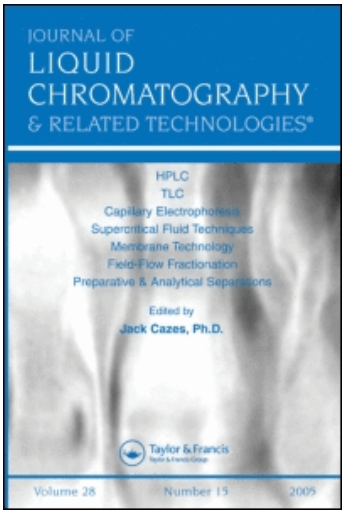

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Purification of Glucosyltransferase from *Streptococcus sobrinus* Cell Culture Medium by Combined Use of Batch Extraction and Countercurrent Chromatography with a Polymer Phase System

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Purification of Glucosyltransferase from *Streptococcus sobrinus* Cell Culture Medium by Combined Use of Batch Extraction and Countercurrent Chromatography with a Polymer Phase System

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ABSTRACT

Glucosyltransferase (GTF), a cariogenic factor of *Streptococcus sobrinus* (SS), was extracted from a large quantity of cell culture medium by batch extraction with aqueous polymer two-phase systems (APTPs). The small quantity of the extract was further purified by countercurrent chromatography (CCC) using polyethylene glycol (PEG) 8000-dextran T500 system at pH 9.2, by eluting the impurities with the upper mobile phase

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at 1.0 mL/min. After the elution, the GTF still remaining in the dextran-rich lower stationary phase was collected. The GTF fractions were subjected to hydroxyapatite chromatography to eliminate polymers, such as PEG 8000 and dextran T500. Purified fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and GTF enzyme activity, which rose over 270 fold from that in the original culture medium.

Key Words: Countercurrent chromatography (CCC); Hydroxyapatite chromatography; Glucosyltransferase; Aqueous polymer two-phase systems; *Streptococcus sobrinus*.

INTRODUCTION

Glucosyltransferase (GTF) is an enzyme produced from oral bacterial flora of various streptococcal species causing dental caries in humans.[1] GTF, produced by *Streptococcus sobrinus* has been studied by many dental researchers. The water-insoluble glucans (WIGs) were made from sucrose by GTF, in particular, WIG mediate the accumulation of the cariogenic bacteria on tooth surface, causing the aggregation of bacteria as a dental plaque, which leads to dental caries.[2,3] Since GTF is not commercially available in a pure form, these researchers have used a culture medium that contains GTF released from *S. sobrinus*, as the standard enzyme for their studies. In this particular species, about 75% of GTF is released into the culture medium, which makes purification of the GTF more difficult. In the past, purification of various types of GTF was carried out using the conventional chromatographic methods. Purification of the GTF-I and GTF-SI from the culture medium of *S. milleri* was carried out by the combined use of hydroxyapatite and gel filtration chromatography, with a Toyopearl HW-55 or Toyopearl HW-65 column, after ammonium sulfate precipitation and dialysis.[4] Purification of *S. mutans* extracellular GTF from the cell-free culture supernatant was performed by combined use of gel filtration chromatography, with a Bio-Gel A-5, A-15 column and affinity chromatography with Amino ethyl Bio-Gel P-60 column, after ethanol precipitation and ultra-filtration.[5] However, the recovery of the GTF from the culture medium was not satisfactory due to the irreversible adsorption to the column packing materials. In particular, GTFs have some dextran-binding domains leading to the irreversible adsorption onto the cross-linked dextran, such as Sephadex and Sephacryl beads.[6] Thus, it is highly desirable to develop a purification method, which can produce a high yield of GTF without loss of enzyme activities from *S. sobrinus* culture medium.

Aqueous–aqueous polymer phase systems for the partitioning of biological macromolecules were first established by Albertsson in 1950s.[7] Partition of macromolecules, such as proteins[8–12] have been carried out by the use of various aqueous polymer phase systems available, dextran–polyethylene glycol (PEG) and PEG–potassium phosphate systems have been most commonly used for partition of biological samples.

Chromatographic partitioning of proteins with polymer phase systems can be performed by countercurrent chromatography (CCC)[13] using a type J-coil planet centrifuge (CPC), a spiral disk assembly CPC, and a cross-axis CPC. A type J-CPC has been successfully used for the separation of plasmid DNA using PEG–potassium phosphate two-phase system.[14] A spiral disk assembly provides satisfactory retention of the stationary phase for polar solvent systems, including aqueous polymer two-phase (APTP) systems.[15] The cross-axis CPC is one of the CCC systems that provide reliable retention of the stationary phase of viscous aqueous polymer phase systems used for protein separations.[16–20] The cross-axis CPCs have been successfully used for the separation and purification of a variety of physiological proteins, including histones and serum proteins,[21] recombinant uridine phosphorylase,[22] human lipoproteins,[23] lactic acid dehydrogenase,[24] chicken egg white proteins,[25] cholinesterase,[26] and single-strand DNA binding proteins.[27] Because of the protective effects of high polymer–salt concentrations, proteins can maintain their integrity at room temperature for a relatively long period of time, so that the purification may be performed without cooling the column or collected fractions. Recently, we described the purification of GTF from the cell-lysate of *S. mutans* by CCC with an aqueous polymer two-phase system.[28]

This paper describes successful purification of GTF from a large volume of culture media of *S. sobrinus* using a batch extraction followed by high-speed CCC with an aqueous–aqueous polymer two-phase system.

EXPERIMENTAL

Apparatus

The cross-axis CPC (X-axis) used in the present study has a unique feature among the CPC systems available, in that it provides reliable retention of the stationary phase for viscous polymer phase systems. The detailed design of the X-axis CPC was described elsewhere.[18,29] The present apparatus holds a pair of horizontal rotary shafts, symmetrically mounted one on each side of the rotary frame, at a distance of 10 cm from the centrifuge axis as shown in Fig. 1. A spool-shaped column holder is mounted on each rotary shaft

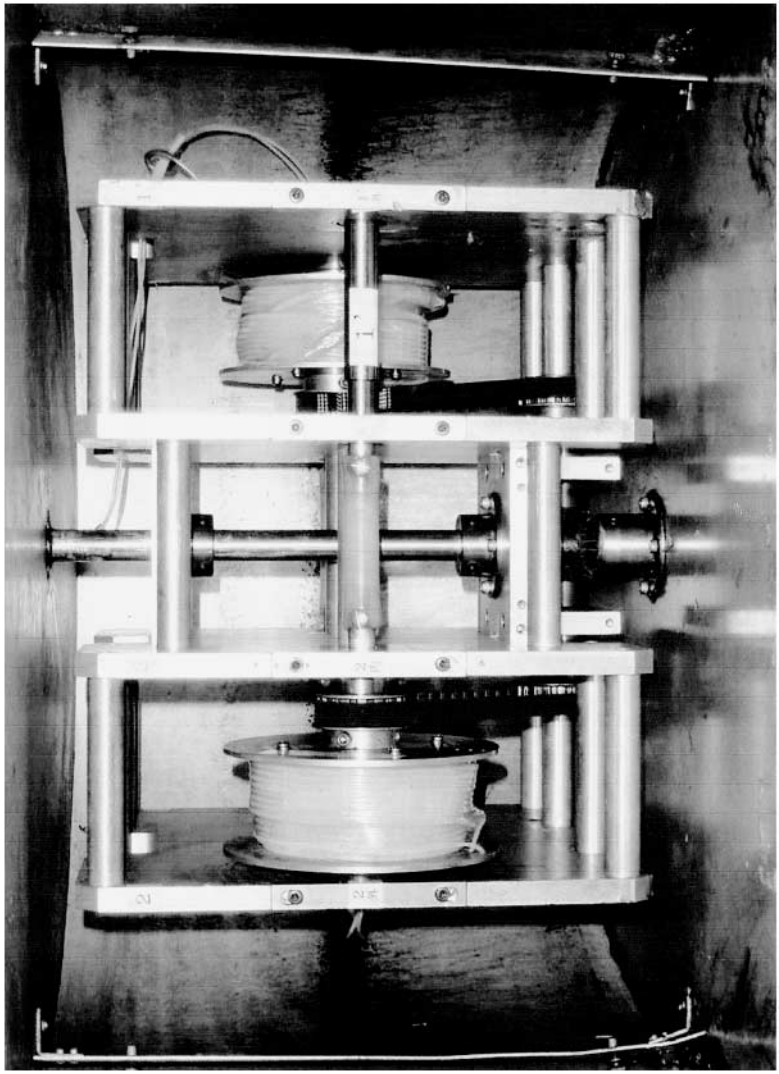

Figure 1. Photograph of type XL cross-axis coil planet centrifuge.

at an off-center position 10 cm from its mid-point. The multilayer coil separation column was prepared from a 2.6 mm I. D. polytetrafluoroethylene (PTFE) tube (Zeus Industrial Products, Raritan, NJ) by winding it onto a 5.0 cm diameter holder hub, forming three layers of left-handed coils between a pair of flanges spaced 5 cm apart. A pair of columns mounted on the rotary frame was connected in series with a flow tube (PTFE, 0.85 mm I.D.), resulting in the total capacity of 145 mL. The speed of the apparatus is regulated at 400 rpm with a speed control unit (Bodine Electric, Chicago, IL).

Reagents

PEG 8000 (average molecular mass = 8000) and dextran T500 (Mw = 460500, Mw/Mn = 2.2) were purchased from Wako Pure Chemicals (Osaka, Japan) and Amasham Biosciences (Tokyo, Japan), respectively. Other chemicals were all of analytical reagent grade.

S. sobrinus 6715 (sero type g) was kindly provided by Dr. I. Nasu from Nihon University, School of Dentistry, Matsudo, Japan.

Preparation of *S. sobrinus* Culture Medium Containing GTF

S. sobrinus 6715 was grown for 24 hr at 37°C in 3 L of TTY broth[30] composed of 1.5% (w/v) trypticase soy broth (BD, MD, USA), 0.4% (w/v) bacto tryptose (BD), 0.4% (w/v) yeast extract (Sigma, MD), 0.2% K_2HPO_4, 0.4% (w/v) KH_2PO_4, 0.2% (w/v) Na_2CO_3, 0.2% (w/v) NaCl, and 1.0% (w/v) glucose. The cultured medium of *S. sobrinus* cells was centrifuged at 3500*g* for 20 min at 4°C. The supernatant was filtered with a DISMIC 13HP filter cartridge (Advantec Toyo, Tokyo, Japan). The filtrate was used as the culture medium for the subsequent studies.

Measurement of GTF Enzymatic Activity

GTF enzymatic activity was estimated from the amount of WIG produced from sucrose by GTF. For a rapid assay of GTF solution in the culture medium and chromatographic fractions,[31] 100 μL of sample solution was incubated in 2 mL of 0.5 M potassium phosphate buffer (pH 6.0) containing 1% (w/v) sucrose and 0.05% (w/v) sodium azide in the presence of primer dextran T10 (20 μM), for 18 hr at 37°C. After incubation, the amount of WIG in the mixture was subjected to nephelometry

for determination of the increased absorbance at 550 nm (A_{550}) using a V-530 UV-VIS spectrophotometer (JASCO, Tokyo, Japan). In addition, for determination of the specific activity of purified GTF, an aliquot of WIG was measured by the phenol–sulfate method.[5,32] A 10 μL amount of the purified GTF solution was incubated in 2 mL of 0.1 M potassium phosphate buffer (pH 6.0) containing 1% (w/v) sucrose and 0.05% (w/v) sodium azide for 18 hr at 37°C. After incubation, the mixture was centrifuged at 19000*g* for 10 min at 4°C. The precipitated WIG was rinsed with 50% (v/v) ethanol containing 0.1 M potassium phosphate buffer (pH 6.0) three times and sonicated with 0.5 mL of 1 M sodium hydroxide solution for 20 min. The solution was incubated with 0.5 mL of 5% (w/v) phenol solution and 2.5 mL of concentrated sulfuric acid for 30 min at room temperature. After incubation, the absorbance of the solution was measured at 490 nm. Finally, using a calibration curve made from the standard glucose solutions, the amount of WIG produced by GTF was expressed as the concentration of glucose, where one unit (1 U) of GTF was defined as the amount of enzyme required to convert 1.0 μmol of glucose residue of the sucrose molecule into WIG per minute.

Preparation of PEG 8000-Dextran T500 Aqueous Two-Phase Solvent Systems for Concentration of Culture Media of *S. sobrinus*

In order to concentrate a large volume (500 mL) of culture medium into 10 mL of sample solution without losing the target enzyme, three different polymer phase systems were prepared: 4.4% PEG 8000–6.0% dextran T500, 6.0% PEG 8000–3.0% dextran T500, and 7.5% PEG 8000–1.0% dextran T500, each having different volume ratio between the two phases.

CCC Sample Preparation

Batch extraction of GTF in the aqueous polymer two-phase system performed in the PEG 8000 and dextran T500 system, having the minimum volume of the dextran-rich lower phase, efficiently concentrates GTF since GTF strongly favors its into the lower phase. The lower phase was collected, and used as the sample solution after a proper amount of PEG 8000 was added to meet the composition of the polymer phase system used for CCC separation.

CCC

In each experiment, the CCC column of the X-axis CPC was first entirely filled with the dextran-rich lower phase of 4.4% PEG 8000–6.0% dextran T500–10 mM potassium phosphate buffer at pH 9.2 as a stationary phase. A 10-mL-volume of sample solution prepared, as described above, was injected into the column using an EYELA type SV6000 sample injector (Tokyo Rikakikai, Tokyo, Japan). The PEG 8000-rich upper phase was eluted through the column at 1.0 mL/min flow rate by an EYELA LP-1100 pump (Tokyo Rikakikai), while the apparatus was rotated at 400 rpm. The effluent from the outlet of the column was continuously monitored with an EYELA UV 9000 absorbance monitor (Tokyo Rikakikai) at 220 nm, and fractionated into test tubes using a fraction collector (LKB Instruments, Bromma/Stockholm, Sweden). After most of the impurities present in the sample solution were eluted from the column, the apparatus was stopped and the column contents containing the target enzyme were collected by admitting with air using an EYELA SMP-23 cassette tube pump (Tokyo Rikakikai). Finally, an aliquot of each fraction was diluted with distilled water and the absorbance measured at 220 nm with a Shimadzu UV-1200 spectrophotometer. The GTF activity of each fraction was also subjected to the enzymatic assay described above.

Hydroxyapatite Chromatography of CCC Fractions

In order to eliminate the polymers (PEG 8000 and dextran T500) present in the purified GTF fractions, the pooled fractions were diluted 10 times with 100 mM potassium phosphate buffer at pH 7.0, and subjected to hydroxyapatite chromatography. The LC system for hydroxyapatite chromatography consisted of an L-7150 pump (Hitachi, Tokyo, Japan), S-310A model-II UV-detector (Soma Kogaku, Tokyo, Japan), D-2500 chromato-integrator (Hitachi), and SF-2120 fraction collector (Advantec, Tokyo, Japan). Bio-Gel HTP DNA grade (Bio-Rad Labs, Richmond, CA, crystal size 10–250 μm) was suspended with 100 mM potassium phosphate buffer (pH 7.0) and, after swelling, slurry-packed into the column (25 × 2 cm I.D., 78 mL bed volume). The sample solution prepared above, was loaded onto the hydroxyapatite column and eluted stepwise with 150 and 500 mM of potassium phosphate buffer at a flow-rate of 1.0 mL/min. The eluate from the column was continuously monitored at 220 nm and fractionated into the test tube (3 mL/tube).

Analysis of Hydroxyapatite Chromatography Fractions

The GTF activity of each fraction was subjected to the enzymatic assay. The fractions containing GTF were pooled, dialyzed against 10 mM potassium phosphate buffer (pH 7.0), and concentrated using a Centriprep YM-10 centrifugal filter device (Millipore, Bedford, MA).

Protein profiles of the purified GTF fractions were characterized by dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli.[33] Total protein contents in the purified GTF fractions were quantified by the Bradford dye-binding colorimetric method[34] using a Coomassie Protein Assay Reagent Kit (PIERCE, Rockford, IL). A 1.0 mL amount of the test solution was mixed with 1.0 mL of Coomassie Reagent in a test tube. After thorough mixing of each tube, each mixture was measured by absorbance at 595 nm using a V-530 UV-VIS spectrophotometer (JASCO). A calibration curve of the concentration of standard bovine serum albumin vs. the absorbance at 595 nm, was constructed to quantify the contents of proteins in the fractions.

RESULTS AND DISCUSSION

Batch Extraction of GTF from Culture Medium Using Aqueous Polymer Two-Phase Systems

As mentioned earlier, *S. sobrinus* releases GTF into the culture medium resulting in excessive dilution of the enzyme. This necessitates careful selection of the method to concentrate the target enzyme from 500 to 10 mL or 50-fold without loss of the enzymatic activity. Adsorptive sample loss and deactivation by the conventional chromatographic column support becomes critical at low protein concentration, while the ammonium sulfate precipitation method is less efficient for dilute protein solution. In the previous studies on purification of GTF from *S. mutans*, we found that GTF is almost entirely partitioned into the lower dextran-rich phase of polymer two-phase system, due to its strong affinity to dextran.[28] In the present study, we successfully utilized this partition behavior of GTF both for concentration and subsequent purification of GTF using the PEG–dextran polymer phase system.

Concentration of GTF with the above polymer phase system requires a suitable volume ratio between the two phases, where the volume of the upper phase is much greater than that of the lower dextran-rich phase. A set of polymer phase systems with different PEG/dextran ratios and concentrations were tested to measure the volume ratio between the two phases.

Table 1 shows the composition of PEG 8000–dextran T500 polymer two-phase systems and their relative volume ratio (v/v%). The minimum volume ratio of the lower phase of 2.4 (v/v%) was obtained with 7.5% PEG 8000–1.0% dextran T500 aqueous two-phase system, and by adding 41.0 g of PEG 8000 and 5.5 g of dextran T500 to the 500-mL-volume of culture medium, the GTF was concentrated into 12.0-mL-volume of the lower phase (Fig. 2).

Purification of GTF by CCC

Figure 3 shows a countercurrent chromatogram of the concentrated GTF with the cross-axis CPC using 4.4% (w/w) PEG 8000–6% (w/w) dextran T500–10 mM dibasic potassium phosphate (pH 9.2). The separation was performed at 400 rpm and at a flow-rate of 1.0 mL/min using the upper phase. After a large peak was eluted in the frs. 35–75, the CCC run was stopped, and the equilibrated column content was fractionated by forcing the solvent from the column with air using a peristaltic pump. The solid circles indicate the absorbance at 220 nm in the eluted upper phase, and the open circles those in the stationary lower phase of the column contents. The bar graphs in the chromatogram indicated the GTF enzyme activities, which are solely found in frs. 120–129 obtained from the column content. However, these fractions contain high concentrations of dextran T500, which cannot be easily removed by dialysis or ultrafiltration methods. To eliminate those high concentrations of polymers, CCC fractions

Table 1. Upper and lower volume ratio of PEG 8000–dextran T500 aqueous polymer two-phase systems with different polymer concentrations.

PEG 8000 (w/w%)	Dextran T500 (w/w%)	Upper phase (v/v%)	Lower phase (v/v%)
7.5	1.0	97.6	2.4
7.5	3.0	83.3	16.7
7.5	6.0	71.7	28.3
6.0	1.0	93.3	6.7
6.0	3.0	78.3	21.7
6.0	6.0	63.3	36.7
4.4	1.0	Single phase	
4.4	3.0	75.0	25.0
4.4	6.0	55.0	45.0

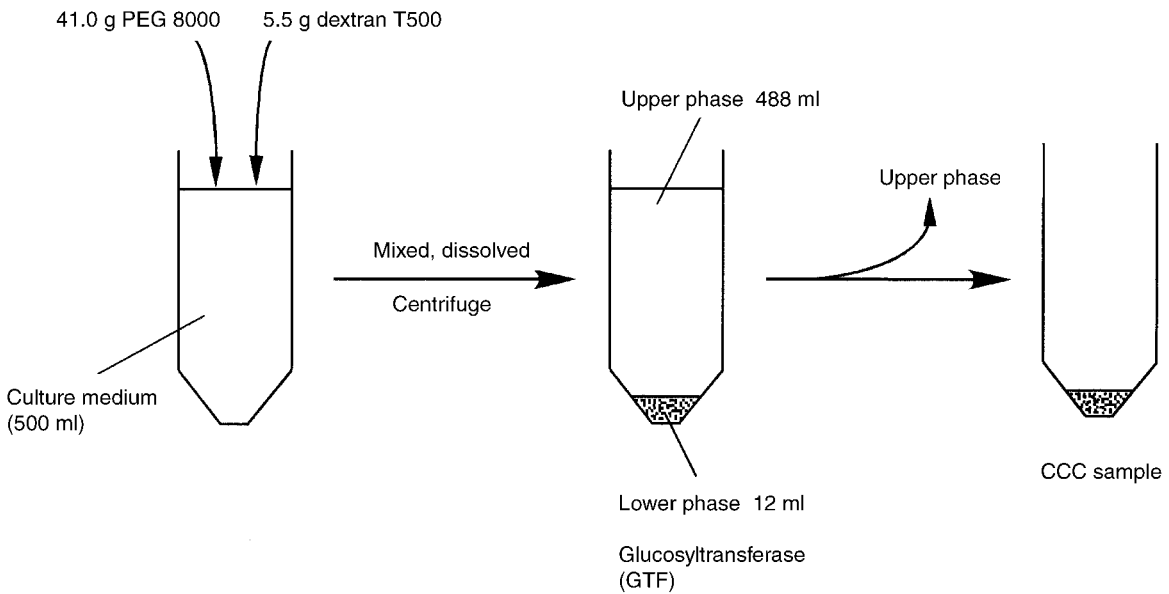

Figure 2. Schematic presentation of the concentration of GTF in the dextran-rich lower phase by batch extraction, using aqueous polymer two-phase system.

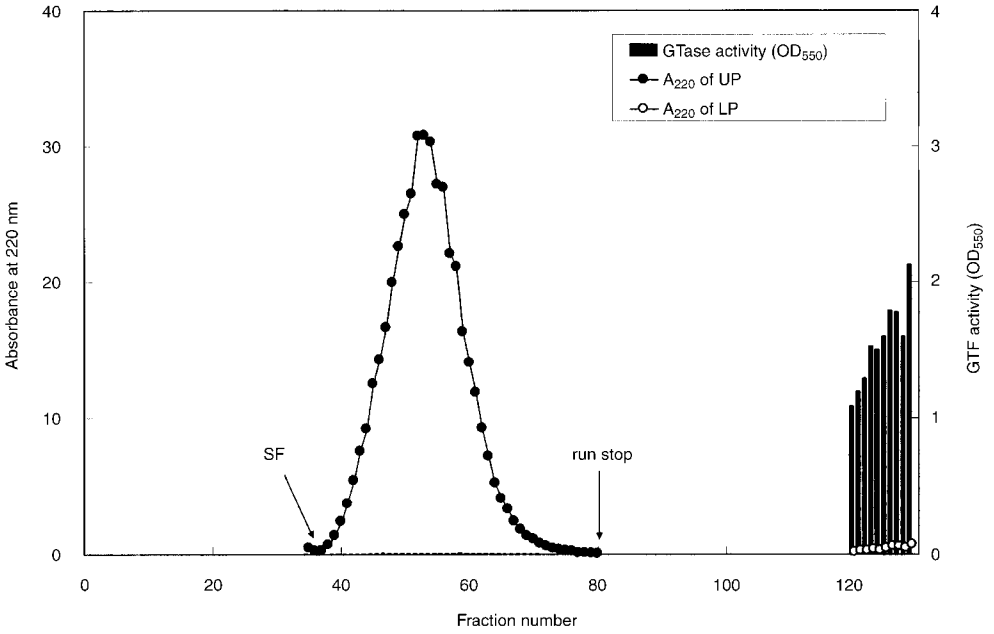

Figure 3. Countercurrent chromatogram of concentrated GTF by batch extraction. CCC conditions: column: a pair of 2.6 mm I.D. PTFE multilayer coils with a total capacity of 145 mL; sample: 10 g of lower phase of batch extraction of culture medium containing 7.5% (w/w) of PEG-8000 and 1.0% (w/w) of dextran-T500; solvent system: 4.4% (w/w) PEG-8000 and 6.0% (w/w) dextran-T500 containing 10 mM phosphate buffer (pH 9.2); mobile phase: PEG-rich upper phase; flow rate: 1.0 mL/min; revolution: 400 rpm; fractionation: 3.0 mL/tube; SF = solvent front.

were diluted with potassium phosphate buffers and subjected to the hydroxyapatite chromatography.

Hydroxyapatite Chromatography of CCC Fractions

Figure 4 shows a hydroxyapatite chromatogram of CCC fractions. Frs. 120–129 in Fig. 3 were collected together and diluted with 10-fold volume of 100 mM potassium phosphate buffer at pH 7.0. About 300-mL-volume of diluted sample solution was loaded onto the hydroxyapatite column (25 × 2 cm I.D.), and eluted, stepwise with 150 and 500 mM phosphate buffer. After the elution of polymers with 150 mM phosphate buffer, GTF still remained in the column and was recovered with 500 mM potassium phosphate buffer (frs. 66–90), thus maintaining their enzymatic activities. These fractions containing GTF were dialyzed, concentrated, and analyzed by SDS-PAGE.

Figure 5 shows the SDS-PAGE profiles of the several samples obtained by the present method (concentration with dextran-rich polymer phase, CCC, and hydroxyapatite chromatography). The lower phase of the aqueous polymer two-phase system prepared by adding PEG 8000 and dextran T500 to the culture medium of the *S. sobrinus* (sample solution) and CCC fractions, contained a small amount of GTF corresponding to the molecular weigh of 150 kDa. Frs. 66–90 obtained by hydroxyapatite chromatography (Fig. 4) and 10-fold concentrated fractions clearly produced GTF bands. No other proteins were found in the hydroxyapatite chromatography fraction after purification by CCC.

Purity and Recovery Rate of GTF after Two Types of Chromatography Steps

The purity and recovery rate of GTF were estimated at each purification step by the protein quantity and the GTF activities. Table 2 shows the purity and recovery rate of GTF in each fraction after two types of chromatography steps. Through the GTF purification process, the purity of GTF was greatly increased while maintaining a reasonable recovery rate. In the first step of purification by the batch extraction in the APTP system, the total proteins were decreased about 1/30 compared with those in the culture medium of *S. sobrinus.* After CCC, the recovery of the total activity of GTF was 70% of that in the culture medium. In the final GTF, purity after hydroxyapatite chromatography was increased about 272 times as that in the large amount of culture medium of *S. sobrinus.*

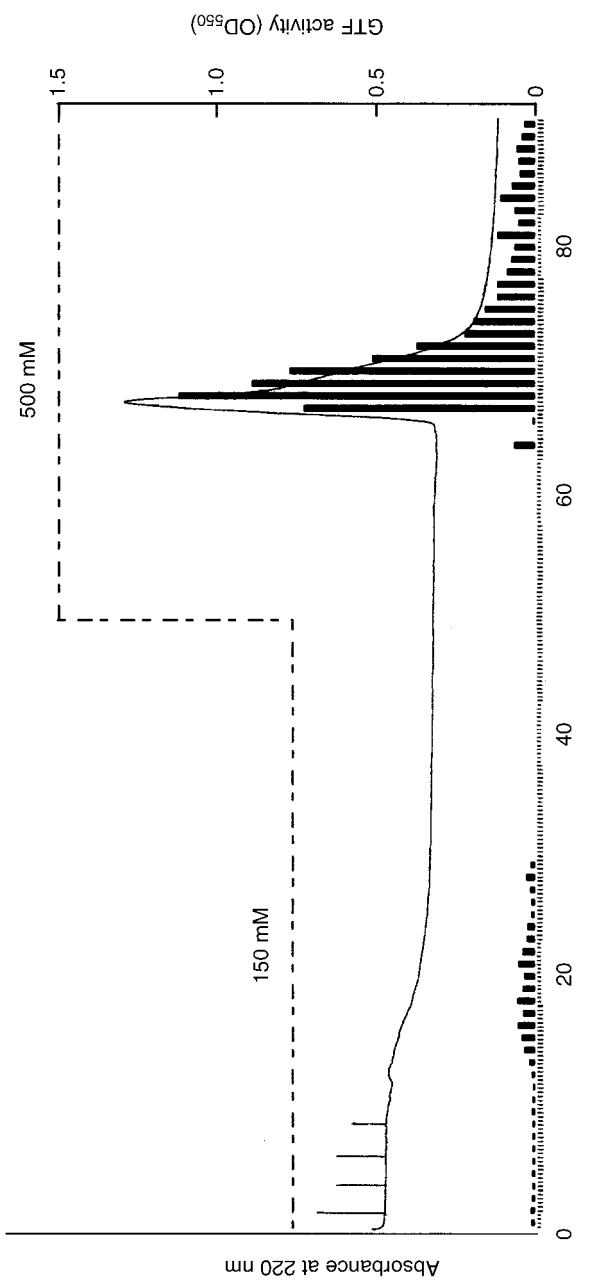

Figure 4. Elimination of polymers and purification of GTF in the CCC fraction by hydroxyapatite chromatography. Chromatographic conditions: column: Bio-Gel HTP DNA grade hydroxyapatite (25 × 2 cm I.D.); sample: 10-fold diluted CCC fraction corresponding to frs.120–129 of Fig. 3; mobile phase: stepwise elution with 150 and 500 mM potassium phosphate buffers at pH 7.0; flow rate: 1.0 mL/min; detection: UV absorbance at 220 nm; fractionation: 3.0 mL/tube. GTF activity (A_{550}) of each fraction is indicated by bar graphs.

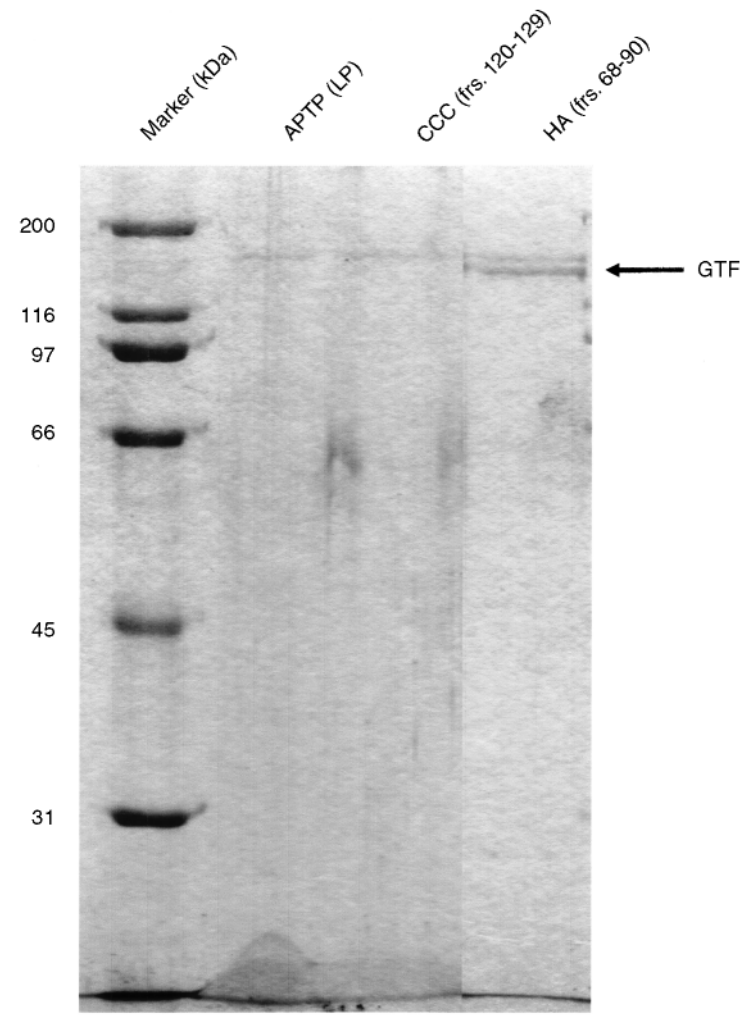

Figure 5. 10% SDS-PAGE profiles of the several purification steps of the GTF.

We assume that the excellent results of GTF purification in the present method is attained by the combined use of batch extraction, CCC, and hydroxyapatite chromatography.

The overall results of the above studies, indicate that the complementary use of the batch extract in the APTP system and the CCC is a very useful method for the purification of GTF released to the large quantities of the

Table 2. Purity and recovery of GTF at different purification steps.

	Total protein[a] (mg)	Total activity[b] (mU)	Specific activity[c] (mU/mg)	Recovery[d] (%)	Purity[e] (fold)
Culture medium (500 mL)	58.3	7.91	0.14	100	1
LP of aqueous polymer two-phase system (10 mL)	2.0	9.26	4.62	117	34
CCC fractions (18 mL)	0.4	5.51	13.51	70	100
hydroxyapatite chromatography fractions (5 mL)	0.07	2.56	36.81	32	272

[a]Total protein content (mg) was measured by Bradford protein assay.
[b]One unit (1U) of GTF was defined as the amount of enzyme required to convert 1.0 mmol of glucose residue from the sucrose molecule into WIG per minute. The amount of WIG was measured by phenol–sulfate method.
[c]Specific activity was expressed in the ratio of total activity (mU) per total protein (mg).
[d]Recovery of GTF was expressed in the percentage ratio of the total activity of each fraction per that of SS culture medium.
[e]Purification efficiency was expressed in the ratio of the specific activity of each fraction per that of SS culture medium.

culture medium of *S. sobrinus.* We hope that the present method will contribute to research on human dental caries and its prevention.

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