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# Purification of serine hydroxymethyltransferase from *Bacillus stearothermophilus* with ion-exchange high-performance liquid chromatography

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ABSTRACT

The gene of serine hydroxymethyltransferase (SHMT) of a thermophilic bacterium *Bacillus stearothermophilus* was expressed in *Escherichia coli*, and SHMT was successfully purified from the crude extract of *E. coli* in two steps while maintaining the enzymatic activity. The purification steps involved ammonium sulphate precipitation followed by high-performance liquid chromatographic separation using the anion-exchange column Fractogel EMD DEAE-650(S). In addition to the DEAE column, three other types of anion- and cation-exchange columns were also studied for their ability to separate SHMT, and the performances of the four columns were compared.

## INTRODUCTION

L-Serine is a key starting material for the enzymatic synthesis of a series of industrially important amino acids such as L-tryptophan, L-tyrosine and L-cysteine, and for the synthesis of manu pharmaceuticals [1–4]. Accordingly, the production of L-serine at low cost is indispensable for successful applications of the synthetic methods. Amino acids are generally synthesized by a chemical, fermentation or enzymatic method. The enzymatic method has the advantage that optically pure amino acids can be specifically produced in a short time. Furthermore, the fact that some of the key enzymes responsible for the amino acid synthesis are made available in fairly large amounts using recombinant DNA technology has made the enzymatic method more attractive.

Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) catalyses the interconversion of glycine and L-serine (reaction, Fig. 1) [5,6]. The physiological role of SHMT is to transfer the $\beta$-carbon of L-serine

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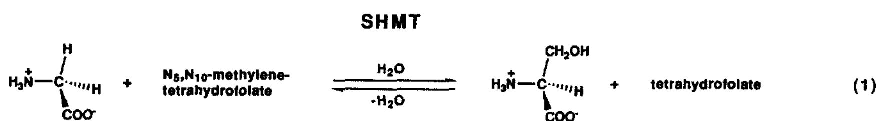


Fig. 1. Interconversion of glycine and L-serine catalysed by serine hydroxymethyltransferase (SHMT) (reaction 1).

to tetrahydrofolate, thereby providing the biosynthesis of amino acids and nucleic acids in cells with one-carbon units. However, reaction 1 catalysed by SHMT is reversible, and glycine can be stereospecifically converted to L-serine using the backward reaction of reactin 1. A multicopy plasmid carrying the *Escherichia coli glyA* gene encoding SHMT has been placed in a *Klebsiella aeronenes* strain and SHMT obtained from the strain has been used to produce L-serine [7,8]. Recently, the SHMT gene of a thermophilic bacterium *Bacillus stearothermophilus* has been cloned and expressed in *E. coli* [9]. The use of thermostable SHMT has the potential to make it possible to operate bioreactors for the production of L-serine at elevated temperatures, thereby avoiding possible contamination by sundry bacteria. However, the purification procedure for thermostable SHMT which is necessary for preliminary characterization of the enzyme before its application to the bioreactors has not been established.

In this paper, we report the application of high-performance liquid chromatography (HPLC) to the purification of SHMT of *B. stearothermophilus* from the crude extract of *E. coli* harbouring a plasmid encoding the SHMT gene. The enzyme can be purified to near homogeneity in two steps from the crude extract while maintaining the activity.

## EXPERIMENTAL

### *Expression of SHMT gene in E. coli cells*

Construction of an SHMT expression vector (pMS046) in which the SHMT gene of *B. stearothermophilus* was inserted in the *SmaI* site of pUC18 will be published elsewhere [9]. A host JM101 was transformed with pMS046 following the method of Sambrook *et al.* [10]. Transformed cells were grown in an LB medium containing ampicillin (50 $\mu$g/ml) with shaking at 37°C for 21 h. Cells were collected by centrifugation at 2400 *g* for 15 min. To confirm the expression of the SHMT gene in the transformant, proteins were analysed by sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) as follows: harvested cells ($\approx$ 1 mg) were resuspended in GTE buffer (50 $\mu$l) containing glucose (9 g/l), Tris (25 m*M*) and EDTA (10 m*M*) and disrupted by 0.2 *M* NaOH–1% SDS (50 $\mu$l). After centrifugation at 22 000 *g* for 20 min, the supernatant (50 $\mu$l) was analysed by SDS-PAGE.

For SDS-PAGE, samples were denatured by brief heating at 100°C in the presence of SDS and 2-mercaptoethanol and subjected to electrophoresis on a 12.5% stacking polyacrylamide gel. Electrophoresis was performed at a constant voltage (2 V/cm). Gels were stained by Coomassie brilliant blue, destained and photographed.

### *Purification*

*Preparation of crude enzyme.* Cells were grown and harvested as described above and resuspended in five volumes of a 1% glycine solution. The harvested cells were disrupted by sonication with an Astrason Model XL2020 ultrasonic processor (Heat Systems, Farmingdale, NY, USA) (output 475 W, 12 × 10 s with 20-s interval) at 0°C. Cell debris was removed by centrifugation at 16 000 *g* for 12 min and the supernatant recovered (fraction I).

*Ammonium sulphate precipitation.* Solid ammonium sulphate was added stepwise to fraction I (typically 50 ml) and precipitates recovered by centrifugation at 30, 50, 60 and 70% saturation, respectively. Each protein fraction obtained by the ammonium sulphate precipitation was dissolved in a 1% glycine solution (2 ml). The resulted solutions were assayed for activity and analysed by SDS-PAGE (see *Expression of SHMT gene in E. coli cells*). The fractions (30–60% saturation) containing SHMT were combined (fraction II).

*Ion-exchange HPLC.* Fraction II was purified by ion-exchange HPLC. Four types of Fractogel EMD ion-exchange columns (150 × 10 mm I.D., particle size 25–40 $\mu$m) (Merck, Darmstadt, Germany), including TMAE-650(S) (strong anion exchange),

DEAE-650(S) (weak anion exchange), $SO_3^-$-650(S) (strong cation exchange) and $COO^-$-650(S) (weak cation exchange) were tested for their ability to separate SHMT. HPLC separation was performed using a liquid chromatograph consisting of a Model L-6000 pump (Hitachi, Tokyo, Japan) and a Model L-3000 photodiode-array three-dimensional detector (Hitachi, Tokyo, Japan). Gradient elution was carried out at a flow-rate of 1 ml/min using 20 m*M* phosphate buffer (pH 7.5) (eluent A) and 20 m*M* phosphate buffer (pH 7.5) containing 1 *M* NaCl (eluent B). The gradient profiles used are shown in the figures by broken lines. All HPLC operations were performed at room temperature. SHMT purified by HPLC is designated as fraction II (for detail, see under results and discussion).

*Determination of activity*

SHMT catalyses not only the interconversion of glycine and L-serine (reaction 1 but also the conversion of β-phenylserine to benzaldehyde (reaction, Fig. 2) [11]. As benzaldehyde has a distinctive absorption band at 250 nm (ε = 15 000), reaction 2, catalysed by SHMT, can be easily followed by measuring the increase in absorbance at 250 nm. In a typical assay, the reaction mixture (2 ml) for the determination of the enzymatic activity contained an appropriately diluted enzyme solution (100 μl), β-phenylserine (0.5 m*M*), pyridoxal phosphate (PLP, 50 μ*M*) and phosphate buffer (125 m*M*, pH 7.5). Incubation was performed at 25°C for 15 min. UV spectra were taken using the same assay mixture as a reference except that the enzyme solution was omitted in the reference misture. One unit is defined as an activity that produces 1 μmol of benzaldehyde per min under these conditions.

The protein concentration was determined by the method of Lowry *et al.* [12]. UV spectra were measured on a DU-68 spectrometer (Beckman, Palo Alto, CA, USA) with a 50-μl microcell.

RESULTS AND DISCUSSION

SDS-PAGE analysis of proteins produced in *E. coli* cells was first performed to confirm the overproduction of SHMT. For JM101 transformed with pMS046 carrying the SHMT gene, a distinctively intense band was observed (Fig. 3, lane 4), but it was not present for JM101 without transformation or JM101 transformed with a control plasmid pUC19 carrying no insert (Fig. 3, lanes 2 and 3). The calculated molecular weight based on the migration distance was 45 000 (Fig. 4), which was very close to that reported for a subunit of *E. coli* SHMT (molecular weight 46 500 [13], 45 265 [14]), whose active form is a dimer consisting of two identical subunits. These results strongly suggest that the SHMT gene was expressed in the *E. coli* cells transformed with pMS046.

A crude extract (fraction I) was prepared from JM101/pMS046 and the enzymatic activity of fraction I was determined as described under Experimental. The amount of benzaldehyde fromed by SHMT increased linearly with time up to 15 min (data not shown). The specific activity of fraction I was 0.010 U/mg. Fraction I was treated with ammonium sulphate and precipitates were analysed by SDS-PAGE. SHMT was precipitated in the fractions with ammonium sulphate concentrations between 30 and 60% saturation. The precipitated crude SHMT in these fractions was dissolved in a 1% glycine solution and the fractions were combined (fraction II). The specific activity of fraction II was 0.012 U/mg.

For analytical purposes, fraction II was diluted ten-fold and 100 μl of the sample (0.114 mg as protein) were separated by HPLC equipped with a weak anion-exchange Fractogel EMD DEAE-650(S) column. Fig. 5 shows a typical chromatograph obtained. To analyse the major peaks 1–4 in the chromatogram, fraction II was applied to

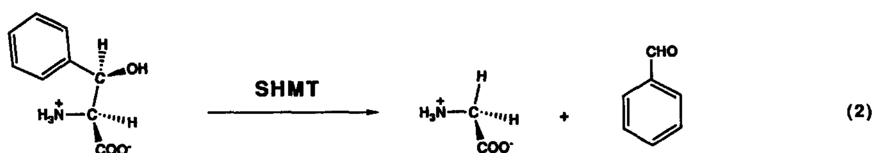


Fig. 2. Conversion of β-phenylserine to benzaldehyd catalysed by SHMT (reaction 2).

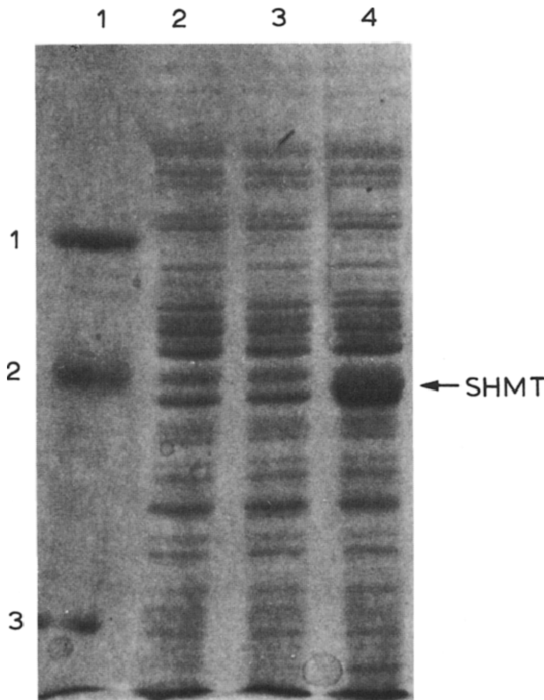


Fig. 3. SDS-PAGE analysis of proteins produced in *E. coli* cells. Lane 1, molecular weight marker [1, bovine serum albumin (molecular weight 66 000); 2, ovalbumin (45 000); 3, trypsinogen (24 000)]; lane 2, JM101; lane 3, JM101 transformed with a control plasmid pUC19; lane 4, JM101 transformed with PMS046 bearing the SHMT gene.

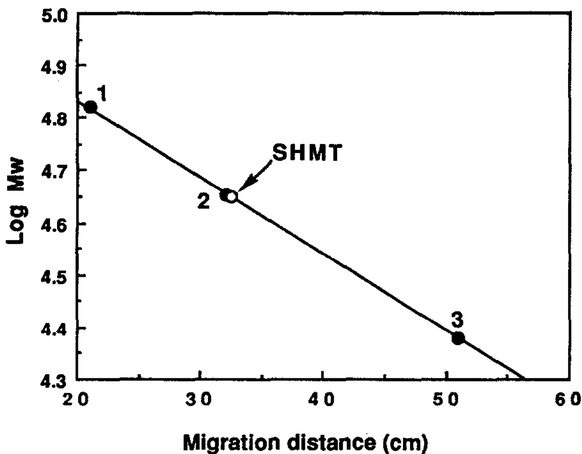


Fig. 4. Molecular weight determination of SHMT of *B. stearothermophilus*. The molecular weight of SHMT (open circle) was determined by SDS-PAGE on a 12.5% slab gel with reference proteins of known molecular weights (1, bovine serum albumin; 2, ovalbumin; 3, trypsinogen). Data points were taken from Fig. 3.

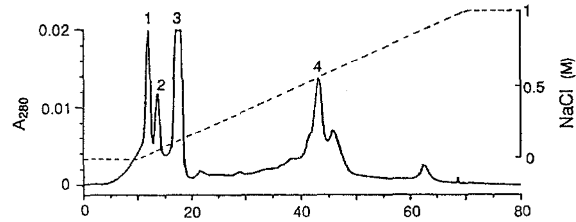


Fig. 5. HPLC analysis of crude SHMT. Fraction II obtained by ammonium sulfate precipitation was diluted ten-fold and 100 $\mu$l of the sample (0.114 mg as protein) were injected. Column, Fractogel EMD DEAE-650(S) (150 × 10 mm I.D.); eluent A, 20 m*M* phosphate buffere (pH 7.5); eluent B, 20 m*M* phosphate buffer (pH 7.5) containing 1 *M* NaCl; gradient, 0% B (0–10 min), 0–100% B (10–70 min), 100% B (70–80 min); flow-rate 1 ml/min.

HPLC system without dilution (100 $\mu$l, 1.14 mg as protein) and peaks 1–4 were collected. There was no significant change in an elution profile arising from the increase in the load of the sample (data not shown). When the activity of each peak was assayed, only peak 4 showed any activity. According to the colour development by Lowry's method, peaks 1–3 contained small amounts of proteins compared with peak 4 containing SHMT. For more efficient separtion of SHMT, fraction II was concentrated by ultrafiltration and 100 $\mu$l of the sample (4.3 mg as protein) were separated by HPLC using a slightly modified gradient (Fig. 6a). Eluted fractions were collected every minute and assayed for protein concentration and enzymatic activity. By comparison of the elution peaks of SHMT (peak 4 in Fig. 5 and the peak indicated by SHMT in Fig. 6a), it can be seen that the separation of the last half of peak 4 in Fig. 5 was improved with the modified gradient in Fig. 6a. It is also clear that the major contaminating species eluted between 5 and 20 min had strong UV absorption at 280 nm, but contained very small amounts of proteins (Fig. 6b). These results indicate that the DEAE column can efficiently remove contaminating components other than protein which are not detectable in SDS-PAGE analysis. The eluted fractions containing SHMT were pooled (fraction III). The specific acticity of fraction III was 0.031 U/mg.

When the UV spectrum of fraction III was measured, a weak absorption band was observed around 425 nm (Fig. 7). By comparison with the spectrum of authentic PLP, the absorption was as-

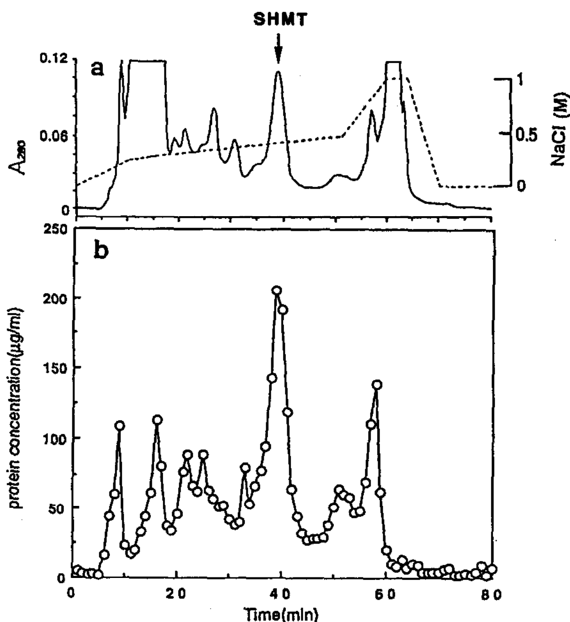


Fig. 6. HPLC elution profiles of crude SHMT monitored by (a) absorption at 280 nm and (b) protein concentration. Fraction II was concentrated by ultrafiltration and 100 μl of the sample (4.3 mg as protein) were injected. (b) Eluted fractions were collected every minute and assayed for protein concentration by the method of Lowry *et al.* [12]. Column, Fractogel EMD DEAE-650(S) (150 × 10 mm I.D.); eluent A, 20 m*M* phosphate buffer (pH 7.5); eluent B, 10 m*M* phosphate buffer (pH 7.5) containing 1 *M* NaCl; gradient 0–25% B (0–10 min), 25–50% B (10–50 min), 50–100% B (50–60 min), 100% B (60–70 min), 100–0% B (70–80 min); flow-rate 1 ml/min.

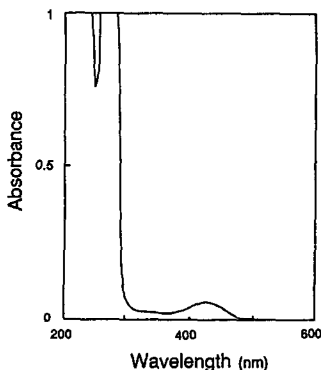


Fig. 7. UV spectrum of purified SHMT. SHMT purified with the DEAE column (fraction III) was concentrated by ultracentrifugation and the UV spectrum was measured.

signed to PLP bound to SHMT. It has been reported that SHMT purified from eukaryotic and prokaryotic cells contains PLP as a co-factor. The ratio of the adsorptions at 425 nm (PLP) and 280 nm (protein) determined for fraction III was 0.04, which was considerably smaller than those (0.11–0.23) reported for SHMT purified from various origins [15–18]. This implied that PLP was released from the SHMT holoenzyme during HPLC separation, and that purified SHMT in fraction III was present in part in the form of an apoprotein. Thus the apparent recovery of the SHMT activity after the HPLC separation was as low as 21% (Table I). To explore the possibility of reconstituting the enzymatic activity, fractions II and III were incubated with PLP (0.5 m*M*) at 42°C for 2 h, and the enzymatic activity was assayed. For fraction II obtained by the ammonium precipitation, no increase in the specific activity was observed after incubation (Table I). In contrast, the specific activity of fraction III obtained by the HPLC separation increased approximately fivefold, and the total activity of fraction III after reconstitution was virtually the same as that of the loaded sample. These results clearly indicate that the partial release of PLP from SHMT occurred during the HPLC separation; however, full enzymatic activity could be reconstituted by the post-reaction incubation with PLP. The interaction between the negatively charged phosphate group of PLP and the anion-exchange matrix in the DEAE column must be responsible for the release of PLP from the enzyme.

It has been also confirmed by SDS-PAGE analysis of fractions I–III that the HPLC purification with the DEAE column is a very effective step for the purification of SHMT (Fig. 8). Although a few minor bands were still noticeable in the SDS-PAGE analysis of fraction III, SHMT was purified to near homogeneity after the HPLC purification.

When SHMT was prepared by this method, the increase in the specific activity was at most fifteenfold: the specific activities of fractions I, II and III were 0.010, 0.012 and 0.147 (after reconstitution), respectively. This is because the specific activity of the crude extract (fraction I) was already high due to the overproduction of SHMT in *E. coli* cells bearing plasmid pMS046. However, the major contaminating components other than protein, as wel as proteins, were able to be efficiently removed by this method, as shown in Fig. 4.

TABLE I

PURIFICATION OF SHMT WITH FRACTOGEL EMD-DEAE-650(S) COLUMN

Fraction II concentrated by ultrafiltration (100 $\mu$l, 4.3 mg as protein) was separated on the DEAE column and the enzymatic acitvity was assayed as described under Experimental.

| Sample | Fraction | Total activity (U) | Recovery of activity (%) | Specific activity (U/mg) |
|---|---|---|---|---|
| Before HPLC | II | 0.052 | 100 | 0.012 |
| purification | II + PLP[a] | 0.048 | 92 | 0.011 |
| After HPLC | III | 0.011 | 21 | 0.031 |
| purification | III + PLP[a] | 0.051 | 98 | 0.147 |

[a] Fraction II or III incubated with PLP to reconstitute SHMT holoenzyme.

In addition to the weak anion-exchange column (described above), three other types of ion-exchange columns, including Fractogel EMD TMAE-650(S) (strong anion-exchange), $SO_3^-$-650(S) (strong cation exchange) and $COO^-$-650(S) (weak cation exchange) were tested for their ability to separate SHMT using fraction II (4.3 mg as protein). Elution peaks containing SHMT were identified by independent injections of marker SHMT purified by the DEAE column (fraction III). The elution profile for the strong anion-exchange column (TMAE) was similar to that for weak anion exchange column (DEAE) shown in Fig. 4, so that major contaminating specied were eluted in an early region of the gradient (retention time 7–15 min). Based on this result, it is concluded that the strong anion-exchange column (TMAE) can essentially separate SHMT as well as the weak anion-exchange column (DEAE). In contrast, the weak and strong cation-exchange columns did not separate SHMT as well as the anion-exchange columns. A typical elution profile obtained with the weak cation-exchange column [$COO^-$-650(S)] is shown in Fig. 9, and a similar profile was obtained for the strong cation-exchange column [$SO_3^-$-650(S)]. Most of the components present in the injected samples were eluted in the front regions of the gradient for both the cation-exchange columns. SHMT was found to be eluted in the first major peak (marked by an asterisk in Fig. 9) under these conditions. As the integrated areas of the first peaks containing SHMT were too large to account for SHMT alone, it is

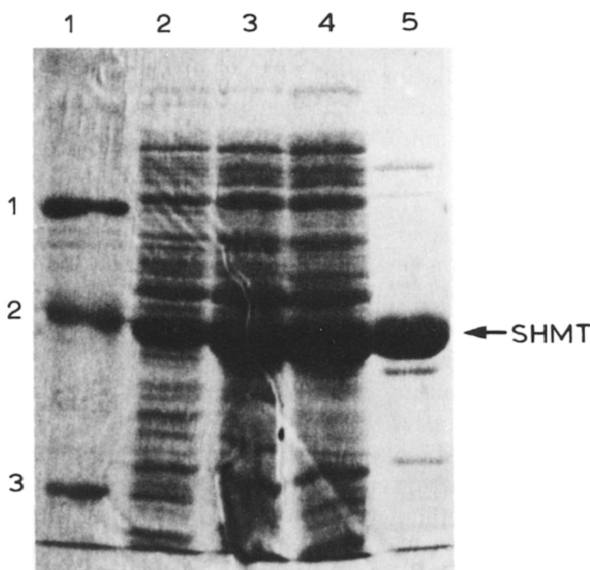


Fig. 8. SDS-PAGE analysis of fractions I–III. Lane 1, molecular weight marker (see Fig. 1 for numbers); lane 2, crude extract of JM101/pMS046 prepared by the alkaline SDS method (see under Experimental); lane 3, fraction I (crude extract); lane 4, fraction II (ammonium sulfate precipitate); lane 5, fraction III (HPLC purified fraction).

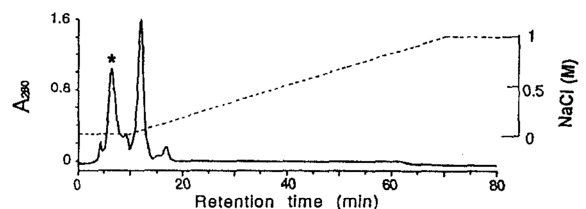


Fig. 9. Separation of crude SHMT by a weak cation-exchange column. Sample, concentrated fraction II (100 $\mu$l, 4.3 mg as protein); column, Fractogel EMD $COO^-$-650(S) (150 × 10 mm I.D.). Other HPLC conditions were as in Fig. 5. SHMT was eluted in the peak marked by an asterisk.

evident that contaminating components were co-eluted with SHMT with these columns.

In summary, the SHMT gene of *B. stearothermophilus* has been expressed in *E. coli*, and the enzyme has been efficiently purified from the crude extract by HPLC with a weak anion-exchange column [Fractogel EMD DEAE-650(S)]. Release of PLP bound to the SHMT holoenzyme as a co-factor occurred during the HPLC separation, but a full enzymatic activity was reconstituted by post-reaction incubation with PLP. With respect to the separating ability of SHMT, the performance of a strong anion-exchange TMAE column was comparable with the DEAE column, whereas those of cation-exchange $SO_3^-$ and $COO^-$ columns were not so satisfactory. In conventional methods, it takes many steps to purify SHMT from various origins [11,18–20]. The application of this method using HPLC coupled with the anion-exchange columns to the purification of SHMT should facilitate the purification process.

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