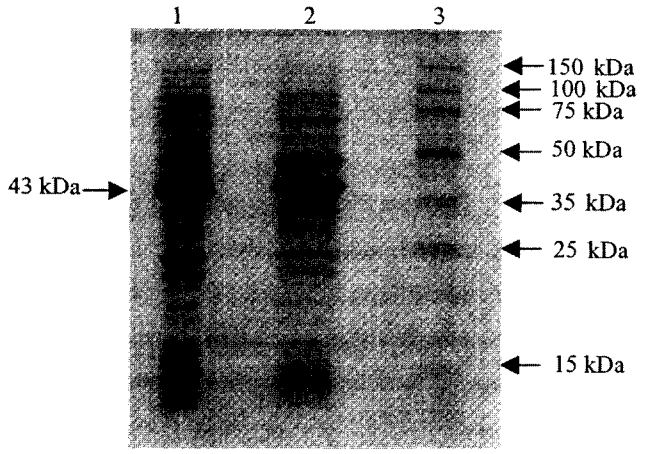

Fig. 3. SDS-PAGE analysis of overexpressed dTDP-glucose 4,6-dehydratase.
Lanes 1, 2, and 3 contained crude soluble protein, ammonium sulfate precipitated fraction after dialysis, and protein marker (Novagen), respectively.

hygroscopic qualities, and is capable of immobilizing bioactive substances such as drugs and enzymes [8, 9]. The repeated production of dTDP-4-keto-6-deoxy-D-glucose using immobilized enzyme by the former method is herein described, and its efficiency with the latter one is compared.

Materials and Methods

Chemicals

Sepharose (Q) was purchased from Pharmacia (Uppsala, Sweden). Cyanogen bromide, chitosan and xanthan were obtained from Aldrich Company (Milwaukee, WI, U.S.A.). dTDP-D-glucose and nicotinamide adenine dinucleotide (NAD^+) were purchased from Sigma (St. Louis, MO, U.S.A.). The dTDP 4-keto-6-deoxy-D-glucose used in the assay was provided by GenChem (Daejon, Korea). All the chemicals used were reagent grade. The *rfbB* gene (1.5 kb) harboring recombinant plasmid (pPR1162) was obtained from Prof. Peter R. Reeves, University of Sydney. The *E. coli* XL1-Blue MRF was purchased from Stratagene (La Jolla, U.S.A.). The Xpree™ protein expression system was purchased from Invitrogen Corporation (Carlsbad, CA, U.S.A.).

Enzyme Preparation

E. coli BL21(DE3), a lysogen of bacteriophage DE3 that carries the T7 RNA polymerase gene under the control of the inducible *lac UV5* promoter, was transformed with pPR1162 as described by Sambrook *et al.* [16]. Transformed cells were cultured at 37°C until A_{600} of 0.5-0.7 and IPTG was added to a final concentration of 0.4 mM. The cells were harvested by centrifugation, washed twice with ice-cold 50 mM Tris-Hcl buffer (pH 7.6) containing 1 mM EDTA, 1 mM $MgCl_2$ and 1mM DTT, and stored at -20°C for 6 h. The frozen cells were thawed, disrupted using an ultrasonicator and then the soluble protein (crude extract) was separated by centrifugation. The crude extract was precipitated with ammonium sulfate twice, firstly 70% and then 60% (wt/vol), and resuspended in Tris-HCl buffer. The excess salt was removed by dialysis for 10 h using the same buffer at 4°C and concentrated by centrifugation using Centricons YM-3 (Millipore). The concentration of enzyme taken for the immobilization (3.32 mg/ml) was determined by the Bradford assay method [4]. The expression of enzyme (43 kDa) was observed on the SDS-polyacrylamide gel (Fig. 3).

Activation of Sepharose with Cyanogen Bromide

The sepharose was activated by slightly modified method proposed by Bickerstaff [3]. The pre-swollen sepharose (40 ml) was washed with distilled water (4x50 ml) and finally suspended in 40 ml distilled water. Sodium carbonate solution (50 ml, 2 M) was added to the beaker containing sepharose. Cyanogen bromide (2 ml, 5 M) was added into the beaker under stirring condition, and then the mixture was stirred for an additional 10 min. The activated sepharose was filtered through sintered glass, washed with sodium bicarbonate (100 ml, 1 M), washed several times with Tris-HCl buffer (pH 7.6) and stored in 40 ml of same buffer at 4°C.

Immobilization of Enzyme

Ammonium sulfate precipitated enzyme fractions (200 µl each in vial) were mixed with the same volume of activated sepharose gel (wet wt. 0.125 g) and kept overnight under shaking condition for the coupling. After 16 h of coupling reaction, the mixture was centrifuged, the

supernatant was taken to determine the concentration of free enzymes, and the enzyme immobilized-sepharose was used for the assay after washing several times with buffer. For each experiment, two replicates were studied and an average of the two was taken as the result. For the entrapment, chitosan-xanthan gel was prepared by the method proposed by Bickerstaff [3]. The enzyme and 0.65% xanthan solutions (wt/vol) were mixed in the same proportion to make 1 ml final solution. The mixture was stirred for 15 min and introduced drop wise into the glass vial containing 2 ml of 0.55% chitosan solution (wt/vol) under shaking condition (200 rpm). The hydrogel was separated from the remaining liquid, and the gel was washed with Tris-Hcl buffer for two times (2 ml each). Thus, formed gel (510 mg) was taken for the assay. For the reference, 500 µl buffer was mixed with xanthan in place of the enzyme.

Enzyme Assay

The oxidation-reduction catalyzed by immobilized dTDP-D-glucose 4,6-dehydratase was determined by the formation of dTDP-4-keto-6-deoxy-D-glucose using a UV spectrophotometer comparing the absorbances with the standard sample at 318 nm as described by Vara and Hutchinson [21]. A UV-spectrum of the reaction mixture using a crude enzyme is shown in Fig. 4. The suspension buffer was removed by centrifugation from the vial containing immobilized sepharose. The enzyme assay was carried out in 100 µl containing 0.75 mM dTDP-D-glucose, 0.75 mM NAD^+ and 50 mM Tris-Hcl buffer (pH 7.6). The mixture was incubated at 37°C for 30 min, centrifuged for 5 min, and the supernatant (80 µl) was taken to the vial containing 750 µl sodium hydroxide (100 mM). The alkali-treated mixture was further incubated for 20 min, filtered through 0.45 µm Whatman filter, and taken for the spectrophotometric analysis. For the control

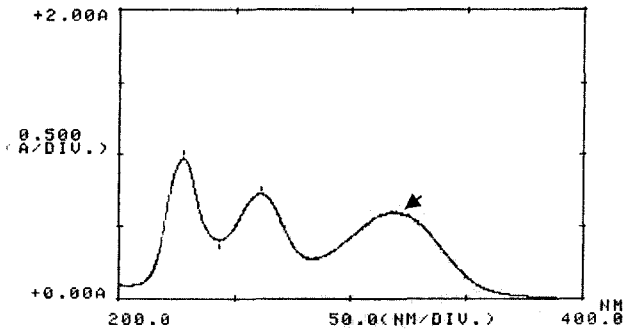

Fig. 4. UV-Spectrum of reaction mixture using ammonium sulfate precipitated enzyne.
The arrow represents the absorption due to the 4-keto-6-deoxy-D-glucose at 318 nm and the remaining absorptions are due to the NAD^+. The wavelength and absorbance ranges were set as 200 to 400 nm and 0 to 2, respectively. The assay was carried out according to the method described by Vara and Hutchinson [20].

samples, the activated sepharose was used in place of the enzyme-immobilized sepharose. The enzyme assay for the enzyme-entrapped hydrogel was carried out in 200 µl solution containing 510 mg enzyme-immobilized gel, 0.75 mM dTDP-D-glucose, 0.75 mM NAD^+, and 50 mM Tris-HCl buffer (pH 7.6). The mixture was incubated for 30 min, 80 µl of reaction mixture was put into the vial containing 750 µl of NaOH (100 mM), and the assay was performed by the method as mentioned previously. To study the multiple use of the enzyme containing gel, it was washed twice with Tris-HCl buffer (pH 7.6, 500 µl each) and the enzyme assay was carried out repeatedly. Similarly, the multiple applicability of enzyme-immobilized sepharose was also studied.

RESULTS AND DISCUSSION

dTDP-4-keto-6-deoxy-D-glucose Conversion by Using Enzyme-Entrapped Hydrogel

Enzyme-entrapped hydrogel was prepared as mentioned in the Materials and Methods. The extent of entrapment was not determined due to the turbidity of gel components. Assay was carried out using the enzyme-entrapped hydrogel (510 mg) with the same amount of hydrogel without an enzyme as reference. About 71% conversion of dTDP-4-keto-6-deoxy-D-glucose was recorded in the first batch. When the hydrogel was washed for repeated use, the conversion rate decreased in the following batches and showed only 20% in the fourth batch reaction (Fig. 5).

Covalant Immobilization of Enzyme

Sepharose was activated with cyanogen bromide and coupling was carried out with enzyme under stirring

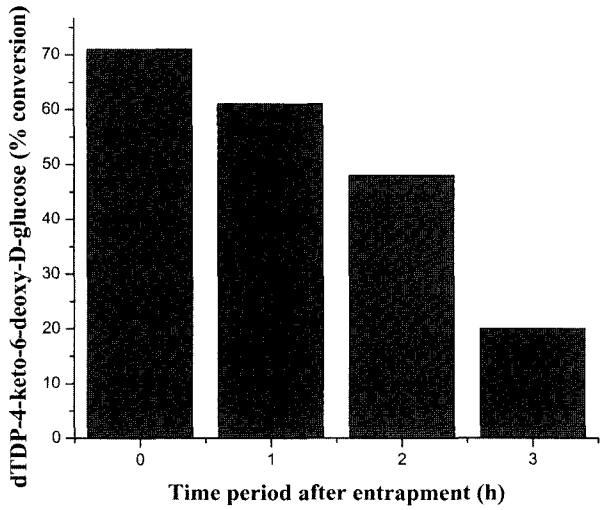

Fig. 5. Conversion profile using the chitosan-xanthan hydrogel.
The same enzyme was repeatedly used in assay for four times.

condition. When the sample was withdrawn immediately after the onset of the coupling reaction, and assayed, about one-third of the enzyme (28% with that of free enzyme used) was covalently linked. The immobilization process was studied until 16 h of coupling. Rapid immobilization was observed during the first hour of coupling, the rate was lowered until 4 h, and significantly low coupling was recorded after 4 h (Fig. 6). The maximum immobilization yield (80.03% with that of the free enzyme used) was recorded after 14 h of coupling with activated support.

dTDP-4-keto-6-deoxy-D-glucose Conversion by the Immobilized Enzyme

The assay of the covalently immobilized enzyme was carried out as mentioned in the Materials and Methods. Significantly low conversion of dTDP-4-keto-6-deoxy-D-glucose (39%) was achieved in the first batch but very high conversions (an average of 78.4%) were achieved in the following batches (Fig. 7). The same enzyme-immobilized sepharose was repeatedly used until seven batches and the assays were carried out until 120 h from the completion of the coupling process. Instead, the conversion of dTDP-4-keto-6-deoxy-D-glucose was exponentially decreased in each proceeding batch from the first batch using the same enzyme-entrapped gel. This shows that the entrapment was weak and it suffered from leakage in every washing step or the enzyme-immobilized gel was not stable under experimental conditions. Although the temperature influences the stability of hydrogel and the size of lamellae, it might not be the cause of such low activities as the assay was carried out at the optimal condition reported so far [7]. Dumitriu and coworkers have reported similar results where the activity of lipase immobilized on the chitosan-xanthan hydrogel was decreased by 17% after washing the enzyme-immobilized gel for 100 min [8]. Therefore, the

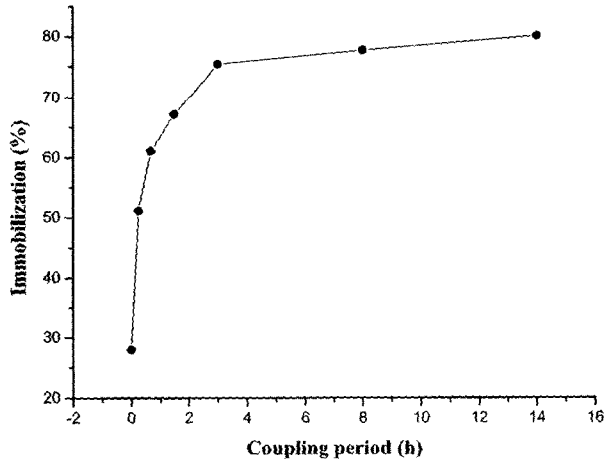

Fig. 6. Immobilization profile using the cyanogen bromide activated sepharose.

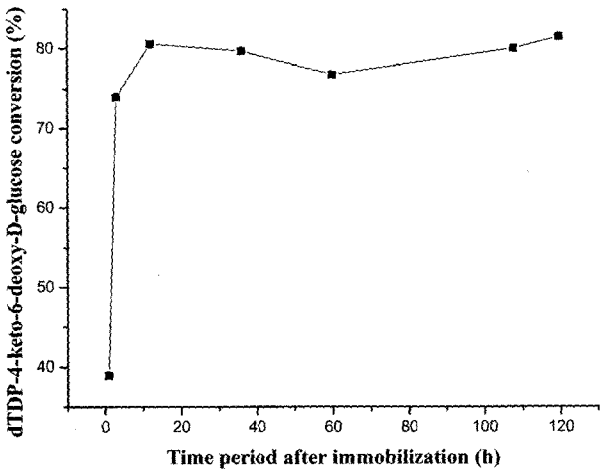

Fig. 7. Conversion profile using the immobilized-enzyme on cyanogen bromide activated sepharose.
The same enzyme was repeatedely used for seven times.

gradual loss of activity could be due to the leakage of enzyme in each washing step. Furthermore, the weak nature of gel makes the situation more complicated for the separation of product from the reaction mixture.

In contrast, the covalently linked enzyme showed very high conversion when it was repeatedly used for seven times until 120 h. This shows that the enzyme is stable under the condition investigated. Temperature and solvent effects on enzyme activity were unpredictable since they could be the consequences of the interaction of a number of factors including alterations in protein conformation generally, or, especially, in the environment of the active site, and changes in chemical reactivity of the substrate [17]. The lowest conversion in the first batch was probably due to the lack of time necessary for proper folding of covalently linked enzyme or due to the swelling property of activated sepharose at different temperature influencing the activity of enzyme. The result is compatible with the result of Kim and coworkers in the production of tagatose using L-arabinose isomerase-immobilized agarose [14]. The specific activity of the immobilized enzyme was 39.42% of that of the free enzyme. Depending on the carrier and method of immobilization, a lower specific activity of the immobilized enzyme than that of the free enzyme might result due to its inactivation by the interaction with support, inaccessibility of the active sites, and by the limitation of access to the substrate [17]. However, 80.03% of the free enzyme was covalently attached to the activated sepharose, which suggests that a considerable amount of dTDP-D-glucose-4,6-dehydratase had been bound in an unfavorable conformation for the catalytic activity. This could arise since the immobilization involved the covalant bond formation between enzyme-amino functionality and cyclic imido-carbamate derivatives of sepharose (Fig. 2); some of these may be in the active

site of the RfbB, as it was found in immobilized bromoperoxidase [17] and L-arabinose isomerase [14]. Instead, the immobilization profile indicated that cyanogen bromide activated sepharose is suitable for the fast linkage and good stability of enzyme.

CONCLUSION

In conclusion, RfbB immobilized on cyanogen bromide activated agarose has better stability than its entrapment in the hydrogel. The stability and recyclability of the covalently immobilized dTDP-glucose 4,6-dehydratase system suggests that it can be applied for the economic production of dTDP-4-keto-6-deoxy-D-glucose.

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