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Purification of glucosyltransferase from cell-lysate of *Streptococcus mutans* by counter-current chromatography using aqueous polymer two-phase system

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Abstract

Counter-current chromatography (CCC) using a cross-axis coil planet centrifuge (X-axis CPC) was applied to the purification of glucosyltransferase (GTF) from a cell-lysate of cariogenic bacteria. The purification was performed using an aqueous polymer two-phase system composed of 4.4% (w/w) polyethylene glycol (PEG) 8000–6% (w/w) dextran T500 containing 10 mM phosphate buffer at pH 9.2 by eluting the upper phase (UP) at 1.0 ml/min. The bacterial GTF in the cell-lysate of *Streptococcus mutans* was selectively retained in the dextran-rich lower stationary phase. The column contents were diluted and subjected to hydroxyapatite (HA) chromatography to remove the polymers from the GTF. Fractions eluted with 500 mM potassium phosphate buffer were analyzed by GTF enzymatic activity as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The GTF purity in the final product was increased about 87 times as that in the cell-lysate with a good recovery rate of about 79% through this purification process.

Keywords: *Streptococcus mutans*; Glucosyltransferase

1. Introduction

Among the oral bacterial flora in human, *Streptococcus mutans* and *S. sobrinus* have been confirmed to be highly cariogenic pathogens [1]. These bacteria produce glucans from sucrose by glucosyltransferase (GTF; EC 2.4.1.5). In particular, water-insoluble glucans (WIG) mediate the accumulation of these cariogenic bacteria on tooth surface, causing the aggregation of bacteria as a dental plaque which finally leads to dental caries [2,3]. Because GTF produced by the bacteria is the most important cariogenic factor in human dental caries, the tertiary structures and the enzymatic activities of the bacterial GTF have been studied by many dental researchers, and for the prevention of dental caries many different kinds of GTF inhibitors from natural sources have been reported in recent years [4–10]. Because purified GTF is not commercially available, many researchers have been used a crude extract of bacterial cells and/or a cultured-media containing GTF as the standard enzyme solutions for their studies.

In the initial studies on molecular genetics, several bacterial genes coded GTF have already been isolated and characterized. Among those, *gtf*B and *gtf*C from *S. mutans* [11,12] and *gtf*I from *S. sobrinus* [13] were the most important genes, coding for the synthesis of water-insoluble glucans GTFs (WIG-GTF) of about 150 kDa. These genes were introduced into the cells of noncariogenic variant (*S. milleri*) or *Escherichia coli*, and the recombinant WIG-GTFs were expressed as intra- or extracellular protein from these transformed cells [11–16]. In the case of *S. mutans*, the WIG-GTF was solubilized by a highly concentrated urea solution from the cell-associated fraction such as a cell-lysate or a cell-extract. Then, GTF was purified from the extracts by a combined use of ion-exchange [7,11,12,15,17,18], chromatofocusing [7,11,12,15], hydroxyapatite (HA) [10,14,18], gel-filtration [14,17] chromatographic methods and other techniques [16,17]. However, the recovery of GTF was not satisfactory in these methods due to the irreversible

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adsorption of GTF to the column packing materials. In particular, GTFs have some dextran-binding domains leading to the irreversible adsorption of cross-linked dextran such as Sephadex and Sephacryl beads [19]. Therefore, it is desirable to find an alternative method which isolates GTF at a high yield without loss of its enzymatic activity.

Counter-current chromatography (CCC) is essentially a form of liquid–liquid partition chromatography. Its unique feature, among other chromatographic systems, is derived from the fact that the method uses no solid support, and the liquid stationary phase is retained in a multilayer coiled tube aided by an Archimedean screw effect induced by a rotating centrifugal force field. For protein separations, several types of cross-axis coil planet centrifuges (X-axis CPCs) have been designed for performing CCC using highly viscous polymer two-phase systems such as polyethylene glycol (PEG)-potassium phosphate buffer system and PEG-dextran system [20–22]. These X-axis CPCs have been successfully used for the separation and purification of a variety of physiological proteins including histones and serum proteins [23], recombinant uridine phosphorylase [24], human lipoproteins [25], lactic acid dehydrogenase [26], chicken egg white proteins [27], cholinesterase [28] and single-strand DNA binding protein [29].

This paper describes the CCC purification of GTF from a *S. mutans* cell-lysate using the X-axis CPC with an aqueous-aqueous polymer phase system composed of 4.4% (w/w) PEG 8000 and 6.0% (w/w) dextran T500. Hydroxyapatite chromatography is complementarily used for eliminating polymers from CCC fractions.

2. Experimental

2.1. Reagents and bacterial strain

Polyethylene glycol 8000 (average molecular mass = 8000) and dextran T500 (Mw = 460, 500, Mw/Mn = 2.2) were purchased from Wako Pure Chem. (Osaka, Japan) and Amersham Biosciences (Tokyo, Japan), respectively. Other chemicals were all of analytical reagent grade.

S. mutans MT8148 (sero type c) was kindly provided by Dr. I. Nasu from Nihon University, School of Dentistry at Matsudo, Japan.

2.2. Preparation of S. mutans cell-lysate

S. mutans MT8148 (SM) was grown for 24 h 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, USA), 0.2% (w/v) 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 SM cells were concentrated by centrifugation at 3500 × *g* and rinsed at three times with 10 mM phosphate buffered saline (PBS; pH 7.4). After a sonication of 1 g (wet weight) of SM cells with 10 ml of PBS, the solution was centrifuged at 25,000 × *g*, and the supernatant was filtered through a DISMIC 13HP filter cartridge (Advantec Toyo, Tokyo, Japan). The filtrate was used as the SM cell-lysate for the subsequent studies.

2.3. Measurement of GTF enzymatic activity

GTF enzymatic activity was estimated from the amount of water-insoluble glucan produced from sucrose by GTF. For a rapid assay of crude GTF solution such as the SM cell-lysate and its chromatographic fractions [10], 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 h 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 the determination of specific activity of the purified GTase, an aliquot of WIG was measured by the phenol-sulfate method [8,17]. A 10 μl amount of the purified GTase 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 h at 37 °C. After incubation, the mixture was centrifuged at 19,000 × *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 1M NaOH 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.

2.4. Preparation of aqueous two-phase solvent systems

Aqueous two-phase solvent systems were prepared by modifying the standard polymer phase systems used for the protein separation [23,31]. The PEG 8000-dextran T500 systems were prepared by dissolving 44 g of PEG 8000 and 60 g of dextran T500 in 10 mM potassium phosphate buffer to make the total mass of 1000 g. The pH of this solvent system was adjusted to the desired value between 4.6 and 9.2. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature. The two phases, consisting of the PEG-rich upper phase (UP) and the dextran-rich lower phase (LP), were separated shortly before use. The % volumes of UP and LP were about 55 and 45%, respectively.

2.5. Measurement of partition coefficient of SM cell-lysate in the two-phase system

The partition coefficient of SM cell-lysate (K_{lysate}) was determined spectrophotometrically by a simple test tube procedure. In each measurement, 2.25 ml of each phase of the equilibrated PEG-dextran two-phase system was delivered into a test tube, to which 0.5 ml of SM cell-lysate, 22 mg of PEG and 30 mg of dextran were added. The contents were thoroughly mixed and centrifuged at 3300 × *g* for 5 min at 4 °C. After two clear layers were formed, an aliquot (usually 0.2 ml) of each phase was diluted 10-fold with distilled water and the absorbance was measured at 220 nm with a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan).

$$K_{lysate} = \frac{A_{220}\ \text{of UP}}{A_{220}\ \text{of LP}} \quad (1)$$

K_{lysate} means that the partition coefficient of total UV absorbent containing protein in SM cell-lysate.

On the other hand, the partition coefficient of GTF in the SM cell-lysate (K_{GTF}) was determined using a different method: after partitioning the SM cell-lysate in the PEG-dextran two-phase system under the above described condition, the GTF activity of each phase was measured by its enzymatic assay (see, the paragraph of GTF enzymatic assay described in this section). The GTase activity of each phase was expressed as the nephelometric absorbance of WIG at 550 nm (A_{550}) produced from sucrose in this assay solution. Then the partition coefficient (K_{GTF}) was calculated from the following equation:

$$K_{GTF} = \frac{\text{GTase activity of UP}}{\text{GTase activity of LP}} = \frac{A_{550}\ \text{of UP assay solution}}{A_{550}\ \text{of LP assay solution}} \quad (2)$$

2.6. CCC apparatus

CCC separation of the SM cell-lysate was performed using a type-XL cross-axis coil planet centrifuge. The apparatus holds a pair of horizontal rotary shafts that are symmetrically mounted one on each side of rotary frame, at a distance of 10 cm from the central axis of the centrifuge. A spool-shaped column holder is coaxially mounted on each rotary shaft at a lateral location 10 cm away from the midpoint. A large multilayer coil was prepared from 2.6 mm i.d. polytetrafluoroethylene (PTFE) tubing by winding it onto a 5 cm diameter holder hub making three layers of left-handed coils between a pair of flanges spaced 5 cm apart. A pair of columns was serially connected on the rotary frame using a flow tube (PTFE, 0.85 mm i.d.) to give a total column capacity of 145 ml. The revolution speed of the apparatus was regulated at 400 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

2.7. CCC separation of SM cell-lysate

In each separation, the multilayer coil separation column of X-axis CPC was first entirely filled with the dextran-rich lower stationary phase. Then the column was rotated at 400 rpm while the PEG-rich upper mobile phase was pumped into the column by an EYELA LP-1100 pump (Tokyo Rikakikai, Tokyo, Japan) at a flow-rate of 1.0 ml/min on head to tail elution mode (P_{II}-H-I) [32]. After the hydrodynamic mixing between the two phases reached an equilibrium in the column, 10 g of SM cell-lysate containing 0.44 g of PEG 8000 and 0.6 g of dextran T500 was injected into the column using an EYELA SV-6000 sample injector (Tokyo Rikakikai). The eluate from the column was continuously monitored at 220 nm with an EYELA UV 9000 absorbance monitor (Tokyo Rikakikai) and fractionated into test tubes (3 ml per tube) using a 2112 Redirac fraction collector (LKB Instruments, Bromma/Stockholm, Sweden). After elution of the impurities in the cell-lysate (75 frs.), the apparatus was stopped and the column contents were fractionated by pushing with air using an EYELA SMP-23 cassette tube pump (Tokyo Rikakikai). Finally, an aliquot of each CCC fraction was diluted with distilled water and the absorbance was measured at 220 nm with a Shimadzu UV-1200 spectrophotometer. The GTF activity of each fraction was also measured by enzymatic assay.

2.8. Hydroxyapatite chromatography of CCC fractions

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, USA, crystal size 10–250 μm) was suspended with 100 mM potassium phosphate buffer (pH 7.0) and, after swelling, slurry-packed in the column (25 cm × 2 cm i.d., 78.5 ml). The CCC fraction containing GTF was diluted 10-fold with 100 mM potassium phosphate buffer at pH 7.0. The diluted fraction was loaded on the HA column and eluted stepwise with 250 and 500 mM of potassium phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. The eluate from the column was continuously monitored at 220 nm and fractionated into test tubes (3 ml per tube).

2.9. Analysis of HA chromatography fractions

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

Protein profiles of the purified GTF fractions were characterized by sodium 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, USA). A 1.0 ml amount of the test solution was mixed with 1.0 ml of the Coomassie Reagent in a test tube. After thorough mixing of each tube, each mixture was measured by the absorbance at 595 nm using a V-530 UV-Vis spectrophotometer (JASCO). The calibration curve of the concentration of standard bovine serum albumin versus the absorbance at 595 nm was constructed to quantify the contents of proteins in the fractions.

3. Results and discussion

3.1. Partition coefficients of SM cell-lysate in the polymer phase system

CCC is a liquid–liquid partition method where the separation is based on the difference in partition coefficient of solutes. For achieving efficient separation of proteins, it is essential to optimize the partition coefficient of each component by selecting the proper composition of the polymer phase system. In the PEG-phosphate buffer two-phase systems, some proteins in the cell-lysate are precipitated due to a high concentration of the potassium phosphate buffers. On the other hand, the PEG-dextran polymer phase systems provide high solubility for various macromolecules that would be precipitated in the PEG-phosphate buffer systems.

Partition coefficients of the SM cell-lysate were examined in aqueous polymer two-phase systems of both 16% (w/w) PEG 1000-12.5% (w/w) potassium phosphate system and 4.4% (w/w) PEG 8000-6.0% (w/w) dextran T500 system. The results showed that GTF and some other protein components in the lysate were precipitated in the PEG-phosphate system, whereas proteins were completely dissolved in the PEG-dextran systems which were then used for the determination of the partition coefficient of the cell-lysate and GTF. K_{lysate} and K_{GTF} (see Eqs. (1) and (2), respectively) were individually measured in the 4.4% (w/w) PEG 8000-6.0% (w/w) dextran T500 systems buffered with 10 mM phosphate at various pH values ranging from 4.6 to 9.2. Table 1 lists the partition coefficient of each component. As the pH is increased from 4.6 to 9.2, the K_{lysate} values are sharply increased from 0.18 to 1.89, whereas K_{GTF} values measured at different pHs are below 0.1 indicating that GTF is almost entirely distributed to the dextran-rich lower phase regardless of the pH of the polymer phase system. This strongly suggests that the unilateral distribution of GTF to the lower phase is caused by a strong affinity between the dextran-binding domain of GTF and dextran molecules in lower phase. The maximum difference between K_{lysate} and K_{GTF} was found at pH 9.2 in this PEG 8000-dextran T500 two-phase system (K_{lysate} and K_{GTF} were 1.89 and 0.05, respectively). However, the difference between both K values is too small for one-step extraction of GTF from SM lysate using this solvent system with a good recovery rate. For an effective purification of GTF, either the extraction repeated with several times or one run of CCC separation may be necessary. In the present study, we carried out the purification of GTF from the SM lysate by CCC because of its high efficiency of separation and concentration. PEG 8000 (4.4%)-dextran T10 (6%) containing 10 mM phosphate buffer (pH 9.2) was used as aqueous polymer two-phase system on this CCC separation, and the dextran-rich lower phase was used as stationary phase. For keeping the optimum condition of viscous stationary phase retention in PTFE tubing, X-axis CPC was needed and used as centrifugal unit on this CCC separation system.

Table 1
Partition coefficients of SM cell-lysate and GTF in phosphate buffered PEG 8000-dextran T500 two-phase systems

pH of buffer in two-phase system[a]	K_{lysate}[b]	K_{GTF}[c]
9.2	1.89	0.05
8.0	1.82	0.08
7.0	0.88	0.03
6.0	0.38	0.02
4.6	0.18	<0.01

[a] Two-phase system: 4.4% PEG 8000 and 6% dextran T500 containing 10 mM potassium phosphate buffer.
[b] K_{lysate}: partition coefficient of SM cell-lysate (see, Eq. (1) in Method section) in the PEG-dextran two-phase systems.
[c] K_{GTF}: partition coefficient of GTF activity of the cell-lysate (see, Eq. (2) in Method section) in the PEG-dextran two-phase systems.

3.2. Purification of GTF from SM cell-lysate using CCC and HA chromatography

Fig. 1 shows the CCC chromatogram where solid circles indicate the absorbance at 220 nm in the eluted upper phase and open circles indicate those in the stationary lower phase of the fractionated column contents. The large peak with a front shoulder eluted from frs. 35 to 75 (105–225 ml retention volume) shows no GTF enzyme activity, indicating that it contains almost all protein components other than GTF in the cell-lysate. The bar graphs in the chromatogram indicate the GTF activities which are, as expected from their K values (Table 1), solely found in frs. 115–125 (345–375 ml retention volume) obtained from the column contents. These results indicate that the effective separation of GTF from the bacterial cell-lysate was achieved by this CCC method.

As described above, GTF in the CCC fractions is readily recovered from the PTFE column whereas the strong affinity of GTF to dextran is a severe drawback in gel-filtration chromatography using Sephadex beads as a solid support [19]. However, these CCC fractions contain high concentrations of high dextran T500 which cannot be easily removed

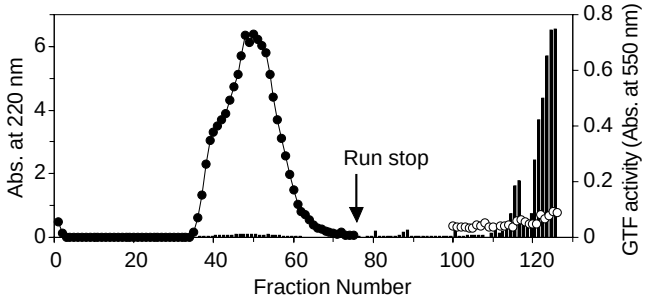

Fig. 1. Separation profile of *Streptococcus mutans* (SM) cell-lysate by CCC using the X-axis CPC. CCC conditions—column: a pair of 2.6 mm i.d. PTFE multilayer coils with a total capacity of 145 ml; sample: 10 g of SM cell-lysate containing 0.44 g of PEG 8000 and 0.6 g of dextran T500; solvent system: 4.4% (w/w) PEG 8000 and 6.0% (w/w) dextran T500 containing 10 mM phosphate buffer (9.2); mobile phase: PEG-rich upper phase; flow rate: 1.0 ml/min; revolution: 400 rpm; fractionation: 3.0 ml per tube. Following the elution of mobile phase for 225 min (i.e. up to fr. 75), dextran-rich stationary phase was pushed out from the column by air pressure and fractionated from #76 to #125. Symbols: solid circle (●): absorbance at 220 nm (A_{220}) of upper mobile phase; opened circle (○): A_{220} of lower stationary phase; black bar: GTF activity (A_{550}) of each fraction.

by dialysis or ultrafiltration. Therefore, a HA chromatography was complementarily used for the elimination of these polymers from the CCC fraction. Several studies have been reported [10,14,18] that GTF strongly adsorbed to HA beads can be recovered by eluting the column with a high concentration of phosphate buffer (e.g. higher than 400 mM) without a serious loss of enzymatic activity.

Fig. 2 shows an HA chromatogram of CCC fractions containing GTF. The CCC fraction was diluted 10-fold with 100 mM potassium phosphate buffer (pH 7.0), which was loaded onto the HA column (25 cm × 2 cm i.d.). Then, eluting the column with 250 mM potassium phosphate washed out all polymers while leaving the GTF strongly adsorbed to the HA column at this ionic strength of potassium phosphate. After all polymers are washed out from the column, the GTF adsorbed on the HA column was recovered by eluting with 500 mM potassium phosphate buffer at pH 7.0 (frs. 65–90) while maintaining their enzymatic activities. These GTF fractions were dialyzed, concentrated, and examined by electrophoresis.

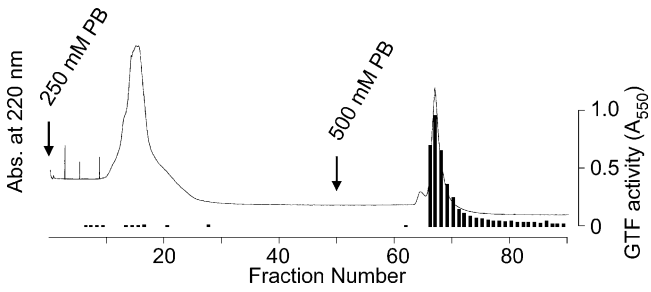

Fig. 2. Purification of GTF from CCC fraction by HA chromatography. Chromatographic conditions: column: Bio-Gel HTP DNA grade hydroxyapatite (25 cm × 2 cm i.d.); sample: 10 fold diluted CCC fraction corresponding to frs. 115–125 on Fig. 1; mobile phase: stepwise elution with 250 and 500 mM potassium phosphate buffers at pH 7.0; flow rate: 1.0 ml/min; detection: UV absorbance at 220 nm; fractionation: 3.0 ml per tube. GTF activity (A_{550}) of each fraction is indicated by black bars.

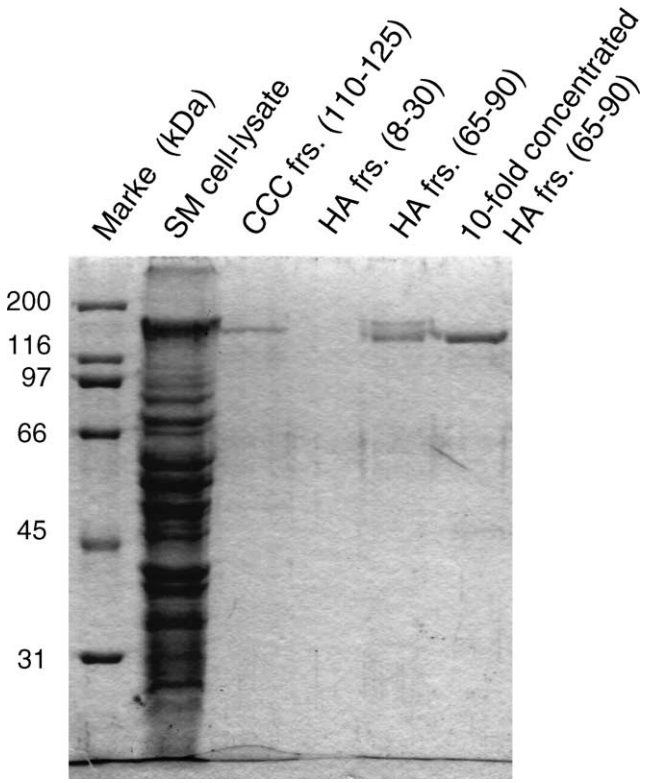

Fig. 3. SDS–10% polyacrylamide gel electrophoretic profile of CCC and HA fractions.

Fig. 3 shows SDS–PAGE profiles of the SM cell-lysate and CCC fractions before and after separation with the HA column. GTF appears as a band corresponding to its molecular mass of about 150 kDa on the gel. The lane of CCC fractions (frs. 110–125) proved that GTF was clearly separated from the other proteins in cell-lysate by CCC in the one-step operation. In the lanes of HA fractions, GTF was only detected in the lane of frs. 65–90 corresponding to the eluate with 500 mM phosphate buffer on the HA chromatogram shown in Fig. 2. On the HA chromatogram, the eluate with 250 mM phosphate (HA frs. 8–30) showed strong UV absorption (A_{220}) without a visible band on the SDS–PAGE gel in Fig. 3. This result may suggest that protein-free impurities such as DNAs, peptides, lipids, etc. are present in these fractions. Finally, the concentrated HA fractions eluted in frs. 65–90 showed a single band of GTF on the gel. The results of the SDS–PAGE analysis (Fig. 3) clearly indicate that the GTF was completely isolated from the SM cell-lysate.

Table 2 shows the purity and recovery rate of GTF in each fraction at two purification steps before and after HA chromatography. Through the GTF purification process, the purity of GTF was markedly increased while maintaining a reasonable recovery rate. In the final product the GTF purity in the concentrated HA fractions was increased about 87

Table 2
Specificity and recovery of GTF purification from SM cell-lysate

GTF fraction (volume)	Total protein (mg)	Total GTF activity (mU)	Specific activity (mU/mg)[a]	Recovery (%)[b]	Purity (fold)[c]
SM cell-lysate (10 ml)	11.350	3.302	0.291	100	1
Fraction after CCC (25 ml)	0.457	2.845	6.23	86	21.4
Fraction after HA (5 ml)	0.103	2.604	25.3	79	86.9

[a] Specific activity was expressed in the ratio of total GTF activity (mU) per total protein (mg).
[b] Recovery of GTF was expressed in the % ratio of the total GTF activity of each fraction per that of SM cell-lysate.
[c] Purification efficient was expressed in the ratio of the specific activity of each fraction per that of SM cell-lysate.

times as that in the cell-lysate with a good recovery rate of about 79% through this two-step purification process.

Among many previous trials of GTF purification, a few reports listed the data of purification efficiency on each separation step. In one case of extracellular GTF purification from SM culture supernatant [17], the GTF purity was increased about 10,000 times but the recovery rate was decreased to 3.8% through the five purification steps which were first EtOH precipitation and following four kinds of column chromatography. In another case of purification of *gtfC* gene product from *E. coli* cells [12], the GTF purity was increase about 25 times with a severe recovery rate of about 0.5% through the four purification steps which were first precipitation with ammonium sulfate, following two kinds of column chromatography and final ultracentrifugation. Compared with their data, it is clearly shown that our method kept on recovering the GTF with high rate during the purification process. We assume that the excellent results of GTF purification in the present method is attained by the combined use of support-free CCC system, high solubility of proteins in the PEG-dextran system, and a strong affinity between GTF and dextran. The present method will yield much larger amounts of purified GTF products if combined with an efficient expression system of recombinant GTF described earlier [11–16]. Production of a large amount of purified GTF will contribute to an advanced research on human dental caries and its prevention. We also think that our CCC method may be effectively used for purification of other proteins in biological fluids with a slight modification.

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