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USE OF BIOSPECIFIC ADSORBENTS WITH POLYSACCHARIDE SPACERS IN AFFINITY CHROMATOGRAPHY

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SUMMARY

The isolation of mouse liver poly(A)-containing mRNA [poly(A)–mRNA] and purification of trypsin and kallikrein using new biospecific adsorbents having dextran, glycogen or amylopectin spacers are described as the first examples of affinity chromatography on such adsorbents. A comparative study of these adsorbents and similar adsorbents without spacers was carried out. The optimum conditions for affinity chromatography of poly(A)–mRNA were considered.

INTRODUCTION

In the preceding paper[1] we described the preparation and properties of a number of affinity adsorbents having polysaccharide spacers. It seemed expedient to demonstrate the advantages of such adsorbents by concrete examples of the isolation and purification of biopolymers of different chemical nature, particularly polyadenylated mRNA (polynucleotide biopolymer) and proteolytic enzymes (protein biopolymers).

The isolation of poly(A)–mRNA on poly(U)–Sepharose (1)[2] or oligo(dT)–cellulose[3] is based on the hybridization of complementary bases. Some non-specific effects of a different nature were involved in the chromatography of poly(A)–mRNA on oligo(dT)–cellulose[4]. The disadvantages of chromatography on poly(U)–Sepharose (1) are the non-quantitative recovery of poly(A)–mRNA and insufficient adsorbent capacity.

At the present time, the immobilized soy bean trypsin inhibitor (SBTI) is widely used for purification of serine proteinases[5]. Highly purified trypsin[6,7] and human serum kallikrein[8,9] have been prepared by means of affinity chromatography on SBTI–Sepharose (2). Attachment of SBTI to Sepharose is generally achieved by the BrCN method[10]. However, direct binding of a high-molecular-weight ligand to a solid support may result in steric hindrance to subsequent biospecific interaction of immobilized ligand with the active centre of the enzyme to be purified. Consequently, a decrease in adsorbent capacity may take place. We believe that the use of a spacer to separate the ligand from the surface of the solid matrix would improve some prop-

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erties of the adsorbent. A recently published paper[11] described the immobilization of SBTI via a monomeric spacer using a glutaraldehyde-activated amino derivative of Sepharose.

In the present work, we describe the first applications in affinity chromatography of biospecific adsorbents having polysaccharide spacers. The isolation of poly(A)–mRNA on poly(U) immobilized on Sepharose via dextran or glycogen spacers as well as the purification of trypsin and human serum kallikrein on SBTI bound to Sepharose via an amylopectin spacer have been performed. The properties of adsorbents with and without polysaccharide spacers are compared.

EXPERIMENTAL

Materials and methods

Most of the materials used in the present study were as specified in the preceding paper[1]. Poly(U) and SBTI were obtained from Reanal (Budapest, Hungary), pig pancreatic trypsin from Spofa (Prague, Czechoslovakia). Human serum kallikrein was partially purified by chromatography on DEAE-Sephadex A-50 and SP-Sephadex C-50 (the method will be published in detail elsewhere). N-Benzoylarginine ethyl ester (BAEE) was obtained from Koch-Light (Colnbrook, Great Britain). O-[N-2-(Aminoethyl)carbamoyl]dextran (AED) (γ^{NH_2} = 12) was prepared as described previously[12]. Nuclear and cytoplasmic RNAs from mouse liver cells were isolated by the phenol–temperature fractionation procedure[13].

Some of the methods used in this work were as described in the preceding paper[1]. DNA-like RNAs were selectively labelled with [^{14}C]orotic acid (30 μCi per mouse) for 60–90 min by the use of the actinomycin D-induced block of rRNA synthesis. For the determination of radioactivity, an RNA carrier (100 μg/ml) and trichloroacetic acid (final concentration 5%) were added to the samples. The precipitates were then collected on HUFS filters, and after drying were measured in a Nuclear Chicago Mark 2 liquid scintillation spectrometer. Sedimentation coefficients were calculated as described previously[14].

Estimation of the ligand content in the adsorbents

The concentration of the protein ligand was determined by the differential method. In the case of a polynucleotide ligand the solubilization method[15] was used. A mixture of 0.2 ml of a Sepharose derivative and 12 ml of 1 *N* NaOH containing 0.1% $NaBH_4$ was heated for 2 h at 75–80°C. The mixture was then centrifuged for 15 min at 7000 rpm (3000 *g*). The absorbance of the supernatant was measured at 260 nm.

AED–Sepharose (3)

A suspension of 18 ml of BrCN-activated Sepharose (2.5 g of BrCN per 10 ml gel)[1] in 18 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl, pH 9, and 2.7 g AED (γ^{NH_2} = 12)[12] was stirred for 16 h at 4°C. The gel was then washed with 1 l water, 200 ml of 0.2 *M* NaCl and 200 ml water and stirred with 20 ml of 1 *M* ethanolamine (pH 9) for 2 h at 20°C. The gel was washed with water (200 ml), suspended in an equal volume of water and the mixture was stirred with two or three drops of acetic acid and 20 mg EDC for 72 h at 20°C. After washing with 200 ml of 0.1 *N* HCl and 500 ml of water, the AED–Sepharose (3) was stored as an aqueous suspension at 4°C in the presence of 0.02% NaN_3.

Poly(U)–AED–Sepharose (4)

A solution of 2 g BrCN in 1 ml acetonitrile was diluted to 5 ml with water and added to a suspension of 10 ml "AED–Sepharose" (3) in 10 ml of 5 *M* potassium phosphate buffer, pH 11.9. The suspension was vigorously stirred for 5 min at 4°C. Then the gel was quickly washed with 100 ml of 0.1 *M* $NaHCO_3$, 100 ml water and 100 ml of 0.1 *M* potassium phosphate buffer, pH 7.5. The gel was immediately suspended at 4°C in a solution of 15 ml poly(U) in 10 ml of the same buffer and stirred for 16 h at 4°C. The sorbent was washed with 500 ml of 0.1 *M* potassium phosphate buffer, pH 7.5, and 100 ml water and then stirred with 1 *M* ethanolamine (10 ml) for 2 h at 20°C and pH 9.0. Finally, the gel was washed with water.

Poly(U)–glycogen–hydrazidosuccinyl-Sepharose (5)

Glycogen–hydrazidosuccinyl-Sepharose (10 ml) prepared as described previously[1] was activated with 2 g BrCN and then stirred with a solution of 15 mg poly(U) in 10 ml of 0.1 *M* potassium phosphate buffer, pH 7.5, for 16 h at 4°C. The gel was washed with 500 ml of the same buffer and 100 ml water, stirred with 3 mg acetic acid hydrazide in 10 ml water for 2 h at 20°C and washed with 500 ml water. The adsorbent (5) was stored in 0.1 *M* potassium phosphate buffer (pH 7.5) in the presence of 0.02% NaN_3.

Affinity chromatography of poly(A), poly(U) and poly(A)–mRNA

The chromatography of different samples on the adsorbents 1, 4 and 5 was carried out using a glass column (7.0 × 0.6 cm) at a flow-rate of 15 ml/h. The buffers used for sample application and elution were: a, hybridization buffer, 0.02 *M* Tris–HCl, pH 7.0, 0.3 *M* NaCl, 0.001 *M* ethylenediaminetetraacetate (EDTA); b, 0.02 *M* Tris–HCl, pH 7.6, 0.001 *M* EDTA. Fractions were assayed for absorbance at 260 nm or radioactivity.

SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6)

Adsorbent 6 was prepared by means of activation of amylopectin–hydrazidosuccinyl-Sepharose[1] (10 ml) with 2 g BrCN as described above and subsequent incubation with 100 mg SBTI in 10 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl. Washing of the adsorbent and blocking of the excess of active groups were performed as described in the preceding paper[1] for protein immobilization. SBTI–Sepharose (2) was synthesized as described[10]. Adsorbents 2 and 6 contained 7–9 mg SBTI per ml of gel (the differential method).

Determination of antitryptic activity of immobilized SBTI

Aliquots (0.01–0.1 ml) of adsorbents 2 and 6 were adjusted to 1.4 ml with 0.05 *M* Tris–HCl buffer, pH 8.0, and 0.1 ml of 0.01% trypsin in 2.5 m*M* HCl was then added. The mixture was kept for 5 min, and 0.5 ml of 0.1% agarose solution and 1 ml of 1.5 m*M* BAEE were added. The mixture was thoroughly stirred, and the absorbance at 253 nm was measured against a corresponding amount of unsubstituted Sepharose. The antitryptic activity was expressed as the number of inhibitor units per mg of immobilized SBTI: 1 IU = one unit of inhibitory activity, corresponding to the amount of SBTI which inhibits the hydrolysis of 1 μmole of substrate under the conditions described above.

Proteinase assay

Assays for trypsin and kallikrein activities were carried out in 0.05 *M* Tris–HCl buffer (pH 8.0) at 25°C by measurement of the absorption at 253 nm with BAEE as substrate. One unit of activity, E, is defined as the amount of enzyme which hydrolyses 1 μmole of BAEE per min under the standard conditions.

Affinity chromatography of kallikrein and trypsin

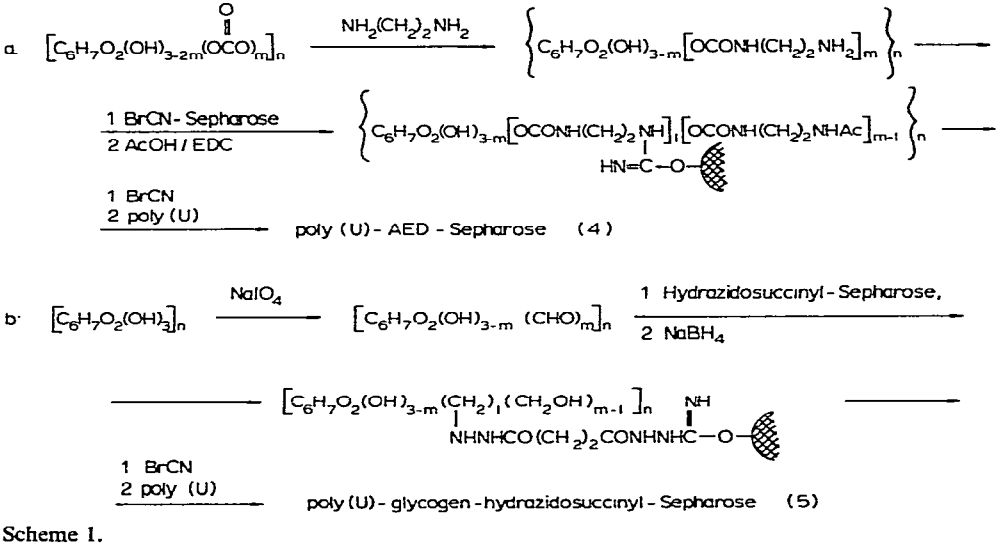
All procedures were performed at 4°C. A solution of partially purified human serum kallikrein was applied to a column (4.0 × 0.8 cm) packed with adsorbent 2 or 6. The column was equilibrated with 0.1 *M* sodium phosphate buffer (pH 6.2) containing 0.2 *M* NaCl. The flow-rate was 12 ml/h. Protein contaminants were eluted from the columns with the starting buffer (eluate I). Kallikrein was eluted with 0.01 *M* HCl containing 1 *M* NaCl (eluate II). Fractions (2.5 ml) were collected at a flow-rate of 48 ml/h. Each fraction was immediately adjusted to pH 8.0 with 3.5 *M* NaOH, and the BAEE-esterase activity was determined.

Affinity chromatography of pig pancreas trypsin was performed under similar conditions.

RESULTS AND DISCUSSION

The adsorbents having polysaccharide spacers (4–6) were prepared according to the methods described in the preceding paper[1], but with some modifications.

In the synthesis of adsorbent 4 (Scheme 1a) AED containing 12 N-2-(aminoethyl)carbamoyl groups per 100 anhydroglucose residues was used for introduction of the polysaccharide spacer into the adsorbent. AED was prepared by condensation of dextran cyclic carbonate with ethylenediamine[12]. The use of AED prevents the presence of unreacted carboxyl groups on the adsorbent[16], in contrast to the method using carboxymethyldextran[1]. The amount of bound dextran (15–20 mg per ml gel)



Scheme 1.

was determined from the difference in nitrogen content (by Kjeldahl's method) of the starting solution of the polysaccharide and of the washings (after dialysis). The active groups on Sepharose were blocked with ethanolamine, and the unreacted amino groups on the polysaccharide spacer were acetylated with acetic acid in the presence of EDC. Acetylation was complete in 72 h, the reaction being monitored by the colour test with trinitrobenzenesulphonic acid.

Attachment of poly(U) to adsorbent 3 was performed after activation of the dextran spacer with BrCN[2]. The content of poly(U) in the adsorbent (1.47 mg per ml gel) was determined by measurement of the absorbance at 260 nm of the starting solution and that of the washings. The solubilization method[15] was also used. Fig. 1 shows the UV spectrum of the solubilized adsorbent (4). It should be noted that complete solubilization of adsorbents with polysaccharide spacers was not achieved under the standard conditions[15]. Centrifugation of the reaction mixture before adsorption measurements was found to be necessary. The increase in stability of agarose beads may be due to the presence of the polysaccharide spacer and to the occurrence of cross-linking during its attachment. However, this did not affect the accuracy of determination of the poly(U) content since the immobilized poly(U) was completely hydrolysed under the conditions used.

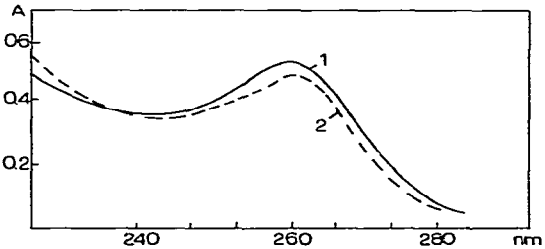


Fig. 1. UV spectra of solubilized adsorbents: 1 = adsorbent 4; 2 = adsorbent 5. The conditions of solubilization are given in the text.

The method used for synthesis of poly(U)–glycogen–hydrazidosuccinyl-Sepharose (5) (Scheme 1b) minimizes the amount of charged groups on the adsorbent, through the use of Sepharose hydrazide. As shown by UV spectrophotometric analysis of the solubilized gel (Fig. 1), adsorbent 5 contained 1.45 mg poly(U) per ml gel. Poly(U)–Sepharose (1) was prepared as described[2], and contained 0.5 mg poly(U) per ml gel.

The chromatographic behaviour of poly(A) and poly(U) on adsorbents 4 and 5 was studied in order to evaluate the capacity and biospecificity of the adsorbents. Efficient adsorption of poly(A) was observed after application of the polynucleotide to the columns in the hybridization buffer of high ionic strength. Columns of the adsorbents 4 and 5 were saturated with poly(A), and the capacity of the adsorbents was estimated by measuring the absorbance at 260 nm of the polynucleotide fraction eluted by the buffer of low ionic strength. Adsorbents 4 and 5 possessed poly(A)-binding capacities of 1.44 and 1.48 mg poly(A) per ml gel, respectively. These values significantly exceed that of adsorbent 1 (0.4 mg/ml). However, a large part of the bound poly(A) (61.1% for adsorbent 4 and 16.8% for adsorbent 5) could not be desorbed from the column under the conditions generally used for elution of poly(A)–

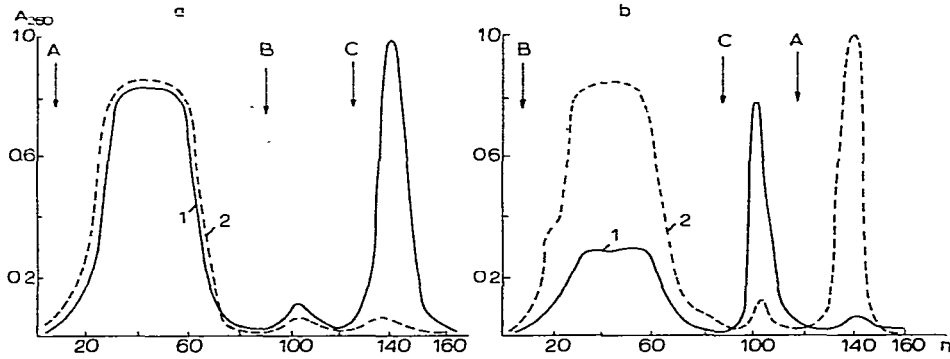


Fig. 2. Chromatography of poly(A) (1) and poly(U) (2) on adsorbent 4 (a and b). Arrows indicate change of the conditions: A, hybridization buffer; B, elution buffer at 20°C; C, elution buffer at 50°C. The polynucleotides (40–60 μg/ml) were applied to the columns in the corresponding buffer.

mRNA[17]. Nevertheless, the initial adsorption of poly(A) was biospecific since poly(U) was not adsorbed on the columns under the same conditions (Fig. 2a).

To achieve a quantitative yield of poly(A) during the elution step, the adsorbents were washed with 90% formamide in the elution buffer in the presence of 0.5% sodium dodecyl sulphate (SDS) at 50°C. The formamide was then washed off, and an aliquot of the gel was solubilized and tested as above. The results show that desorption of poly(A) was quantitative under these conditions.

The behaviour of poly(A) and poly(U) on adsorbents 4 and 5 equilibrated with the elution buffer of low ionic strength was also studied. These conditions were unfavourable for specific base pairing between the poly(A) and poly(U) chains, but, on the other hand, they promoted the non-specific ionic interactions. Considerable adsorption of the polynucleotides was observed on both adsorbents. Moreover, the quantitative desorption of poly(U) was achieved by means of the hybridization buffer (Fig. 2b).

The results of the model experiments (Table I) suggest that electrostatic interactions between the polynucleotide phosphate groups and cationic groups of the adsorbents occur when buffer of low ionic strength is used. These interactions seem to be multivalent since the non-specific binding of adenosine 5′-phosphate to the adsorbents was not observed under the same conditions. The content of poly(A) bound non-specifically to adsorbents 4 and 5 (Table I) may be correlated with the number of cationic groups of these adsorbents. Indeed, some positively charged groups were definitely introduced into adsorbent 4 during the multivalent attachment of AED to BrCN–Sepharose via the primary amino groups. On the other hand, the adsorbent formed after the attachment of succinic acid dihydrazide to BrCN–Sepharose was uncharged at the physiological pH. For this reason, the blocking of residual active groups in adsorbent 5 was carried out by use of acetic acid hydrazide, not ethanolamine.

The undesirable processes of ligand release and of increasing cationic charge during storage of adsorbents based on BrCN-activated polysaccharides have recently been recognized[18]. We found that the recovery of poly(A) from freshly prepared adsorbent 1 was 90%, while after storage of the adsorbent for 1 year at 4°C and pH 7

TABLE I

PROPERTIES OF THE AFFINITY ADSORBENTS 4 AND 5

Capacity of adsorbent 1 was 360 μg poly(A) per ml gel.

Adsorbent	*Capacity*		*Non-specific adsorption of poly(A)* (% of bound polynucleotide)*	*Adsorption of poly(U) under conditions of high ionic strength (μg per ml gel)*	*Adsorption of poly(A) under conditions of low ionic strength (μg per ml gel)*	*Adsorption of poly(U)** under conditions of low ionic strength (μg per ml gel)*
	μg poly(A) per ml adsorbent	*mg poly(A) per mg immobilized poly(U)*				
Poly(U)–AED–Sepharose (4)	1440	0.98	61	0	760	650
Poly(U)–glycogen–hydrazido-succinyl-Sepharose (5)	1480	1.02	16.2	0	440	480

* The fraction not desorbed with the elution buffer at 50°C.

** The bound poly(U) was quantitatively eluted with the hybridization buffer.

the recovery was only *ca.* 50%. Repeated usage of the adsorbent also resulted in deterioration of its properties.

Adsorbent 5 yielded better results than adsorbent 4, and optimal recovery of poly(A) was achieved by desorption of the bound polynucleotide with 90% formamide in the elution buffer containing 0.5% SDS, at 50°C. Under these conditions, the biospecific A–U hybridization was interrupted and also the ion-exchange interactions were diminished.

Isolation of mouse liver cytoplasmic and nuclear poly(A)–RNAs was performed using adsorbent 5 under optimum conditions. Approximately 2% of the total cytoplasmic RNA and 30% of nuclear RNA were bound to the adsorbent. The total RNA content in the fractions was estimated by UV spectrophotometry, and that of non-ribosomal RNA was calculated on the basis of the radioactive label. The desorption of cytoplasmic RNA was carried out with the elution buffer at 20 and 50°C and, finally, with 90% formamide in the same buffer containing 0.5% SDS. The results obtained suggest that quantitative recovery of the bound RNA was achieved when the formamide-containing buffer was used. The eluted cytoplasmic and nuclear poly(A)–RNAs were precipitated by two volumes of ethanol containing 0.2% sodium acetate (final concentration). The labelled material gave a typical pattern for high-molecular-weight RNA under the conditions of centrifugation in a sucrose gradient (10–35%) at 23,000 rpm (64,000 *g*) (rotor SW 25.2) and 4°C.

Quantitative recovery of poly(A)–mRNA from a biospecific adsorbent is of great importance for the study of mRNA metabolism. Non-specific effects, in particular ion-exchange ones, interfere with the affinity chromatography of poly(A)–RNAs. Ion-exchange interactions are of little importance under adsorption conditions (the hybridization buffer of high ionic strength). However, part of the material

may be retained on the column as a result of multipoint interactions between the ionized phosphate groups of RNA and cationic groups of the adsorbent. Part of the electrostatically bound material would again interact biospecifically with the immobilized poly(U) during repeated use of the adsorbent or its regeneration with buffers of high ionic strength. Contamination of the poly(A)–RNA preparations with labelled material from previous experiments may take place during the subsequent elution. Therefore, minimization of the content of positively charged groups on the biospecific adsorbent is especially important for affinity chromatography of poly(A)–mRNA.

In order to demonstrate further the advantages of the adsorbents having polysaccharide spacers, we carried out a comparative study of two bioadsorbents designed for the purification of proteolytic enzymes: SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6) and a similar adsorbent without the polysaccharide spacer (2).

Adsorbent 6 was prepared by coupling of SBTI to amylopectin–hydrazidosuccinyl-Sepharose[1] after activation of the polysaccharide spacer with BrCN. Adsorbent 2 was synthesized by the direct attachment of SBTI to BrCN-activated Sepharose. As shown by the differential method, these adsorbents contained 7–9 mg SBTI per ml of gel. The antitryptic activity of the immobilized SBTI was then estimated by spectrophotometric assay of the BAEE hydrolysis in the gel suspension stabilized by addition of 0.1 % agarose solution. The use of the agarose solution ensured accurate measurements of the absorption. The specific antitryptic activity of SBTI immobilized via the amylopectin spacer was almost twice that of SBTI immobilized directly on Sepharose (Table II). Thus, the use of the polysaccharide spacer preserved 20 % of the activity of immobilized SBTI with respect to that in solution. The presence of the spacer results in a considerable distance between the ligand and the matrix surface, and the native protein conformation is apparently preserved to a greater extent than can be achieved by direct attachment of SBTI to Sepharose.

The capacity of adsorbents 2 and 6 for trypsin and kallikrein was also determined. These enzymes exhibited very similar affinities for the native soy bean inhibitor (inhibition constant *ca.* 10^{-10} *M*[19,20]). The proteinases were applied to the column in 0.1 *M* sodium phosphate buffer (pH 6.2) containing 0.2 *M* NaCl until the column was saturated with the enzyme. After washing the column with the starting buffer, the bound proteinase was eluted with 0.01 *M* HCl containing 1 *M* NaCl. The capacity for both enzymes of the adsorbents having the amylopectin spacer was three

TABLE II

PROPERTIES OF THE AFFINITY ADSORBENTS CONTAINING SBTI

Adsorbent	*Antitryptic activity (IU per mg immobilized SBTI)*	*Capacity for trypsin*		*Capacity for kallikrein*	
		mg per ml gel	*μmoles per μmoles immobilized SBTI*	*mg per ml gel*	*μmoles per μmoles immobilized SBTI*
SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6)	4.4	2.5	0.25	2.8	0.07
SBTI–Sepharose (2)	2.4	0.75	0.08	1.0	0.025

TABLE III

AFFINITY CHROMATOGRAPHY OF PIG PANCREATIC TRYPSIN AND HUMAN SERUM KALLIKREIN ON ADSORBENTS 2 AND 6

Adsorbent	*Fraction*	*Trypsin*						*Kallikrein*					
		Protein		*Activity*		*Yield (%)*	*Purification factor*	*Protein*		*Activity*		*Yield (%)*	*Purification factor*
		mg/ml	*mg*	*Specific (E/mg)*	*Total (E)*			*mg/ml*	*mg*	*Specific (E/mg)*	*Total (E)*		
SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6)	Starting preparation	0.093	14.0	18.0	250.0	–	1	1.04	836.0	0.17	142.0	–	1
	Filtrate + eluate I	0.047	8.2	3.3	27.1	10.8	–	0.94	800.0	0.0	0.0	–	–
	Eluate II (0.01 *N* HCl + 1 *M* NaCl)	0.49	5.3	38.0	202.0	80.8	2.1	0.57	6.4	20.0	128.0	90.0	117
SBTI–Sepharose (2)	Starting preparation	0.093	4.6	18.0	82.8	–	1	1.04	420.0	0.17	71.4	–	1
	Filtrate + eluate I	0.036	2.4	4.2	11.0	13.0	–	0.91	410.0	0.015	6.2	8.6	–
	Eluate II (0.01 *N* HCl + 1 *M* NaCl)	0.137	1.6	38.0	61.0	74.8	2.1	0.26	3.2	20.0	64.0	89.6	117

times that of SBTI–Sepharose (2) (Table II). The increase in the capacity of adsorbent 6 may be due to better accessibility of the reactive centre of the immobilized SBTI to the active centre of the enzyme.

The results of purification of the commercial pig pancreas trypsin and partially purified human serum kallikrein under the conditions described are presented in Table III. The yields, specific activities and purification factors were practically identical for the two adsorbents. The essential advantage of adsorbent 6 over the adsorbent without a spacer lies in the use of much smaller volumes of buffer for elution of the bound enzymes. The increase in the concentrations of trypsin or kallikrein eluted from the adsorbent having the amylopectin spacer (6) is of importance for future studies of these enzymes.

Adsorbent 6 may be used repeatedly (five or six times) without loss of binding capacity or inhibitory activity. The results presented here demonstrate that the introduction of a polysaccharide spacer into a solid matrix improves the properties of a biospecific adsorbent and enables highly efficient purification of biopolymers.

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