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PURIFICATION OF ENZYMES BY HIGH-SPEED GEL FILTRATION ON TSK-GEL SW COLUMNS

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SUMMARY

The purification of enzymes was investigated by high-speed gel filtration on TSK-GEL G3000SWG columns packed with porous silica gel deactivated by chemically bonded hydrophilic compounds. Crude β-galactosidase from bacterial cells and commercial urease were purified *ca.* 15-fold in a single gel filtration. These enzymes were eluted within an hour from the column and the recoveries of enzymatic activity were almost 100% although the operation was carried out at room temperature (22°). Samples up to 100 mg could be applied to the column without loss of separation efficiency.

INTRODUCTION

Since porous glass beads chemically bonded with hydrophilic compounds were developed a few years ago[1], high-speed gel filtration has become possible. Several brands of columns packed with similar types of materials are now commercially available. TSK-GEL SW (Toyo Soda, Tokyo, Japan) is one such column and it has reported that very high resolution can be attained with this column in analytical separations of proteins[2,3]. In this paper, the results of preparative separations of proteins on TSK-GEL SWG columns (SWG columns are preparative columns of large diameter) are described. The degree of purification, recovery and maximum sample loading are investigated.

EXPERIMENTAL

Determination of maximum sample loading

Maximum sample loading was determined using commercial bovine serum albumin as a sample. Gel filtration was performed on a Model HLC-802R (Toyo Soda) liquid chromatograph equipped with two G3000SWG columns (60 × 2.15 cm I.D.) at 22°. The eluent was 0.1 *M* phosphate buffer containing 0.3 *M* NaCl (pH 7). The flow-rates were 4, 8 and 16 ml/min. The detector was a differential refractometer.

Bovine serum albumin solutions (4 ml) of various concentrations were injected and the height equivalent to a theoretical plate (HETP) was measured.

Purification of crude β-galactosidase

Crude β-galactosidase was purified and the degree of purification and the recovery of enzymatic activity were investigated. The crude β-galactosidase was obtained as follows. Cultured bacteria washed with 1/15 *M* phosphate buffer containing 1 m*M* mercaptoethanol and 1 m*M* $MgCl_2$ (pH 6.5) were suspended in the same buffer and disrupted in a French press. Cell debris was removed by centrifugation and $(NH_4)_2SO_4$ was added to the supernatant to give 30% saturation. After the removal of the precipitate by centrifugation, more $(NH_4)_2SO_4$ was added to the supernatant to give 70% saturation. The precipitate isolated by centrifugation was used as a crude sample. Gel filtration was carried out at 22° on the HLC-802R equipped with a variable wavelength UV monitor. The column system consisting of two G3000SWG columns was employed. The separation range for this column system was 10,000–500,000 daltons for globular proteins, as can be seen from the calibration curve in Fig. 1. The eluent was 0.2 *M* phosphate buffer (pH = 6.7). The flow-rate was 5 ml/min and the pressure drop was 25 kg/cm². A 3-ml volume of 2.5% crude β-galactosidase solution (corresponding to 75 mg protein) was applied to the columns. The total protein concentration in the effluent was detected continuously with the UV monitor at 280 nm. Fractions of 5 ml each were collected and examined for β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside.

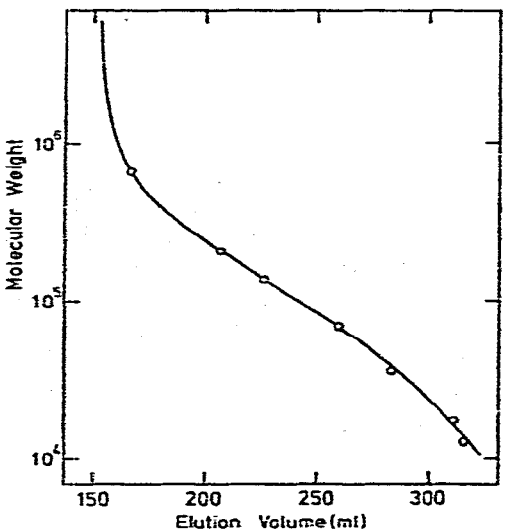

Fig. 1. Calibration curve of G3000SWG two-column system for proteins.

Purification of commercial urease

Commercial urease (from jack bean; P-L Biochemicals, Milwaukee, Wisc., U.S.A.) was purified under the same conditions as crude β-galactosidase, except that 3 ml of a 3% solution (corresponding to 90 mg protein) was applied to the columns. Urease was assayed for enzymatic activity by the colorimetric timing method[5].

Analytical gel filtration of fractions and crude samples

Analytical gel filtration was performed for fractions and original crude samples to examine the degree of purification. Measurements were carried out at 25° on the HLC-802R equipped with a variable wavelength UV monitor. Column systems consisting of two or three G3000SW columns were used. The eluent was 0.1 *M* phosphate buffer containing 0.3 *M* NaCl (pH = 7). The flow-rate was 1 ml/min and the injection volume was 0.2 ml. Detection was made by UV monitor at 220 nm.

Polyacrylamide gel electrophoresis of fractions and crude samples

Fractions and original crude samples were also subjected to electrophoresis to examine the degree of purification. Gels were stained with amido black. A band corresponding to β-galactosidase was identified by colouring the band by immersing another simultaneously obtained gel in the buffer containing *o*-nitrophenyl-β-D-galactopyranoside. A band corresponding to urease was also identified in the same way by using a buffer containing phenol red and urea.

RESULTS AND DISCUSSION

Maximum sample loading

Fig. 2 shows the dependence of HETP on the sample loading for bovine serum albumin at a flow-rate of 8 ml/min. It is evident that samples up to 100 mg could be applied without loss of separation efficiency. Almost the same results were obtained at flow-rates of 4 and 16 ml/min. Generally, the maximum sample loading depends on the sample, especially on its solution viscosity. However, the value of 100 mg obtained for bovine serum albumin may also be approximately valid for other protein samples since globular proteins have almost the same intrinsic viscosities.

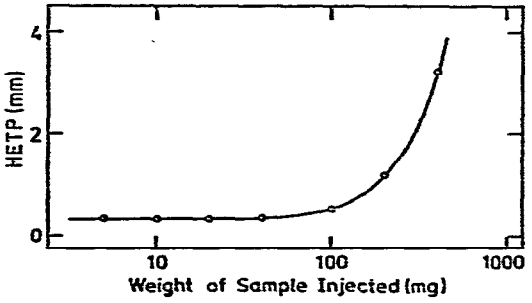

Fig. 2. Dependence of HETP on the sample loading for bovine serum albumin at a flow-rate of 8 ml/min on G3000SWG two-column system.

Purification of crude β-galactosidase

Fig. 3 shows the distributions of total proteins and β-galactosidase in the effluent. β-Galactosidase eluted at *ca.* 45 min. Of the applied β-galactosidase activity, 69% was found in two fractions, 88% in four fractions and the recovery was 93%. Therefore it seems reasonable to assume that there was no deactivation, although the

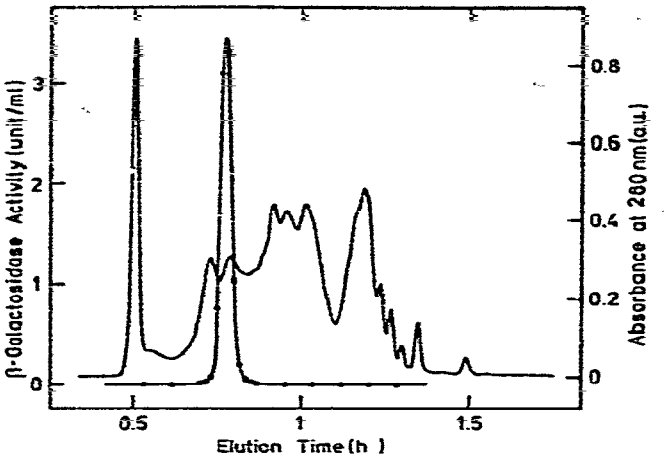

Fig. 3. Purification of crude β-galactosidase on G3000SWG two-column system. ——, Distribution of total proteins monitored by UV absorbance at 280 nm; ○—○, distribution of β-galactosidase monitored by enzymatic activity.

gel filtration was carried out at 22°. This is probably one of the advantages of high-speed gel filtration. The degrees of purification based on specific activity were, respectively, 16.2-fold and 10.5-fold in 69% and 88% yields as shown in Table I. Figs. 4 and 5 show chromatograms of the fraction containing the most β-galactosidase activity and of the crude sample obtained by analytical gel filtration. Only two minor peaks appeared after a major peak in the chromatogram of the fraction, while very

TABLE I

PURIFICATION OF CRUDE β-GALACTOSIDASE ON G3000SWG TWO-COLUMN SYSTEM

Yield (%)	*Degree of purification*
93*	1
88	10.5
69	16.2

* Total recovery.

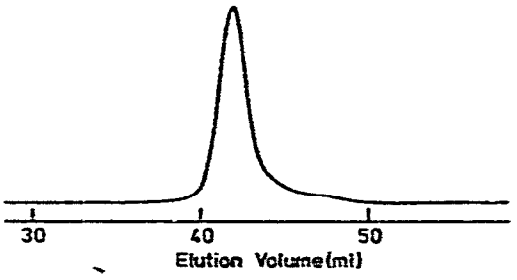

Fig. 4. Analytical chromatogram of the fraction containing the most β-galactosidase activity on G3000SW three-column system.

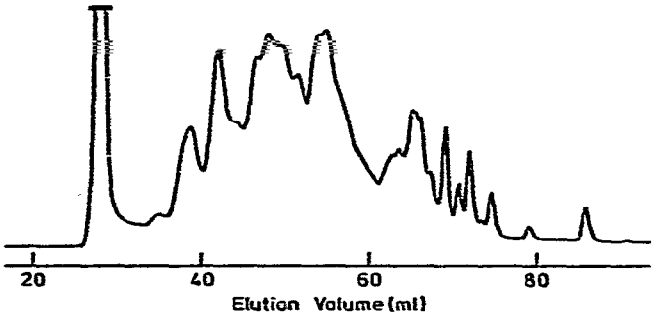

Fig. 5. Analytical chromatogram of the crude β-galactosidase on G3000SW three-column system.

many peaks appeared in the chromatogram of the crude sample. Fig. 6 shows the polyacrylamide gel electrophoresis pattern of the same fraction. One major and two minor bands were observed just as in analytical gel filtration. When another gel was immersed in the buffer containing *o*-nitrophenyl-β-D-galactopyranoside, the band at the position of the major band turned yellow, which shows that the major band corresponds to β-galactosidase. Therefore, it can be concluded that β-galactosidase was purified to a high degree by gel filtration and electrophoresis.

Fig. 6. Polyacrylamide gel electrophoresis pattern of the fraction containing the most β-galactosidase activity.

Purification of commercial urease

Fig. 7 shows the distributions of total proteins and urease in the effluent. Urease eluted at *ca.* 40 min. Of the applied urease activity, 78% was found in four fractions and 91% in six fractions. The recovery of urease activity was 103% on the

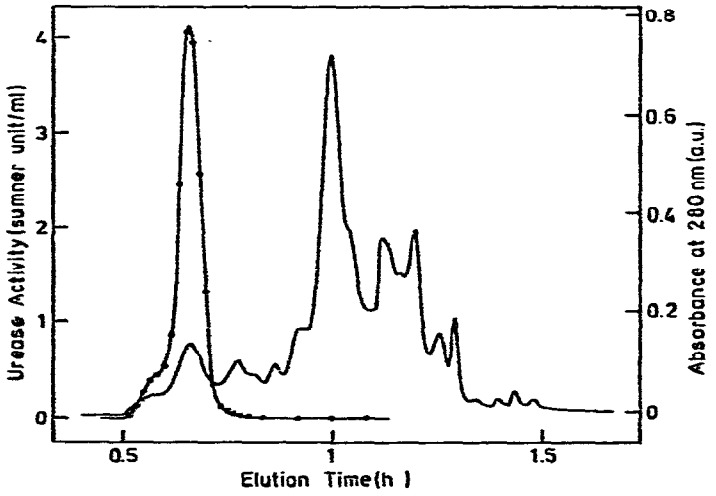

Fig. 7. Purification of commercial urease on G3000SWG two-column system. ——, Distribution of total proteins monitored by UV absorbance at 280 nm; O—O, distribution of urease monitored by enzymatic activity.

TABLE II

PURIFICATION OF COMMERCIAL UREASE ON G3000SWG TWO-COLUMN SYSTEM

Yield (%)	*Degree of purification*
103*	1
91	14.3
78	15.7

* Total recovery.

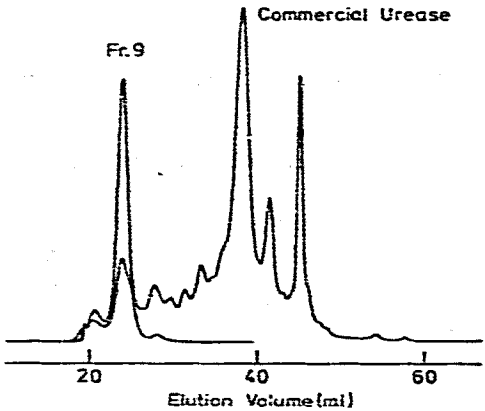

Fig. 8. Analytical chromatograms of the fraction containing the most urease activity (Fraction 9) and commercial urease on G3000SW two-column system.

Fig. 9. Polyacrylamide gel electrophoresis patterns of (A) the fraction containing the most urease activity and (B) commercial urease.

whole, in spite of the labile nature of urease in solution. This is probably due to the short time which the sample remained on the columns. The degrees of purification were, respectively, 15.7-fold and 14.3-fold in 78% and 91% yields as shown in Table II. Fig. 8 shows the chromatograms of the fraction containing the most urease activity (fraction 9) and of the crude sample obtained by analytical gel filtration. A few minor peaks were observed together with a major peak in the chromatogram of the fraction. Fig. 9 shows the polyacrylamide gel electrophoresis patterns of the same fraction and crude sample. Only one band was recognized in the pattern of the fraction, the position of which coincided with that of a pink band which appeared when another gel was immersed in phosphate buffer containing urea and phenol red. Thus, electrophoretically, almost homogeneous urease was obtained from commercial urease containing large amounts of impurities.

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