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Application of two types of CIM tube column for purification of microbial enzymes

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Abstract

Chromatography conditions for two types of convection interaction media (CIM) tube monolithic column, DEAE-8 and C4-8, were investigated using three enzymes from different microorganisms. The enzymes were adsorbed on a CIM DEAE-8 tube column under the same conditions as conventional DEAE columns. The CIM C4-8 tube column required a high concentration of ammonium sulfate compared to the conventional C_4 column for adsorbing the enzymes. The separation of enzymes on the CIM tube column chromatography was not affected at flow rates between 0.15 and 1.25 volumes of the column per min. Both columns were successfully applied to the purification of enzymes from crude enzyme solution. Thus, both CIM tube monolithic columns proved useful in greatly reducing the purification time, and could be used at any stage of enzyme purification.

Keywords: CIM DEAE-8 tube monolithic column; CIM C4-8 tube monolithic column; DEAE-Toyopearl; Butyl-Toyopearl

1. Introduction

We are currently using columns packing particulate materials for purification of proteins such as enzymes. However, column chromatography with these particulate materials has some limitations in terms of separation efficiency and flow rate. Recently, monolithic columns consisting of a single piece of porous materials were developed as new types of column [1,2]. They had a much higher porosity than conventional columns with particulate materials, and exhibited the advantages of reducing backpressure generated by flow and of offering high flow rates [3,4]. Their optimum separation conditions were studied using model materials [5–7], and it was revealed that the shortest separation time was achieved using convection interaction media (CIM) monolithic columns. Until now, CIM monolithic columns have been used for separation of organic compounds, pharmaceuticals, low-molecular-mass biomolecules and large molecules [8–12]. However, these applications were mainly performed using CIM monolithic disc columns (0.3 cm × 1.6 cm diameter) at the final step of purification or for analysis of the purified materials. When we find new enzymes from microorganisms, we must purify a certain amount of them in active form as fast as possible in order to elucidate not only their physicochemical properties, but also their enzymatic and kinetic properties. For the purpose of reducing the purification time, it is preferred that CIM tube monolithic columns (4.5 cm × 1.5 cm diameter) are used from the first stage of the purification.

In the present study, we first investigated the general conditions for chromatography with CIM DEAE-8 and C4-8 tube monolithic columns using partially purified enzyme and crude enzyme solutions. Then we applied the resulting conditions of both columns to the purification of a new enzyme from a crude enzyme solution prepared by disrupting microbial cells.

2. Experimental

2.1. Chemicals and columns

Pyruvic acid, 2-propanol, β-NADH and β-NAD^+ were purchased from Wako (Osaka, Japan). All other chemicals

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used were of analytical grade and commercially available. CIM DEAE-8 tube and CIM C4-8 tube monolithic columns were purchased from BIA Separations (Ljubljana, Slovenia), while DEAE-Toyopearl and Butyl-Toyopearl were from Tosoh (Tokyo, Japan).

2.2. Enzyme preparation

D-Lactate dehydrogenase (EC 1.1.1.28, D-LDH) was obtained from Amano Enzyme (Nagoya, Japan). A crude enzyme solution of secondary alcohol dehydrogenase (S-ADH) was prepared from *Burkholderia* sp. AIU 652 by disrupting their cells according to the method of Isobe and Wakao [13]. A crude enzyme solution of alcohol oxidase (AOD) was prepared as follows. The cells were disrupted with glass beads by a Multi-bead shocker (Yasui Kikai, Osaka, Japan) at 4 °C for 4 min, and the cell debris was discarded by centrifugation. The supernatant obtained was used as the crude enzyme solution of alcohol oxidase.

2.3. Enzyme assay

D-LDH activity was spectrophotometrically assayed by measuring the formation rate of NADH at 340 nm according to the method of Isobe et al. [14]. S-ADH activity was assayed by measuring the formation rate of NADH at 340 and 550 nm according to the method of Isobe and Wakao [13] and following method, respectively. The reaction mixture (0.25 ml) was composed of 0.1% 2-propanol and a color reagent consisting of 2.0 mg of NAD^+, 0.3 mg of nitro blue tetrazolium and 2.0 units of diaphorase (EC 1.8.1.4) per ml of 0.1 M potassium phosphate, pH 8.5, containing 0.05% Triton X-100. The reaction was started by the addition of 50 μl of enzyme solution, and incubated at 30 °C for 10 min. The formation of NADH was measured at 550 nm. AOD activity was assayed by measuring the formation rate of hydrogen peroxide at 30 °C as follows. The reaction mixture (0.95 ml) was composed of 1.7% ethanol and a color reagent consisting of 0.122 mg of 4-aminoantipyrine, 0.643 mg of TOOS and 6.7 units of peroxidase per ml of 0.1 M potassium phosphate, pH 7.0. The reaction was started by the addition of 50 μl of enzyme solution, and the formation of hydrogen peroxide was followed at 30 °C for 5 min by measuring the absorbance at 555 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of NADH or hydrogen peroxide per min under the above conditions.

2.4. Standard conditions of column chromatography

A CIM DEAE-8 tube monolithic column (4.5 cm × 1.5 cm diameter) and DEAE-Toyopearl column (10 cm × 1.4 cm diameter) were used for ion-exchange column chromatography. The same size of C4-8 tube monolithic column and Butyl-Toyopearl column were used for hydrophobic column chromatography. All chromatography was carried out below 10 °C using potassium phosphate buffer, pH 7.0, unless otherwise stated. The protein concentration of chromatography was measured at 280 nm by a Shimadzu UV 2450 spectrophotometer (Kyoto, Japan). The conductivity was measured by a Horiba conductivity meter DS-12 (Kyoto, Japan).

2.4.1. Ion-exchange column chromatography

A CIM DEAE-8 tube monolithic column was equilibrated with 10 mM buffer (1.8–2.0 ms/cm of conductivity), and an enzyme solution deionized to 2.0 ms/cm of conductivity was applied to the column. After the column was washed with 40 ml of 20 mM buffer, the adsorbed enzyme was eluted by a linear gradient with 20 mM buffer and 20 mM buffer containing 0.3 M NaCl (40 ml each), followed by 20 mM buffer containing 0.3 M NaCl at a flow rate of 1.5 ml/min. The same enzyme solutions were applied to a DEAE-Toyopearl column equilibrated with 10 mM buffer, and the adsorbed enzymes were eluted by a linear gradient with the same eluting solution (100 ml each) as CIM DEAE-8 column, followed by 20 mM buffer containing 0.3 M NaCl at a flow rate of 0.2 ml/min.

2.4.2. Hydrophobic column chromatography

A CIM C4-8 tube monolithic column was equilibrated with 10 mM buffer containing 1.0 or 1.5 M ammonium sulfate (their conductivities were 115–120 ms/cm and 170–175 ms/cm, respectively). Enzyme solution adjusted to 120 or 170 mS/cm of conductivity with ammonium sulfate were applied to the above CIM C4-8 tube monolithic column, and the adsorbed enzyme was eluted by a linear gradient with 10 mM buffer containing 1.0 or 1.5 M ammonium sulfate and 10 mM buffer containing 0.5 M ammonium sulfate (40 ml each) at a flow rate of 1.6 ml/min. The same enzyme solutions were applied to a Butyl-Toyopearl column (10 cm × 1.4 cm) equilibrated with 10 mM buffer containing 1.0 or 1.5 M ammonium sulfate, and the adsorbed enzymes were eluted by a linear gradient with the same eluting solution (75 ml each) as CIM C4-8 column at a flow rate of 0.18 ml/min.

2.5. Purification of S-ADH with CIM C4-8 and DEAE-8 tube monolithic columns

All preparations were carried out below 10 °C using potassium phosphate buffer, pH 7.0, unless otherwise stated.

2.5.1. Preparation of crude enzyme solution

Cells of 100 ml culture of *Burkholderia* sp. AIU 652 were disrupted with glass beads in 10 mM buffer, and the supernatant (50 ml) was collected by centrifugation at 10 000 rpm for 10 min. Then ammonium sulfate (10.45 g) was added at 35% saturation to the supernatant, and the precipitates thus formed were discarded by centrifugation at 10 000 rpm for 10 min.

2.5.2. CIM C4-8 tube monolithic column chromatography

A half volume of the supernatant was applied to a CIM C4-8 column equilibrated with 10 mM buffer containing 1.5 M ammonium sulfate at a flow rate of 1.5 ml/min. After the column was washed with 50 ml of 10 mM buffer containing 1.5 M ammonium sulfate, adsorbed S-ADH was eluted by a linear gradient with 10 mM buffer containing 1.5 M ammonium sulfate and 10 mM buffer containing 0.5 M ammonium sulfate (40 ml each), and the active fractions were collected. Another half volume of the enzyme solution was chromatographed under the same conditions, and active fractions of both chromatographies were combined, and deionized to 2.0 ms/cm by ultrafiltration.

2.5.3. CIM DEAE-8 tube monolithic column chromatography

The deionized enzyme solution was applied to a CIM DEAE-8 column equilibrated with 10 mM buffer, and the column was washed with 50 ml of 20 mM buffer. The adsorbed enzyme was eluted by a linear gradient with 20 mM buffer and 0.3 M NaCl (40 ml each) at a flow rate of 2.5 ml/min. The active fractions were collected and deionized by ultrafiltration.

2.5.4. Blue-Sepharose column chromatography

The deionized enzyme solution was applied to a Blue-Sepharose column (6 cm × 1.4 cm) equilibrated with 10 mM buffer, and the column was washed with 50 ml of 0.1 M buffer. The adsorbed enzyme was eluted by a linear gradient of 0.1 M buffer and 0.1 M buffer containing 10 mM NAD^+ (50 ml each) at a flow rate of 0.2 ml/min, and the active fractions were deionized to 1.5 mS/cm of conductivity.

2.5.5. Hydroxyapatite column chromatography

The deionized enzyme solution was applied to a hydroxyapatite column (6 cm × 1.4 cm) equilibrated with 10 mM buffer, and the column was washed with 50 ml of 10 mM buffer. S-ADH was eluted by a linear gradient with 10 mM buffer and 0.1 M buffer (50 ml each) at a flow rate of 0.1 ml/min, and the active fractions were concentrated by ultrafiltration.

2.5.6. Gel filtration on Toyopearl HW 55 column

The concentrated enzyme solution was applied to a Toyopearl HW 55 column (50 cm × 1.0 cm) equilibrated with 20 mM phosphate buffer. The active fractions were collected and concentrated by ultrafiltration.

3. Results and discussion

3.1. Chromatography conditions for CIM DEAE-8 tube monolithic column

The chromatography conditions for a CIM DEAE-8 tube monolithic column were investigated using a partially purified enzyme solution of S-ADH. When 30 ml of that solution was applied to a CIM DEAE-8 tube monolithic column equilibrated with 10 mM buffer, S-ADH was completely adsorbed on the column, and the enzyme peak was eluted at around 10 ms/cm of conductivity. In the DEAE-Toyopearl column

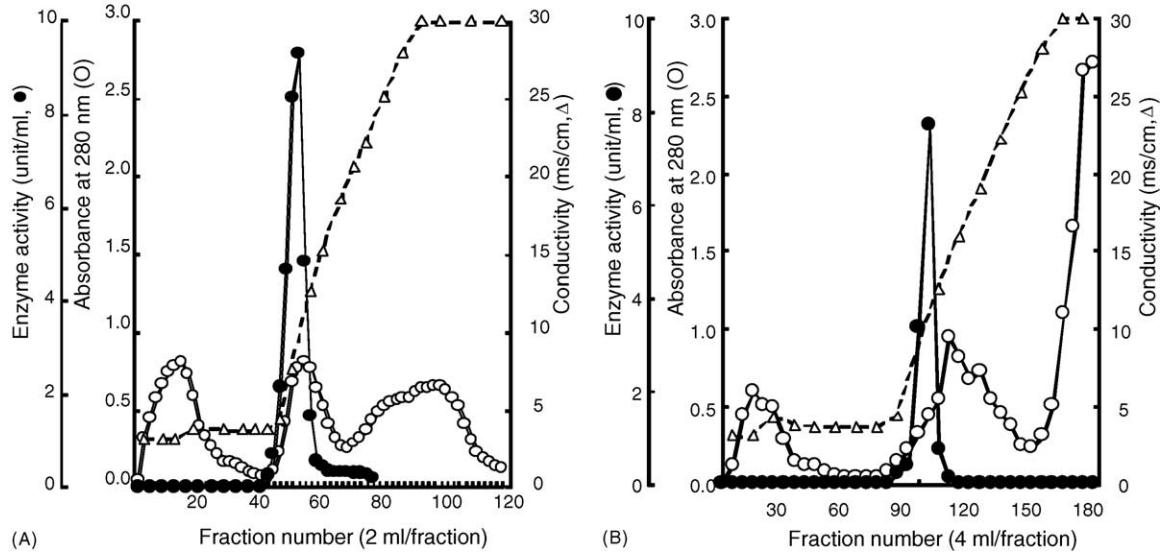

Fig. 1. Chromatography of CIM DEAE-8 tube monolithic column and DEAE-Toyopearl column. (A) A CIM DEAE-8 tube monolithic column was equilibrated with 10 mM buffer (1.8–2.0 mS/cm of conductivity). After a partially purified S-ADH solution was applied to the column, the column was washed with 40 ml of 20 mM buffer, and the adsorbed enzyme was eluted by a linear gradient with 20 mM buffer and 20 mM buffer containing 0.3 M NaCl (40 ml each), followed by 20 mM buffer containing 0.3 M NaCl at a flow rate of 1.5 ml/min. (B) A DEAE-Toyopearl column was also equilibrated with 10 mM buffer, and the adsorbed enzymes were eluted by a linear gradient with the same eluting solution (100 ml each) as the CIM DEAE-8 column, followed by 20 mM buffer containing 0.3 M NaCl at a flow rate of 0.2 ml/min. Enzyme activity was assayed at 550 nm by 2-propanol: β-NAD^+: diaphorase method.

chromatography, the S-ADH was also completely adsorbed on the column under the same conditions, and then eluted at a conductivity of around 10 ms/cm. Thus, the S-ADH was adsorbed and eluted under similar conditions in both columns, whereas the elution patterns of proteins were different from each column chromatography (Fig. 1). These results indicate that a CIM DEAE-8 tube monolithic column was used under the same conditions as the conventional DEAE column, although the interaction of some proteins with the DEAE group on a CIM-8 tube monolithic column might be different from that on a conventional column. Thus, different kinds of proteins might be separated using both columns, and such a combination of both columns might also be useful for the purification of enzymes.

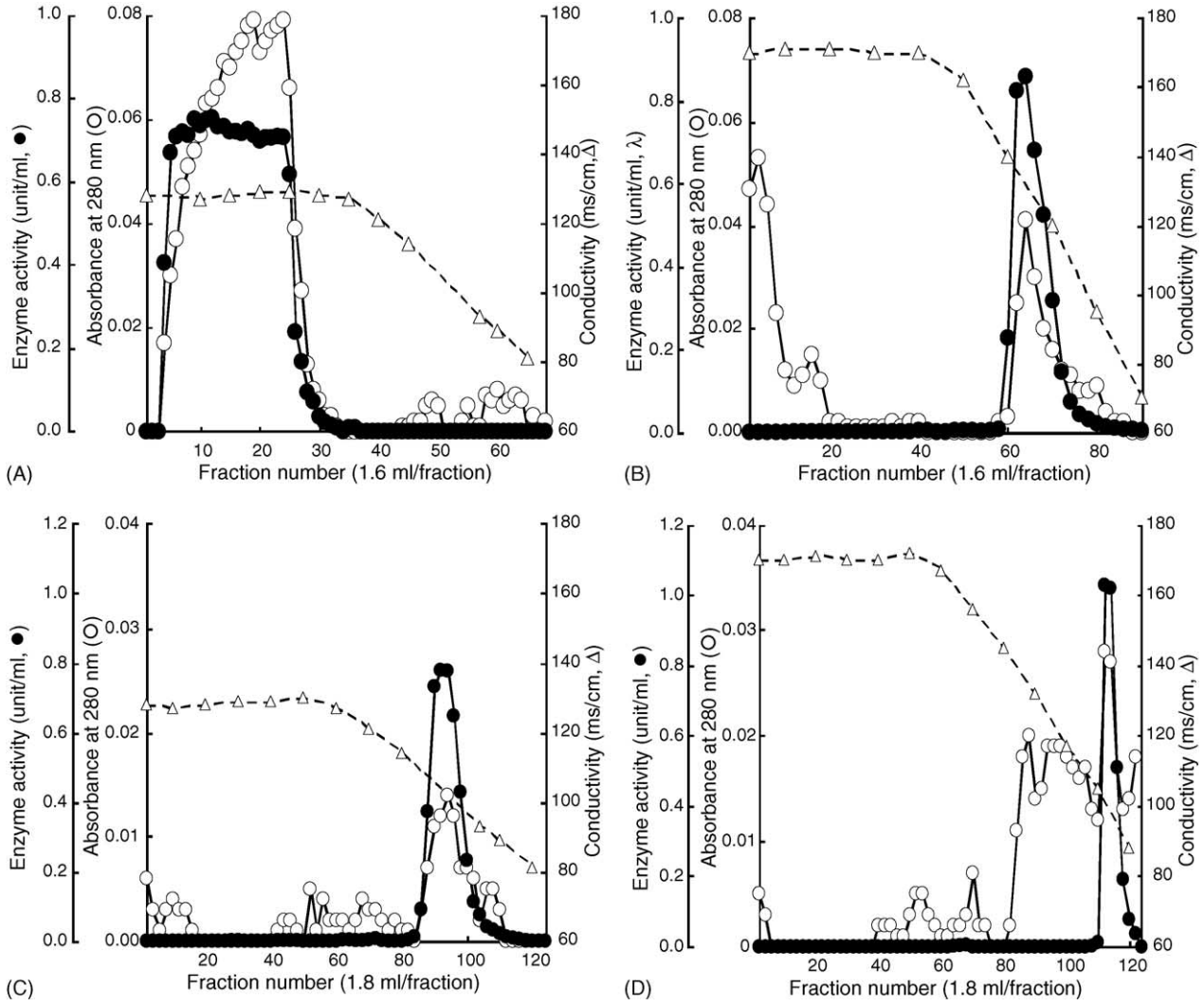

Fig. 2. Effects of ammonium sulfate concentration on adsorption of S-ADH on CIM C4-8 tube monolithic column and Butyl-Toyopearl column. (A) A partially purified S-ADH solution adjusted to 120 ms/cm of conductivity with ammonium sulfate was applied to a CIM C4-8 tube monolithic column equilibrated with 10 mM buffer containing 1.0 M ammonium sulfate (the conductivity: 115–120 mS/cm), and the column was washed with 10 mM buffer containing 1.0 M ammonium sulfate. The elution was carried out by a linear gradient with 10 mM buffer containing 1.0 M ammonium sulfate and 10 mM buffer containing 0.5 M ammonium sulfate (40 ml each) at a flow rate of 1.6 ml/min. (B) The enzyme solution adjusted to 170 ms/cm of conductivity with ammonium sulfate was applied to the CIM C4-8 tube monolithic column equilibrated with 10 mM buffer containing 1.5 M ammonium sulfate (the conductivity was 170–175 ms/cm), and the column was washed with 10 mM buffer containing 1.5 M ammonium sulfate. The adsorbed enzyme was eluted by a linear gradient with 10 mM buffer containing 1.5 M ammonium sulfate and 10 mM buffer containing 0.5 M ammonium sulfate (40 ml each) at a flow rate of 1.6 ml/min. (C) The enzyme solution adjusted to 120 mS/cm of conductivity with ammonium sulfate was applied to a Butyl-Toyopearl column (10 cm × 1.4 cm) equilibrated with 10 mM buffer containing 1.0 M ammonium sulfate, and the column was washed with 10 mM buffer containing 1.0 M ammonium sulfate. The adsorbed enzyme was eluted by a linear gradient with the same eluting solution (75 ml each) as (A) of CIM C4-8 column at a flow rate of 0.18 ml/min. (D) The enzyme solution adjusted to 170 ms/cm of conductivity with ammonium sulfate was applied to the Butyl-Toyopearl column (10 cm × 1.4 cm) equilibrated with 10 mM buffer containing 1.5 M ammonium sulfate, and the column was washed with 10 mM buffer containing 1.5 M ammonium sulfate. The adsorbed enzymes were eluted by a linear gradient with the same eluting solution (75 ml each) as (B) of CIM C4-8 column at a flow rate of 0.18 ml/min. Enzyme activity was assayed at 550 nm by 2-propanol: β-NAD^+: diaphorase method.

3.2. Effects of ammonium sulfate concentration on adsorption of enzyme on CIM C4-8 column

The effects of ammonium sulfate concentration on the adsorption of enzyme were investigated using a CIM C4-8 column and a Butyl-Toyopearl column. When 10 ml of a partially purified enzyme solution of S-ADH containing 1.0 M ammonium sulfate was applied to a CIM C4-8 tube monolithic column equilibrated with 10 mM buffer containing 1.0 M ammonium sulfate, S-ADH was not adsorbed on the column (Fig. 2A). However, when 10 ml of an enzyme solution containing 1.5 M ammonium sulfate was applied to the CIM monolithic column equilibrated with 10 mM buffer containing 1.5 M ammonium sulfate, S-ADH was completely adsorbed on the column, and eluted at a conductivity of around 135 ms/cm (Fig. 2B). On the other hand, when the same enzyme solutions of S-ADH were applied to a Butyl-Toyopearl column equilibrated with 10 mM buffer containing 1.0 and 1.5 M ammonium sulfate, S-ADH was adsorbed on the Butyl-Toyopearl column at both ammonium sulfate concentrations, and the adsorbed S-ADHs were eluted at conductivity of around 100 ms/cm from both columns (Fig. 2C and D). In addition, elution patterns of proteins from a CIM C4-8 tube monolithic column were also different from those of Butyl-Toyopearl column chromatography. When the crude enzyme solutions of AODs were applied to both columns under the same conditions, the AODs were also adsorbed on a Butyl-Toyopearl column equilibrated with 1.0 and 1.5 M ammonium sulfate. On the other hand, the AODs were adsorbed on the CIM C4-8 tube monolithic column equilibrated with 1.5 M ammonium sulfate, but not on the CIM monolithic column equilibrated with 1.0 M ammonium sulfate. The peak of AODs A and B were eluted at approximately 120 and 140 ms/cm of conductivity, respectively (data not shown). These results indicate that the hydrophobic interaction of enzyme with the C4 group on a CIM tube monolithic column might be rather weak compared to that of a Butyl-Toyopearl column, and that a higher concentration of ammonium sulfate was required for adsorbing the proteins on a CIM C4-8 tube monolithic column. Thus, it was concluded that a CIM C4-8 tube monolithic column should be equilibrated with the buffer containing 1.5 M ammonium sulfate as the standard condition for adsorbing proteins.

Table 1
Effects of flow rate on adsorption and elution of D-LDH on CIM DEAE-8 column chromatography

Flow rate (ml/min)	Adsorbed enzyme amounts (mg)	Peak conductivity (mS/cm)
1.24	3.9	33–34
2.5	3.8	32–33
5.0	3.8	36
10.0	3.9	35–36

3.3. Effects of flow rate on adsorption and separation of enzyme

Four milligrams of the purified D-LDH (40 ml) was applied to a CIM DEAE-8 tube monolithic column equilibrated with 10 mM buffer at a flow rate from 1.24 to 10 ml/min, and the column was washed with 50 ml of 10 mM buffer containing 0.15 M NaCl at the same flow rates. All amounts of the enzyme applied were adsorbed on the column, and were not eluted under the above conditions. Then the adsorbed enzyme was eluted by a linear gradient with 10 mM buffer containing 0.15 and 0.5 M NaCl (50 ml each) at the same flow rate. The adsorbed enzyme was eluted at almost same conductivity in the above flow rates (Table 1). Thus, although the flow rates of CIM DEAE-8 tube monolithic column chromatography varied more than 10 times faster than that of the conventional column chromatography, the adsorbed amounts of enzyme and the conductivity for eluting the enzyme were not affected by flow rates. These results indicate that the CIM 8 tube monolithic column was useful in reducing the purification time.

3.4. Application of CIM C4-8 and DEAE-8 tube monolithic columns for purification of S-ADH

On the basis of the above results, we applied CIM C4-8 and DEAE-8 tube monolithic columns for the purification of S-ADH from a crude enzyme solution prepared by disrupting the cells of *Burkholderia* sp. AIU 652 with glass beads. The S-ADH was purified to homogeneity from the crude enzyme according to the procedure described in the Experimental section (Table 2, Fig. 3). Thus, both CIM 8 tube monolithic columns proved useful for the purification of enzymes from

Table 2
Summary of purification of S-ADH from *Burkholderia* sp. AIU 652

Step	Activity (unit)	Protein (mg)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Cell-free extract	115	260	0.44	100	1
CIM C4-8	105	21	5.0	91	11
CIM DEAE-8	82	4.0	21	71	47
Blue-Sepharose	73	1.7	42	63	95
Hydroxyapatite	60	0.64	94	52	213
Toyopearl HW 55	49	0.47	105	43	239

Enzyme activity was assayed at 340 nm by 2-propanol: β-NAD^+ method. Specific activity was expressed as units per milligram of protein.

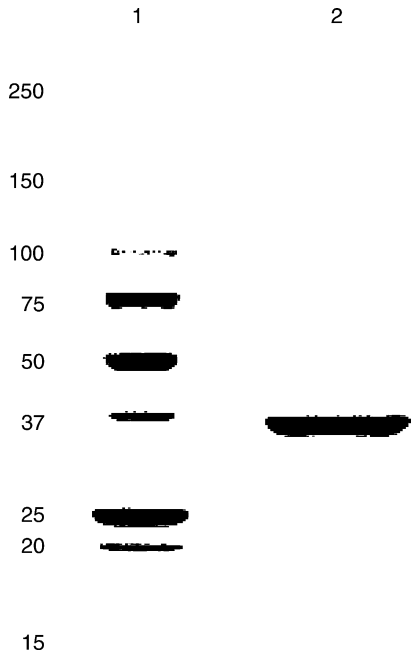

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of S-ADH purified from *Burkholderia* sp. AIU 652. Lines 1 and 2 indicate standard proteins and purified S-ADH, respectively.

crude enzyme solution in a similar manner as the conventional columns, and the purification time was greatly reduced using CIM 8 tube monolithic columns from the first stage of enzyme purification.

4. Conclusion

Using three kinds of enzymes from different microorganisms, the general conditions of chromatography for two types of CIM tube monolithic columns, DEAE-8 and C4-8, were investigated for their effectiveness in purifying a target enzyme from a crude enzyme solution of microorganisms The microbial enzymes were adsorbed on a CIM DEAE-8 tube column under the same conditions as on conventional DEAE columns, whereas the CIM C4-8 tube column required a high concentration of ammonium sulfate for adsorbing the enzymes compared to the conventional C4 column. Thus, it was concluded that CIM DEAE-8 and C4-8 tube monolithic columns should be generally equilibrated with 10 mM buffer and 10 mM buffer containing 1.5 M ammonium sulfate, respectively, for adsorbing proteins. When the flow rates of the CIM DEAE-8 tube monolithic column (column volume: 8 ml) varied between 1.24 and 10 ml/min (more than 10 times faster than that of conventional column chromatography), the adsorbed enzyme amounts and conductivity for eluting the enzymes were not affected by the flow rates. In addition, both CIM DEAE-8 and C4-8 tube monolithic columns could be used for the purification of a target enzyme from a crude enzyme solution prepared by disrupting microbial cells with glass beads. Thus, both CIM tube monolithic columns could be used at any stage of enzyme purification, and proved useful in reducing the purification time.

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