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Short communication

# Characterization of weak hydrophobic composite sorbents and their application to the isolation of bacterial lectin

A.E. Ivanov*, L.S. Zhigis, E.M. Rapoport, O.E. Lisyutina, V.P. Zubov

*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117871 Moscow V-437, Russian Federation*

**Abstract**

Composite sorbents based on wide-pore glass and silica coated with N-butyl polyacrylamide (butyl-PA-glass and butyl-PA-silica) were studied. The surface tension of butyl-PA-silica is 50–55 $mJ/m^2$ as evaluated by the sedimentation volume technique. The linear dependences of log $k'$ on ammonium sulphate concentration were determined by isocratic chromatography of the dipetide Trp–Trp and lysozyme on butyl-PA-glass. Both solutes were shown to have a weaker retention on butyl-PA-glass than on butyl-Toyopearl 650C. This weaker retention is beneficial in the purification of sialic acid-binding lectin from *Bacillus subtilis*.

## 1. Introduction

Hydrophobic-interaction chromatography (HIC) of proteins on weak hydrophobic stationary phases gained popularity as a technique allowing quantitative recovery of complex biologically active macromolecules [1–3], which might be inactivated by the stronger hydrophobic interactions employed in reversed-phase chromatography [4,5]. Water-soluble polymers, such as polyethylene glycols or polyvinylalcohol, were successfully used as weak hydrophobic ligands grafted to silica [1,2] or to agarose matrices [3] to prepare sorbents with a moderate hydrophobic character.

Recently we described the preparation of a weak hydrophobic sorbent based on silica or porous glass chemically modified with adsorbed layers of N-butyl polyacrylamide (butyl-PA-sorbents). The method of synthesis included a covalent adsorption of poly($p$-nitrophenyl acrylate) to $\gamma$-aminopropylsilicas followed by amidation of the ester groups of the polymer with $n$-butylamin [6]. Conditions of adsorption and desorption of standard proteins and proteins from culture fluids resembled those found for commercial butyl-Toyopearl 650C gel (TOSOH, Tokyo, Japan) [6,7]. Further experimental studies revealed, however, a marked difference in the selectivity of protein separations accomplished on the above sorbents, in spite of their nominal chemical functionality [8]. This paper deals with further characterization of butyl-PA-sorbents and their use for purification of sialic acid-binding lectin (SBL) from *Bacillus subtilis*.

Owing to their unique carbohydrate binding characteristics, lectins serve as valuable tools for the separation and characterization of glycoproteins and glycopeptides. Purification and characterization of lectins themselves are therefore of

* Corresponding author.

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great interest. The usual methods such as gel-permeation or ion-exchange chromatography are not suitable for purification of SBL because of either poor resolution or low activity of the recovered sample. Recently we have found the optimal conditions for purification of bacterial SBL by one-step HIC on butyl-PA-glass [8]. We describe herein the use of the same method for the semipreparative isolation of SBL from the crude extract.

## 2. Experimental

Wide-pore glass MPS-2000VGKh (mean pore diameter 2000 Å, particle size 0.16–0.31 mm) was purchased from GOZ VNII NP (N. Novgorod, Russian Federation). Fine silica gel (mean pore diameter 500 Å, particle size 5 $\mu$m) was from YERONEM (Yerevan, Armenia). Weak hydrophobic sorbents were synthesized from the above carriers according to the method described in Ref. [6]. Butyl-Toyopearl 650C gel was from TOSOH (Tokyo, Japan). Trp–Trp dipeptide and lysozyme were from Serva (Heidelberg, Germany). The surface tension of butyl-PA-silica was measured by the sedimentation volume technique as previously described [9]. In brief, $64 \pm 0.5$ mg of the packing was weighed into a 1-ml Eppendorf tube. The particles were dispersed by gentle mechanical stirring in 0.4 ml of suspending liquid and quantitatively transferred into calibrated glass tubes ($50 \times 3.5$ mm I.D.). After 3–4 days of sedimentation the heights of the deposits were recorded. The surface tensions of ethanol–water mixtures as estimated in Ref. [10] were used.

Isocratic hydrophobic-interaction chromatography was performed in $1 \times 9$ cm glass columns at a flow-rate of 1.0 ml/min controlled by peristaltic pumping. A 2-mg sample of lysozyme or 0.5 mg of Trp–Trp dipeptide in 1 ml of 0.1 *M* phosphate buffer (pH 7.0), containing varied concentrations of ammonium sulphate, was applied to the column and eluted with the same solution. A LKB Uvicord S II was used for detection of absorbing fractions ($\lambda = 280$ nm) during chromatography.

Gradient hydrophobic-interaction chromatography of the crude lectin extract was performed on butyl-PA-glass packed into a $21 \times 1.5$ cm I.D. column at a flow-rate of 1.0 ml/min. A 34-mg sample of the liophilized extract was dissolved in 20 ml of 10 m*M* sodium phosphate containing ammonium sulfate (60% of saturation concentration) and applied to the column previously equilibrated with the same buffer. The column was washed with 50 ml of the starting buffer and then with a gradient of ammonium sulfate (from 60% saturation to 0%, 120 ml). Two-milliliter fractions were collected and assayed for absorbance at 280 nm and hemagglutinating activity. Active fractions were dialysed by ultrafiltration on PM-10 membranes (Amicon, Netherlands). The agglutination assay was performed as previously described [8]. Electrophoresis was carried out under non-reducing conditions in 4–30% acrylamide [11]. Proteins were stained with silver nitrate.

## 3. Results and discussion

### 3.1. Surface tension of butyl-PA-silica

Among the characteristics of a hydrophobic chromatographic packing, its surface tension ($\gamma_{SV}$) seems to be of primary importance. It was shown that the Helmholtz free energy of protein adsorption is determined by the surface tensions of the packing, the solvent and the protein [12]. For protein adsorption from phosphate buffered saline or from other aqueous solutions of salts with a surface tension ca. 73 mJ/m$^2$ (at 20°C) the free energy of adsorption becomes more negative as the surface tension of the packing decreases. Consequently, the adsorbed amount of protein increases [12]. This might explain why low-energy packings such as alkylsilicas ($\gamma_{SV}$ = 30–40 mJ/m$^2$, [9]) often display an almost irreversible adsorption of large proteins and why these proteins show poor recovery at the elution step [4,5]. On the other hand, polymer ligands such as polyethylene glycol or acylated polyethyleneimine provide the relevant packings with a higher surface tension ($\gamma_{SV}$ = 46–53 mJ/m$^2$ [9])

and thus ensure milder conditions for the quantitative recovery of proteins [1,5].

A simple technique based on measurement of the sedimentation volumes of fine chromatographic packings was proposed for evaluation of their surface tension [10]. The surface tension of the suspending liquid, in which the sedimentation volume is maximal, was thought to be equal to the surface tension of the packing. We applied this technique to the evaluation of the surface tension of butyl-PA-silica (5 $\mu$m particle size). Fig. 1 shows the dependence of the sedimentation volume on the composition of ethanol solutions in water exhibiting different surface tensions. The maximum corresponds to a value of 50–55 mJ/m$^2$. This by far exceeds the surface tensions of butylsilicas ($\gamma_{SV}$ = 35–41 mJ/m$^2$) known from literature [9]. Possibly, the hydrophilic polyacrylamide chains of the bonded phase contribute to the higher surface tension of butyl-PA-silica. The packing may be regarded, therefore, as a weak hydrophobic sorbent.

### 3.2. *Isocratic chromatography of peptides and proteins*

A characteristic feature of hydrophobic-interaction chromatography is the possible isocratic elution of proteins with rather high retention factors $k'$. By increasing the salt concentration the retention factor increases such that the dependence of log $k'$ on molarity often becomes linear. The limiting slope of this plot is given by the hydrophobic-interaction parameter (HIP) accounting for the interaction area between the protein and the sorbent, the surface tension increment of the salt and the dipole moment of the protein molecule [1]. For a given protein solute eluted at various concentrations of ammonium sulfate HIP is a measure of the contact area between the solute and the sorbent employed. The intercept of the log $k'$–$M$ plots gives the logarithm of the retention factor of the solute in the absence of salt in the eluent.

Fig. 2 shows the log $k'$–$M$ plots obtained for two solutes: Trp–Trp dipeptide and lysozyme on butyl-Toyopearl 650 and butyl-PA-glass sorbents. In spite of the fact that they both contain the same functional groups, i.e. butyl groups, the two sorbents strongly differ in the retention of solutes. Both the Trp–Trp dipeptide and the

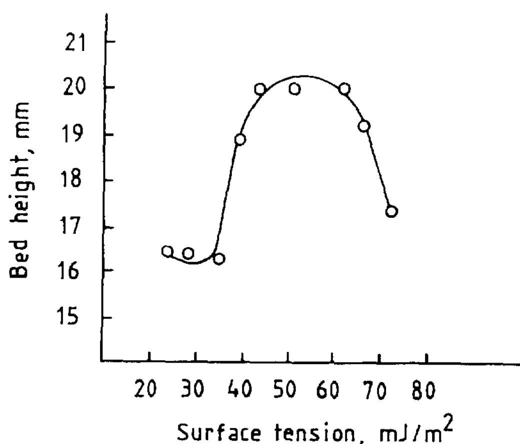


Fig. 1. Sedimentation volumes of butyl-PA-silica (5 $\mu$m particle size, 500 Å pore diameter) in aqueous ethanol solutions with varied surface tension.

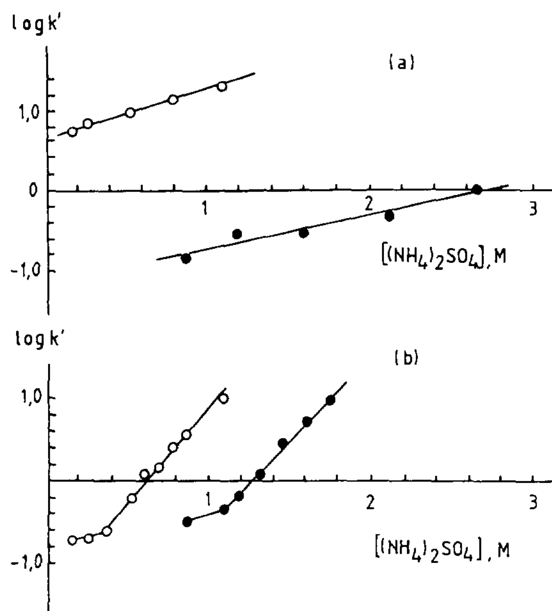


Fig. 2. Logarithm of the retention factor (log $k'$) of Trp–Trp dipeptide (a) and lysozyme (b) plotted versus molar concentration of ammonium sulfate. Isocratic hydrophobic-interaction chromatography on butyl-Toyopearl 650C (empty circles) and butyl-PA-glass (filled circles). For conditions see Experimental.

lysozyme adsorb stronger to butyl-Toyopearl 650. However, the contact areas of lysozyme adsorbed to butyl-PA-glass and to butyl-Toyopearl 650 are almost the same. The weaker retention of the protein on butyl-PA-glass is possibly due to a higher surface tension of this packing. On the other hand, the contact areas of the dipeptide with both sorbents are 4–6 fold lower than those found for lysozyme, owing to the larger size of the molecule. From a practical point of view, some of the advantages of butyl-PA-glass may only be revealed during the isolation of highly hydrophobic proteins, such as bacterial lectins [8].

### *3.3. Semipreparative isolation of bacterial lectin on butyl-PA-glass*

The separation profile of the crude lectin extract is shown in Fig. 3. Almost all contaminants eluted in the flow-through fraction (fraction 1, Fig. 3) or within the descending ammonium sulfate gradient (fractions 2,3), whereas the lectin was eluted with 10 m*M* sodium phosphate (pH 6.8) as a single peak (fraction 4). The latter fraction exhibits almost all the initial hemaglutinating activity. No such activity was found in fractions 1–3. From the protein content in the crude and purified lectin samples we concluded that more than 98% of the contaminants were removed so that one-step HIC provided good purification of active SBL. A 1.8 mg amount of purified lectin was obtained.

Gradient SDS-PAGE carried out under non-reducing conditions (Fig. 4) showed the presence of lectin (80 kDa) in the purified sample (lane 4, Fig. 4), whereas the lower-molecular-mass contaminants were efficiently removed by HIC. SDS-PAGE under reducing conditions revealed two lectin subunits (43 and 36 kDa, data not shown). One cannot exclude, therefore, that the lower-molecular-mass band in Fig. 4 (40 kDa) represents one of the lectin subunits.

The better resolution of the crude lectin sample obtained with butyl-PA-glass as compared with butyl-Toyopearl 650C was reported by us previously [8]. Now we may assume that it is the weak hydrophobic character of butyl-PA-glass (or its high surface tension) that allows the separation of the hydrophobic lectin from the other proteins, since the latter elute already at high ammonium sulfate concentrations during the described HIC procedure.

As early as in 1972 Er-El et al. studied the use of homologous series of alkyl agaroses ($C_1$–$C_6$) for the purification of glycogen phosphorylase b [13]. By varying the alkyl chain length it was

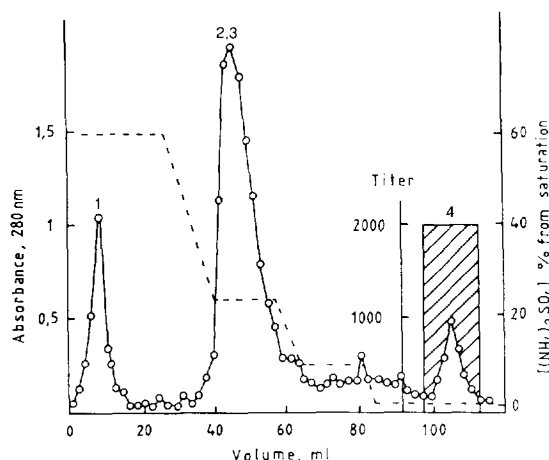


Fig. 3. Hydrophobic-interaction chromatography of sialic acid-binding lectin on butyl-PA-glass column. The dashed line indicates the concentration of ammonium sulfate in the eluent. The titer denotes the hemagglutinating activity of lectin. Fractions 2 and 3 denote the first and the second half of the major peak, respectively. For conditions see Experimental.

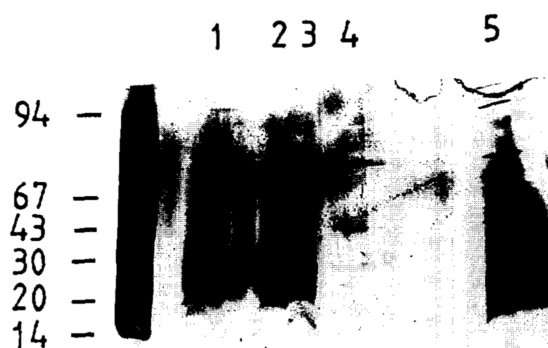


Fig. 4. SDS-PAGE of the chromatographic fractions eluted from butyl-PA-glass column, non-reducing conditions with 4–30% gradient gel; silver staining. The left lane shows the molecular mass markers. Lanes 1–4 correspond to the chromatographic fractions 1–4 of Fig. Lane 5 shows the crude lectin extract.

possible to adjust the tightness of the protein adsorption and to avoid strong retention, which subsequently requires drastic conditions for elution possibly causing denaturation of the eluted protein. The present study shows that besides the alkyl chain length, the chemical nature of the base polymer may be an important variable in searching for the optimal conditions for the separation of proteins.

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