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Production of Cyclodextrin Homologues Using Aqueous Two-Phase System

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Cyclodextrin homologues (CDs), produced by cyclodextrin glycosyltransferase (CGTase), were simultaneously partitioned in aqueous two-phase system (ATPS). Partition coefficients of CDs were measured in PEG/salt and PEG/dextran systems. Phosphate, citrate, sulfate were tested as salt. ATPS of PEG/salt and PEG/dextran had the partition coefficients of the CDs, larger than unity. However, PEG/dextran system was observed better than PEG/salt as CGTase activity decreased sharply with salt concentration. Enzymatic reaction occurred mainly in PEG-rich bottom phase because of the low partition coefficient of CGTase. The resulting CDs transferred to the PEG-rich top phase, obeying the diffusional partition. In the ATPS of 7% PEG (M.W. 20,000) and 9% dextran (M.W. 40,000), 7 mg/ml of CDs were obtained in top phase at 4.5 hours.

Key words: cyclodextrin homologues, aqueous two-phase system, simultaneous production and separation, CGTase

INTRODUCTION

Cyclodextrin homologues are oligosaccharides, synthesized from starch through cyclization by cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) [1]. There are many kinds of CD derivatives, but α-, β- and γ-CD, composed of 6, 7 and 8 glucopyranose units, respectively, are the major products. Recently the usages of CDs expanded not only as an additive for medicinal products, foodstuffs and cosmetics [2], but also as a separating media in separation of enantiomers [3] and other processes. The numbers of usages of CD derivatives are also increasing [4]. CDs production in aqueous solution is affected by many factors, such as the product inhibition on CGTase [5] or product degradation by CGTase and so on. Thus, modified processes to overcome these disadvantages are needed, and ATPS may become a solution.

Aqueous two-phase systems consist of two immiscible fluids in a bulk water solvent [6]. High water content in such systems can give a mild condition for large scale separation of biological materials, allowing the stabilization of biomaterials. Hence ATPS can be used in many processes as a first step of isolation, a carrier system in extractive bioconversion, additional preparative applications, analytical applications and so forth [7]. ATPS can be constructed by the combination of PEG as one polymer and salt or other polymer such as dextran as a second polymer. Until now, ATPS was mainly used for the separation of proteins. In this study, simultaneous enzymatic conversion by CGTase and separation of CDs was tried to circumvent the product inhibition and simplify the separation process of CDs using aqueous two-phase system. Partition coefficients of CDs and CGTase were tested in the ATPS of PEG/salt and PEG/dextran. CGTase and CDs showed an opposite behavior in their partitioning in PEG/dextran system. The favorable partition of CDs to PEG-rich phase (separation phase) from dextran-rich phase (reaction phase) enabled us to carry out a simultaneous reaction and transfer of CDs.

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MATERIALS AND METHODS

Aqueous Two-Phase System

Aqueous two-phase system was constructed with PEG and salt or dextran. Various kinds of salts were tested for the PEG/salt system. As a salt, 10% (w/w) potassium phosphate (Shinyo pure chemicals co.), 15% (w/w) ammonium sulfate or magnesium sulfate (Oriental chemical industry co.) or 15% (w/w) sodium citrate (Shinyo pure chemicals co.) was used with 10% (w/w) PEG. PEG/dextran system was constructed with 7% (w/w) polyethylene glycol (PEG) (M.W. 20,000, Yakuri co.) and 9% (w/w) dextran T40 (M.W. 40,000, Pharmacia co.) with 0.15% (w/w) phosphate salt. The operating conditions for PEG/dextran system followed partially the work of Min [8]. Sodium phosphate (Na_2HPO_4-NaH_2PO_4) was used in PEG/dextran system to keep pH 6.0. The final concentration of phosphate was 10 mM. The binodal curve of phase diagram was determined by turbidometric titration method [9]. The compositions of the binodal curve were decided by the points of turning turbid when one of 20% (w/w) PEG or dextran solution was added to the other. Total concentrations of each polymers were calculated from the amount of solution added.

For the enzymatic production in ATPS, 2 ml (444 unit) of CGTase (Sewon co.) was mixed with 4% soluble starch (Difco co.) in the PEG/dextran system of 200 ml. Volume ratio of the bottom and top phase was 1:1 during the reaction. The solution was mixed with agitator (top phase) and magnetic stirrer (bottom phase) at the

speed of 100 rpm. Temperature was set at 55°C using water bath.

Enzyme Stability

The effect of salt concentration on CGTase stability was tested in the 10% PEG solution. Enzyme solutions were kept for 3, 6, and 18 hours in various concentrations of salt solutions at 55°C, before measuring the enzyme activity. Relative activity of CGTase was calculated by comparing the total amounts of CDs produced by the treated enzyme solution and the fresh solution. One unit of CGTase was defined as the amount of CGTase that can produce 1 mg of β-CD during 20 minutes in 10 mM phosphate solution at 55°C, pH6.0.

Analysis

Concentrations of CDs were measured by HPLC system (Orom tech.) using the carbohydrate column (Waters co.) and RI detector (Waters co.). Eluent was a mixture of water and acetonitrile with the ratio of 35: 65. CGTase concentration was measured following the Bradford method (Bio-rad co.) [10].

RESULTS AND DISCUSSION

Aqueous Two-Phase System

Partition coefficients of CDs were higher than unity in all experimental cases, though there were some variances between the cases (Fig. 1). Partition coefficient of α-CD was higher than γ-CD in every cases. In the PEG/salt systems, partition coefficients of α-and γ-CD were higher than β-CD, while partition coefficient of β-CD was highest among the homologues in the PEG/dextran system. Between the two PEG/sulfate systems, ammonium sulfate at higher concentration (1.14 M) gave lower partition than magnesium sulfate (0.51 M), which is considered due to the lower valence charge of ammonium ion. In salt solutions, CGTase showed low

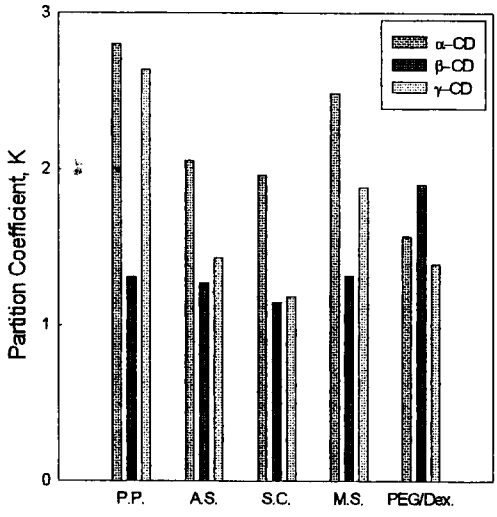

Fig. 1. Partition coefficients of α-, β-, γ-cyclodextrin in PEG/salt and PEG/dextran ATPS. (P.P.: 10% potassium phosphate (0.74 M), A.S.: 15% ammonium sulfate (1.14 M), S.C.: 15% sodium citrate (0.61 M), M.S.: 15% magnesium sulfate (0.51 M)).

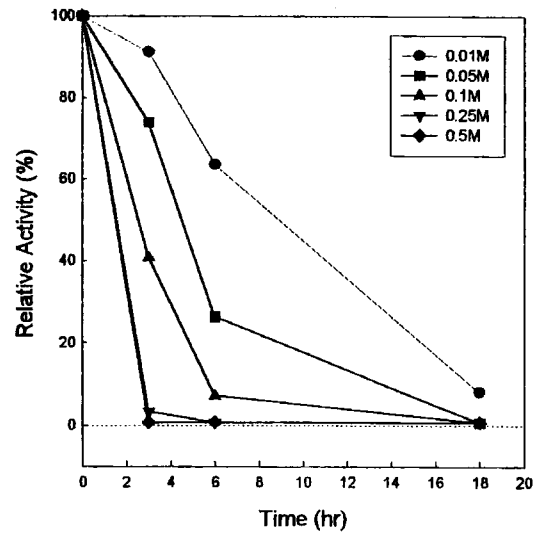

Fig. 2. Stability of CGTase with the phosphate concentrations.

stability (Fig. 2). With the increasing salt concentration, the relative activity of CGTase decreased drastically. After 3 hours, only 3.4% of relative activity remained in 0.25 M salt solution. In the 0.5 M or higher salt solutions, the relative activity decreased to less than 1% in 3 hours (data not shown for concentrations higher than 0.5 M). In the 0.01 M salt solution, CGTase can keep more than 90% of its relative activity after 3 hours, but it decreased to about 60% after 6 hours. Usually, for the enzymatic reaction, it is recommended to maintain the low salt concentration. However, it is normal in the PEG/salt ATPS to use salt concentration, higher than 0.5 M [11]. Considering the enzyme instability and the fact that many commercial CGTases produce more β-CD than α- and γ-CD, PEG/dextran system looks most promising with its highest partition for β-CD. The partition coefficients in the PEG/dextran system were 1.6, 1.9 and 1.4 for α-, β-, and γ-CD, respectively (Fig. 1).

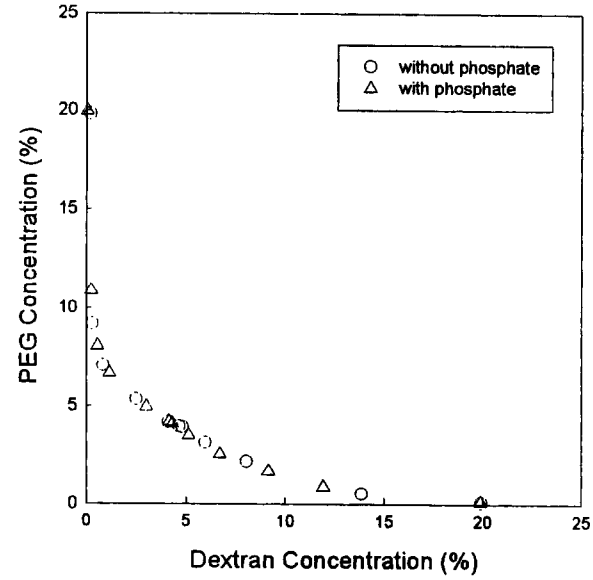

Fig. 3. Binodals of PEG (M.W. 20,000) and dextran (M.W. 40,000) aqueous two-phase system with and without 0. 15% phosphate at 55°C.

Binodal curves were constructed to survey the phase-forming characteristics of ATPS (Fig. 3). Data points display the phase forming compositions of this system at 55°C. Two binodal curves with and without 0.15% phosphate did not show difference from each other. Hence, comparing the phase forming concentrations, 0.15% phosphate is considered not to give much effect on the system. However, it is assumed that the tie lines of the phase diagram between the two cases can be different each other, which is under study at the present time.

Reaction and Transfer of CDs in ATPS

Based upon the properties of ATPS shown in Fig. 1 to 3, an ATPS using 7% PEG (M.W. 20,000) and 9% dextran (M.W. 40,000) was constructed for the simultaneous reaction and transfer of CDs. CGTase and starch (a substrate, used for the CDs production) were observed to distribute more in the bottom phase. Fig. 4 shows the time course of the concentrations of CDs in ATPS. During the first 5 minutes of reaction, the concentrations of the three CDs increased quickly in the bottom phase. After 5 minutes and on, the concentrations of CDs in the bottom phase decreased gradually while the concentrations in the top phase increased. The concentrations of CDs in both phases changed continuously to reach the ratios of 2.52, 2.70 and 1.71 for α-, β-, γ-CD, respectively, at 4.5 hours. The total concentrations of CDs in the top and bottom phase were 7.34 and 2.95 mg/ml, respectively. It is noted that the concentrations of α- and β-CD in the top phase were still increasing at 4.5 hours. Judging from the shapes of concentration change in the earlier period, a quick rise and fall in both phases, this simultaneous reaction and transfer system is a reaction limited process, particularly true with γ-CD.

Experimental data shown in Fig. 4 were redrawn to give the contents of each CDs in the whole system, and the total CDs in each phases in Fig. 5 and Fig. 6, respectively. In Fig. 5, we see the decreases of CDs after the initial 5 minutes except the case of β-CD. It is believed due to the degradation of CDs by CGTase, the extents of which are in the order of γ-CD, α- and β-CD. Although CDs are named in the order of their molecular weights (number of glucose units), such trend of degradation seems not too strange, considering the fact that the solubility of β-CD is the lowest among the three homologues. The total CDs in the bottom phase decreased remarkably after 5 minutes, while that in the top phase showed a steady increase (Fig. 6). This trend is resulting from the combined effects of the reaction, degradation and the mass transfer between the phases. However, the major influence is assumed to come from the degradation effect, with a consequent deduction that PEG protects CDs better than dextran. The decrease of total CDs in both phases in the same period is a strong evidence, supporting such an assumption. The degradation of CDs in the polymer solution by CGTase is being examined by the authors at the present time.

As a result, ATPS was applied to the simultaneous reaction and transfer of CDs, the success of which depends partially on the control of degradation. A precise description of the system should be based upon the

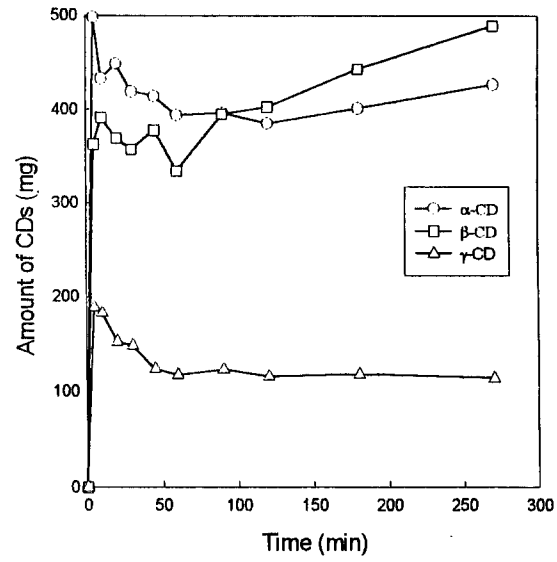

Fig. 5. Changes of the contents of each CDs in ATPS.

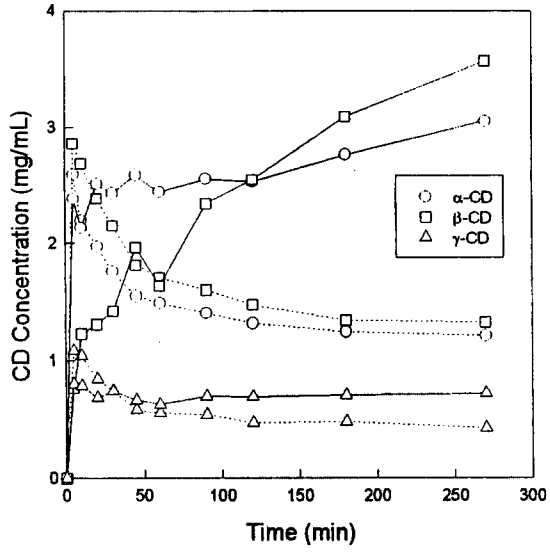

Fig. 4. Concentration changes of CDs in top (——) and bottom (···) phase of ATPS.

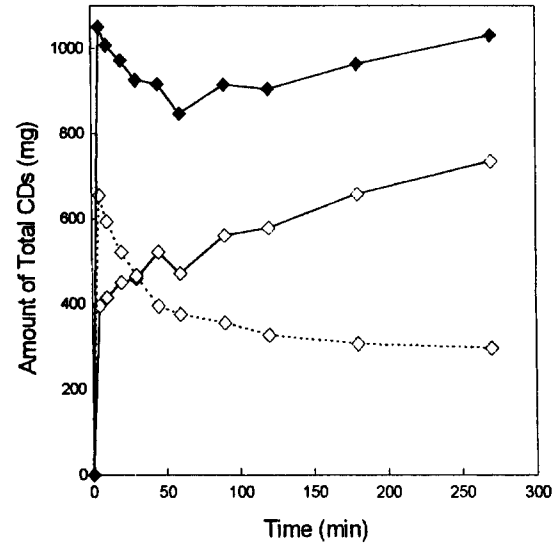

Fig. 6. Total CDs in the top (-◇-), bottom (··◇··) and both (-◆-) phases.

thorough understandings of the enzyme reaction and degradation mechanisms of CGTase. Recovery of CDs from PEG-rich phase is under detailed study for the better economics of this system.

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