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AFFINITY ADSORBENTS WITH POLYSACCHARIDE SPACERS

PREPARATION AND PROPERTIES

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SUMMARY

Soluble branched and neutral polysaccharides may be used as polymeric hydrophilic and inert spacers in affinity adsorbents. A series of methods for preparation of such adsorbents were developed. These methods involve introduction of a definite number of reactive functional groups into a polysaccharide molecule with subsequent coupling of the modified polysaccharide to a solid matrix, activation of the polysaccharide spacer and, finally, covalent binding of ligands of various chemical nature. The conditions are specified for the preparation of biospecific adsorbents containing hemoglobin, ribonuclease, poly(U), uridine, hexamethylenediamine and L-lysine as ligands and dextran, glycogen and amylopectin derivatives as spacers. The adsorbents having polysaccharide spacers were characterized by higher ligand concentrations and stability than analogous adsorbents without such spacers.

INTRODUCTION

Considerable progress has recently been achieved in the isolation and purification of biopolymers by affinity chromatography[1,2]. At the same time, however, certain disadvantages of this method can now be seen more clearly. The most important complications accompanying the biospecific purification are non-specific (hydrophobic, electrostatic) biopolymer–adsorbent interactions[3] and a gradual leakage of ligand from the support during chromatography[4].

The first phenomenon may be due to the use of hydrophobic spacers in biospecific adsorbents, while the second is inherent in the activation of polysaccharide supports by BrCN. The latter disadvantage becomes critical when very small amounts of protein are purified, for instance, in the case of protein receptors. Tesser *et al.*[5] have suggested that the leakage of ligands from the carriers can be prevented by the use of polymeric spacers. Such affinity adsorbents were first prepared by Wilchek[6]. Polypeptides and proteins were used as the spacers[7]. Such spacers would render the adsorbent highly stable due to a large number of binding points between the spacer and the solid support. Disruption of one or several spacer–support linkages would not lead to

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the release of the whole spacer and, consequently, of the coupled ligand. However, the multifunctionality of the protein spacers imparts undesirable ion-exchange properties to the adsorbent and leads to non-specific binding of biopolymers.

We have used branched water-soluble and neutral polysaccharides as polymeric hydrophilic and inert spacers in affinity adsorbents[8]. The non-specific binding during biochromatography was considered to be due to the combined action of hydrophobic and charged groups of the adsorbent[9]. Therefore, the polysaccharide spacers were expected to increase the biospecificity of the purification process, *i.e.*, to exclude or minimize hydrophobic and electrostatic interactions. In addition, such spacers should retain other advantages of polymeric spacers: (1) enable the location of a ligand far from the surface of a solid matrix; (2) increase the stability of the adsorbent; (3) provide a high ligand concentration on the adsorbent and possibly increase the adsorbent capacity. The use of polysaccharide spacers also provides a wider choice of chemical methods for subsequent covalent attachment of a ligand.

The present paper describes a series of chemical methods for attachment of water-soluble polysaccharides to solid supports and for subsequent ligand immobilization on the polysaccharide spacer. The syntheses of several new affinity adsorbents having polysaccharide spacers are presented, and some properties of the adsorbents are compared with analogous adsorbents without such spacers.

EXPERIMENTAL

Materials and methods

Sepharose 4B, Sephadex G-50 and Dextran T20 were obtained from Pharmacia (Uppsala, Sweden), Bio-Gel P-300 from Bio-Rad Labs. (Richmond, CA, U.S.A.), pancreatic ribonuclease, human hemoglobin and poly(U) from Reanal (Budapest, Hungary), dicyclohexylcarbodiimide (DCC) from Chemapol (Prague, Czechoslovakia), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Sigma (St. Louis, MO, U.S.A.), BrCN from Serva (Heidelberg, G.F.R.) and hexamethylenediamine from Koch-Light (Colnbrook, Great Britain). All other chemicals were from Soyuzreactiv (Moscow, U.S.S.R.).

Acetonitrile was distilled several times over P_2O_5 and then over K_2CO_3. Carboxymethyldextran (substitution degree, $\gamma = 5$, *i.e.*, 5 carboxymethyl groups per 100 anhydroglucose residues) was prepared as described[10]. Glycogen was isolated from rabbit liver as described in ref. 11.

UV spectra were recorded on SF-4 or Specord spectrophotometers. The chromatography was carried out on a Mini-Rac collector and a Uvicord-2 densitometer (LKB, Stockholm, Sweden). The BrCN activation of Sepharose was based on previous methods[12,13].

Ribonuclease was assayed as described previously[14]. Protein concentrations were determined by the method of Lowry *et al.*[15] or spectrophotometrically at 280 and 400 (for hemoglobin) nm.

Estimation of ligand contents in the adsorbents

The concentration of immobilized ligands was determined by: (a) a differential method (measuring the difference between the ligand content in the starting solution and in the filtrate and washings after coupling of the ligand to the support); (b) a

solubilization method[16] (measuring the UV absorption of gel aliquots solubilized by warming at 30°C in 80% acetic acid for 2 h); (c) a suspension method in gel[12] (measuring the UV absorption of a suspension of trinitrophenylated derivatives of NH_2-containing adsorbents in 0.05% agarose solution).

In accordance with method c, a slurry of 1 g (wet weight) of a washed NH_2-containing Sepharose, 4 ml of water, 10 ml of 2 *M* potassium borate buffer, pH 9.2, and 5 ml of a 0.1% solution of trinitrobenzenesulphonic acid (TNBS) containing 0.1% $NaHCO_3$ were stirred for 2 h at 37°C. The mixture was then filtered, the gel was thoroughly washed with water and dried at a water pump for 3 min. The samples of the TNBS derivatives obtained (0.15–0.90 g wet weight) were suspended in 1.5 ml of 0.1% agarose solution and 1.5 ml of 2 *M* potassium borate buffer, pH 9.2, containing 0.1% $NaHCO_3$. The absorption at 420 nm was then measured against an analogous suspension containing the corresponding amount of unsubstituted Sepharose. The calculation of the NH_2-group content was carried out as previously described[17].

Aminoethyl-Sepharose (1)

Sepharose (20 ml) was washed with water (200 ml) and transferred to a vessel containing 20 ml of 5 *M* potassium phosphate buffer, pH 11.9. A BrCN solution (prepared from 5 g of BrCN in 2.5 ml acetonitrile and subsequently diluted with 10 ml water) was added to the suspension for 2 min at 4°C with constant stirring. The mixture was vigorously stirred for another 2 min at 4°C, and the gel was then transferred to a glass filter and rapidly washed with 200 ml of cold water and 200 ml of cold 0.1 *M* $NaHCO_3$. The BrCN-activated Sepharose thus prepared was then stirred with 20 ml of an aqueous solution of ethylenediamine (2 mmoles per ml of packed volume), which had previously been adjusted to pH 10 with 6 *N* HCl. The gel was separated and washed with 200 ml of 0.1 *M* $NaHCO_3$, water, 200 ml of 0.1 *N* HCl and water. As shown by method c, adsorbent 1 contained 2.5 μequiv. of NH_2-groups per g wet weight.

Hydrazidosuccinyl-Sepharose (2)

BrCN-activated Sepharose (20 ml) (described above) was stirred for 5 h at 20°C with 20 ml of a saturated aqueous solution of succinic acid dihydrazide, adjusted to pH 8.0–8.5 with 6 *N* HCl. On completion of the reaction the gel was washed with water until the washings gave a negative reaction with TNBS. Finally the gel was washed with 200 ml of 0.2 *M* NaCl and 200 ml water.

Hemoglobin–dextran–Sepharose (6)

Step A. A 1.48-ml volume of 50% aqueous ethylenediamine, water (2 ml) and pyridine (5 ml) were added to a cooled solution (4°C) of carboxymethyldextran (3) (2 g, $\gamma = 5$)[10] in 6 ml water. DCC (1.91 g) in 5 ml pyridine was then added to the stirred solution, and the reaction mixture was stirred for 48 h at 20°C. The mixture was then diluted in three volumes of water, a precipitate was separated and the filtrate was extracted with diethyl ether (3 × 30 ml). The aqueous phase was evaporated, and the residue was dissolved in 20 ml water and applied to a column (volume 100 ml) packed with Sephadex G-50 and equilibrated with water. The excluded fractions were evaporated to 30 ml, the pH value was adjusted to 8–9 and the polymer was precipitated by two volumes of ethanol. The precipitate was separated and triturated first with 96% ethanol and then with absolute ethanol. The product was dried over P_2O_5 at

80°C/0.1 mmHg for 5 h, yielding 1.2 g (60.1 %) of aminoethylamidocarboxymethyldextran (4). Found: N 1.03 %.

Step B. A suspension of BrCN-activated Sepharose (8 ml) in 8 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl was stirred with 150 mg of polysaccharide 4. The Sepharose was then washed with water (100 ml). Measurement of the nitrogen content in washings by Kjeldahl's method indicated that 114 mg of the dextran derivative 4 were covalently bound to Sepharose. The gel was washed and then stirred in water with one drop of acetic acid and 2 mg of EDC at 20°C for 16 h. The "dextran–Sepharose" (5) thus prepared was used in the next step.

Step C. Seven millilitres of 2 *M* Na_2CO_3 and a solution of BrCN in acetonitrile (2 mg of BrCN per ml) were added to a cooled suspension of 7 ml "dextran–Sepharose" (5) in 7 ml water. The suspension was vigorously stirred at 4°C for 2 min and then filtered. The gel was washed with 100 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl and immediately added to a solution of hemoglobin (40 mg) in 8 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl. The mixture was stirred for 20 h at 4°C, after which the bioadsorbent (6) was separated and washed with 250 ml $NaHCO_3$ + 0.5 *M* NaCl, then with 50 ml of 0.1 *M* sodium acetate buffer + 1 *M* NaCl (pH 4) and 50 ml of 0.1 *M* sodium-borate buffer + 1 *M* NaCl (pH 8) (the last two washings were repeated three times). As shown by analytical data, adsorbent 6 contained 5.2 mg of protein per ml of gel. Direct coupling of hemoglobin with BrCN-activated Sepharose was carried out by the standard method[18]. The dark brown adsorbents were stored as aqueous suspensions in the presence of 0.02 % NaN_3.

Uridine–dextran–Sepharose (7)

A 10-ml volume of "dextran–Sepharose" (5) prepared as described above (B) was activated with 2.5 g BrCN and washed with 50 ml of cold 0.1 *M* phosphate buffer (pH 7.0). The gel was mixed with a solution of uridine (242 mg) in 10 ml of the same buffer, stirred for 16 h at 4°C, washed with 100 ml of water and then stirred with 10 ml of 1 *M* ethanolamine (pH 9) for 2 h at 20°C. The adsorbent was washed with water (50 ml), 0.2 *M* NaCl (50 ml) and water (50 ml). The UV spectrum of the solubilized gel showed that the adsorbent 7 contained 12 μmoles of uridine per ml of gel.

Ribonuclease–dextran–Sepharose (9)

Step A. Aminoethyl-Sepharose (1) (6 ml) was added to a solution of carboxymethyldextran (3) (0.5 g) in 6 ml water, the pH was adjusted to 4.7–5.0 with 1 *N* HCl and a solution of EDC (20 mg) in 3 ml water was gradually added. The mixture was stirred for 48 h at 20°C in the range pH 4.7–5.0, pH adjustments being made with 1 *N* HCl during the first hour. The gel was filtered off, and washed with water (100 ml), 0.1 *M* $NaHCO_3$ (100 ml) and water again (100 ml). A suspension of the gel in 6 ml water was stirred with a drop of acetic acid and 2 mg EDC for 16 h at 20°C. The residual carboxy groups were blocked with ethanolamine in the presence of EDC. The adsorbent (8) was washed with 1 l water and used in the next step.

Step B. A solution of $NaIO_4$ (130 mg) in 2 ml water was gradually added to a suspension of "dextran–Sepharose" (8) in 5.5 ml of sodium phosphate buffer, pH 6.0. The mixture was stirred in the dark for 1 h at 20°C. The activated gel was washed with 100 ml water, and a solution of ribonuclease (10 mg) in 10 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl was added. The suspension was stirred for 20 h at 20°C and

100 mg of $NaBH_4$ in 5 ml water were then added gradually. Stirring was continued for 1 h at 20°C, at which point the gel was washed as described for sorbent 6. The adsorbent (9) contained 6 mg protein per ml of gel as revealed by the differential spectrophotometric method as well as by enzyme activity measurements in the starting protein solution and in the washings after protein coupling. For comparative purposes, "ribonuclease–Sepharose" was also prepared by the standard method. Adsorbent 9 was stored for a long time at 4°C as an aqueous suspension in the presence of 0.02% NaN_3.

Glycogen–hydrazidosuccinyl-Sepharose (10)

Step A. A solution of $NaIO_4$ (331 mg) in 3 ml water was gradually added to a solution of glycogen (2.41 g) in 15 ml water, with stirring and cooling in the dark. The mixture was stirred for 1.5 h at 20°C in the dark, the pH (6.5) being adjusted with 0.1 *M* NaOH. The mixture was then diluted to 35 ml with 0.1 *M* sodium acetate buffer, pH 4.8, and used in the next step.

Step B. Hydrazidosuccinyl-Sepharose (2) (30 ml) was washed with 150 ml of 0.1 *M* sodium acetate buffer, pH 4.8, and stirred with 35 ml of the solution of periodate-oxidized glycogen (see *Step A*) for 16 h at 4°C. The gel was then washed with 5 l water, 200 ml of 2 *M* NaCl and 200 ml water and suspended in 30 ml of 0.1 *M* $NaHCO_3$. Solid $NaBH_4$ (600 mg) was added to the suspension at 4°C, and the mixture was stirred for 3 h at 4°C. The gel was then washed with 1 l of water. The washings after glycogen coupling were concentrated *in vacuo* at 40°C and used for determination of the amount of unbound ligand by the phenol sulphate method. The gel was stirred with 30 ml of 0.2 *M* acetaldehyde in 0.1 *M* sodium acetate buffer for 2 h at 4°C, washed with water and stirred with 100 mg of $NaBH_4$ for 2 h at 4°C. The adsorbent prepared (10) was washed with 1 l of water.

Aminohexyl-hydrazidoadipinyl–glycogen–hydrazidosuccinyl-Sepharose (11)

Step A. Glycogen–hydrazidosuccinyl-Sepharose (10) (10 ml) was activated with 2.5 g BrCN as described above. The activated gel was washed with 100 ml of cold water and then stirred with 10 ml of a saturated solution of adipinic acid dihydrazide in water (pH 8.0–8.5) for 16 h at 4°C. The gel was washed with 100 ml water, 100 ml of 0.2 *M* NaCl and 100 ml water. It became bright red after treatment with TNBS in potassium borate buffer (pH 9.2)[19].

Step B. The adsorbent prepared as above (10 ml) was washed with 50 ml of 0.5 *N* HCl and stirred with 10 ml of 0.1 *M* $NaNO_2$ in water for 7 min at 4°C. The gel was rapidly washed with cold water and stirred with 10 ml of 0.2 *M* hexamethylenediamine in 0.2 *M* Na_2CO_3 for 8 h at 4°C and pH 9.0–9.2. After washing with 100 ml water, the gel was stirred with 10 ml of ammonium buffer (2 *M* NH_4Cl and 1 *M* NH_4OH, pH 8.8) and then washed with water. The adsorbent (11) contained 3.77 μequiv. of NH_2 groups per g of gel (wet weight).

L-*Lysine hydrazide (12)*

Hydrazine hydrate (8.75 ml, 0.174 moles) was gradually added to a suspension of 2.8 g (0.0174 moles) of the hydrochloride of L-lysine methyl ester[20] in 30 ml methanol. The mixture was stirred for 50 h at 20°C, the reaction being monitored by thin-layer chromatography (TLC) on Silufol [solvent, ethanol–ammonia (8:2)]. The

mixture was evaporated and the residue was treated by 4 *M* hydrogen chloride in dioxan with cooling; the precipitate was collected, washed with diethyl ether and recrystallized from ethanol. After drying the crystals at 60°C/0.1 mmHg, the yield was 1.53 g (54%); m.p. 225°C (lit.[21] 225–228°C), R_F 0.32.

L-Lysine–amylopectin–hydrazidosuccinyl-Sepharose (13)

Amylopectin–hydrazidosuccinyl-Sepharose was prepared as described for glycogen–hydrazidosuccinyl-Sepharose. This sorbent (10 ml) in 10 ml of potassium phosphate buffer, pH 6.0, was activated with a solution of $NaIO_4$ (140 mg) in 1 ml water. The suspension was stirred for 1 h at 20°C in the dark, after which the gel was thoroughly washed with water. It was then stirred with a solution of 1 g of L-lysine hydrazide (12) in 10 ml of 0.1 *M* potassium phosphate buffer, pH 6.0, for 1.5–2.0 h at 20°C and washed with water until the filtrate gave a negative reaction with TNBS. The gel was then suspended in 10 ml water and stirred with 100 mg of $NaBH_4$ in 2 ml water for 0.5 h at 20°C. After washing with water, the adsorbent (13) containing 2 μequiv. of NH_2 groups per g of gel (wet weight) was obtained.

L-Lysine–glutaraldehyde–hydrazidosuccinyl-Sepharose (14)

A suspension of hydrazidosuccinyl-Sepharose (2) (10 ml) in 4 ml water was stirred with 6 ml of 25% aqueous glutaraldehyde for 30 min at 20°C; the gel was washed with 100 ml water and transferred to a solution of 1 g of L-lysine hydrazide (12) (100-fold excess) in 10 ml of potassium phosphate buffer, pH 6. The mixture was stirred for 30 min at 20°C, the pH being maintained at 6.0 with 6 *N* HCl. The gel was filtered off and washed with the same buffer (100 ml) and water (100 ml). The gel was treated with $NaBH_4$ (100 mg) in 10 ml water for 30 min at 20°C and washed with water. The adsorbent (14) contained 0.8 μequiv. of NH_2 groups per g wet weight (the suspension method).

Poly(U)–glycogen–Bio-Gel P-300 (15)

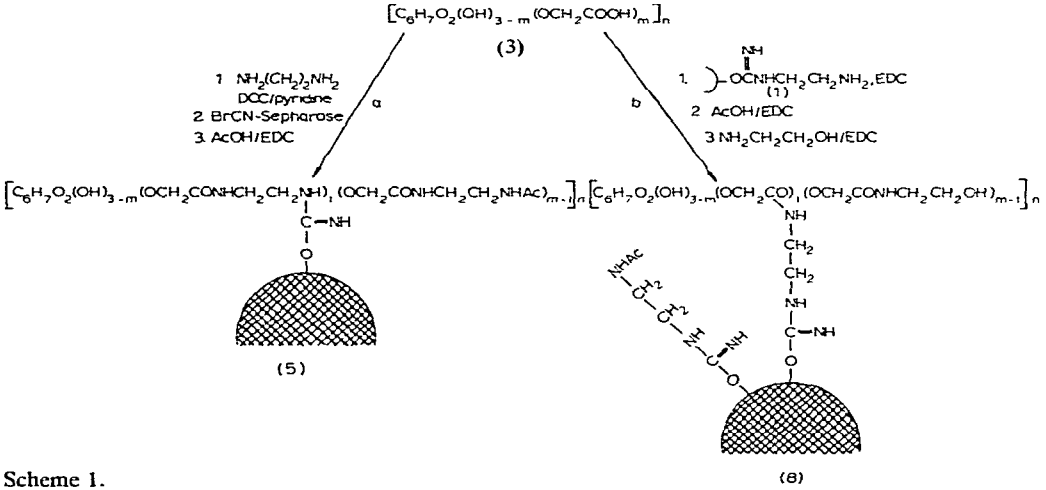
Periodate-oxidized glycogen (2.41 g) was coupled to a hydrazide derivative of Bio-Gel P-300[22] under the conditions described for sorbent 10. After the stabilization of bonds by treatment with $NaBH_4$, followed by blocking the excess of hydrazide groups with acetaldehyde–$NaBH_4$ and activation of a glycogen spacer by BrCN (4 g), the coupling of poly(U) (45 mg) with the gel was carried out in 0.1 *M* potassium phosphate buffer, pH 7.5 (16 h, 4°C). The gel was washed with the same buffer and then treated with ethanolamine to remove the excess of active groups. The adsorbent (15) contained 2 mg of poly(U) per ml of gel (the differential method).

RESULTS AND DISCUSSION

A number of methods have been described for the coupling of polysaccharides to solid supports[3]. The polysaccharides were used mainly as ligands. The general use of water-soluble polysaccharides as spacers in affinity adsorbents has not yet been reported. The methods described here consist in the introduction of reactive functional groups into a water-soluble polysaccharide and subsequent covalent binding of the modified polysaccharide to a support using these reactive groups. Activation of a polysaccharide spacer enabled the immobilization of ligands of different chemical nature.

Affinity adsorbents with dextran spacers

Dextran was chosen as a model water-soluble polysaccharide for the preparation of hemoglobin–dextran–Sepharose (6). The degree of branching of the dextran molecule was due to the presence of ≈5% of α-(1–3) bonds. Dextran was converted into carboxymethyldextran containing 5 carboxymethyl groups per 100 anhydroglucose residues ($\gamma = 5$)[10]. Immobilization of carboxymethyldextran on Sepharose may be carried out by either (a) condensation with an excess of ethylenediamine in aqueous pyridine in the presence of DCC and subsequent coupling with BrCN-activated Sepharose, or (b) condensation with aminoethyl-Sepharose (1) in the presence of the water-soluble carbodiimide, subsequent acetylation of residual free NH_2 groups by acetic acid in the presence of EDC and blocking of unreacted carboxy groups with ethanolamine–EDC (Scheme 1).



Scheme 1.

In the synthesis of adsorbent 6, the introduction of a polysaccharide spacer was carried out by method a. The modified dextran (4) contained 1.03% N, suggesting amidation of all the carboxymethyl groups in the starting compound (3). Polysaccharide 4 was coupled to BrCN-activated Sepharose by the usual method. The amount of dextran coupled to the gel (14.2 mg per ml of gel) was determined by the differential method from the nitrogen content (by Kjeldahl's method). Blocking of residual active groups on Sepharose was carried out by treatment with ethanolamine, and the free NH_2 groups on the polysaccharide spacer were acetylated with acetic acid in the presence of EDC. Attachment of hemoglobin to dextran-Sepharose (5) was performed after activation of the polysaccharide spacer by the BrCN method. Adsorbent 6 contained *ca.* 5.5 mg of protein per ml of gel (the differential method). It may be assumed that the ligand is mainly localized on the polysaccharide spacer; repeated activation of an agarose support by BrCN should proceed to only insignificant degree. The bioadsorbent hemoglobin–dextran–Sepharose (6) may be used for purification of proteolytic enzymes[23]. It was very stable: no leakage of hemoglobin was visible during the storage of the adsorbent in water in the presence of 0.02% NaN_3 for 1 year at 4°C.

The adsorbent uridine–dextran–Sepharose (7) was prepared by a similar method. Dextran–Sepharose (5) was activated with BrCN and stirred with uridine at pH 7.0. Although the chemical structure of adsorbent 7 was not strictly uniform, it may be assumed that uridine couples to a polysaccharide spacer via position 4 of the pyrimidine ring, as is the case in the preparation of polyU–Sepharose[24]. Adsorbent 7 (12 μmoles of uridine per ml of gel) may be used for the purification of enzymes posessing an affinity for the immobilized nucleoside, *e.g.*, uridine kinase[25].

An adsorbent may lose its stability if a low-molecular-weight ligand is singly coupled to a polysaccharide spacer by the BrCN method. In this case gradual leakage of the ligand would take place in accordance with the general mechanism for BrCN-activated polysaccharides[4]. Therefore, we tested another method for the polysaccharide spacer activation, namely periodate oxidation[26], followed by coupling with a ligand containing primary amino groups and stabilization of a ligand–spacer bond by $NaBH_4$. (In the case of a protein ligand the treatment with $NaBH_4$ could be omitted without a decrease in the adsorbent stability.)

Aldehyde matrices prepared by periodate oxidation of polysaccharide supports or glass beads covered with dextran have recently been used[27] for enzyme immobilization by the method of reductive alkylation. Junowicz and Charm[28] described further modifications of periodate-treated polysaccharide supports designed for introduction of reactive hydrazide and azide groups into the matrix. The polysaccharide spacers permitted introduction of a large number of aldehyde groups into an adsorbent by a simple and convenient treatment with periodate.

The biospecific adsorbent ribonuclease–dextran–Sepharose (9), which may be used for purification of enzyme inhibitors, was prepared. In this case the introduction of a polysaccharide spacer into the solid support was achieved by the method b (Scheme 1). Dextran-Sepharose (8) was then treated with sodium periodate, condensed with ribonuclease and the protein–spacer bonds were stabilized with $NaBH_4$. The adsorbent (9) thus prepared containing 6 mg of the enzyme per ml of gel.

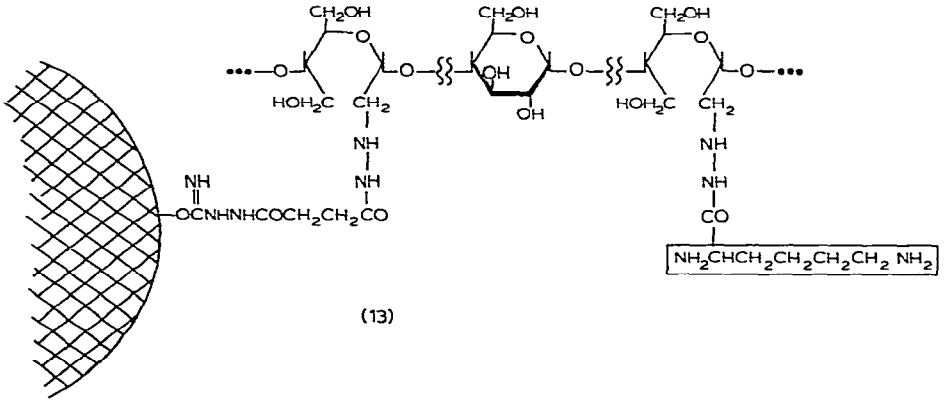
Affinity adsorbents with glycogen and amylopectin spacers

Attachment of ligands or spacers containing NH_2 groups to BrCN-activated polysaccharide supports results in the introduction of a positive charge because of ionization of the isourea groups formed (pK 9.6–10.4)[29,30]. This charge imparts ion-exchange properties to the adsorbent and may interfere with the biospecificity of the purification procedure. In order to avoid the introduction of charged groups we used α,ω-dicarbonic acid dihydrazides for binding water-soluble polysaccharides to the agarose matrix. Thus the treatment of BrCN-activated Sepharose with an excess of succinic acid dihydrazide leads to hydrazide–Sepharose which is uncharged at the physiological pH (pK 4.2)[29]. The polysaccharide spacers can be introduced into this sorbent after water-soluble polysaccharide activation with BrCN or periodate.

This approach was applied to the preparation of aminohexyl-hydrazidoadipinylglycogen–hydrazidosuccinyl-Sepharose (11). A similar adsorbent without the polysaccharide spacer has been successfully used for purification of pig kidney diamine oxidase[31]. Hydrazidosuccinyl-Sepharose (2) was incubated with glycogen which had previously been oxidized by treatment with periodate. The oxidation was carried out for 5% of the anhydroglucose residues of the polysaccharide. After the coupling glycogen, the gel was treated with $NaBH_4$, and the residual

free hydrazido groups were blocked by treatment with acetaldehyde and then with $NaBH_4$. The content of glycogen in the gel was calculated to be 25–30 mg per ml of gel. Then, adipinic acid dihydrazide was coupled to the BrCN-activated polysaccharide spacer. Subsequent attachment of hexamethylenediamine to the hydrazide-adsorbent was carried out via the corresponding azide as described by Klimova *et al.*[31]. At the physiological pH, adsorbent 11 did not contain any additional cationic groups with the exception of the terminal NH_2 groups. The amount of bound hexamethylenediamine (3.77 μmoles/g wet weight) was determined by the suspension method[17].

Various natural polysaccharides as well as synthetic and natural oligosaccharides may be used as spacers in the affinity adsorbents. One of the most readily available branched water-soluble polysaccharides, amylopectin, was used as a spacer for preparation of L-lysine–amylopectin–hydrazidosuccinyl-Sepharose (13). Amylopectin was immobilized on hydrazidosuccinyl-Sepharose (2) as described for immobilization of glycogen on the same sorbent (2). After activation of the amylopectin spacer with sodium periodate, the aldehyde matrix prepared was stirred for 1.5–2.0 h at 20°C and pH 6.0 with L-lysine hydrazide (12) and then treated with $NaBH_4$. It may be assumed that the ligand (12) is mainly attached through the hydrazide group. This immobilization procedure results in the formation of an adsorbent containing 1,5-diaminopentane, substrate of diamine oxidase, as ligand (Scheme 2). Therefore adsorbent 13 could be used for purification of this enzyme.



(13)

Scheme 2.

Adsorbent 13 contained 2 μequiv. of amino groups per g of gel (wet weight). A similar adsorbent having a monomeric spacer, L-lysine–glutaraldehyde–hydrazidosuccinyl-Sepharose (14), was also synthesized for comparison.

Affinity adsorbents having polysaccharide spacers may be synthesized on the basis of various supports including polyacrylamide or glass beads, porous silica gel, Spheron, etc. The preparation of poly(U)–glycogen–Bio-Gel P-300 (15) was considered as an example of such possibilities. Bio-Gel P-300 was converted into the hydrazido-derivative[22]. Then, periodate-oxidized glycogen was coupled to the hydrazido matrix as described for adsorbent 10. Activation of the glycogen spacer by BrCN and subsequent coupling of poly(U) resulted in formation of adsorbent 15 containing 2 mg of poly(U) per ml of gel (the differential method).

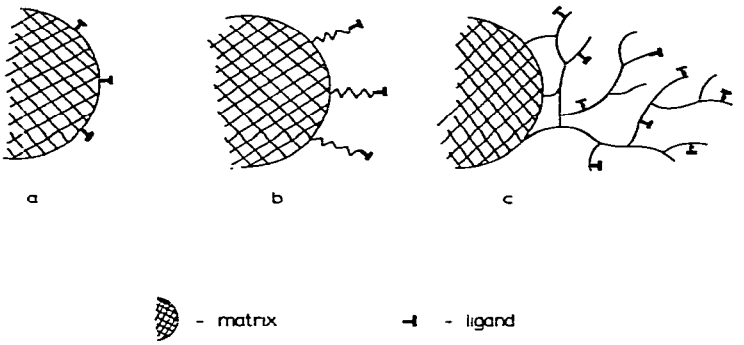


Fig. 1. The different types of affinity adsorbents: a, without a spacer; b, with a monomeric spacer; c, with a polysaccharide spacer.

The experiments described enable the preparation of stable biospecific adsorbents. The amount of the ligands coupled via the polysaccharide spacers (Fig. 1) was considerably higher than that for adsorbents without the polymeric spacer (Table I).

TABLE I

COMPARISON OF THE LIGAND CONTENTS IN THE ADSORBENTS WITH OR WITHOUT POLYSACCHARIDE SPACERS

Adsorbent	*Ligand content*
Hemoglobin–dextran–Sepharose (6)	5.2 mg/ml
Hemoglobin–Sepharose	1.5 mg/ml
Ribonuclease–dextran–Sepharose (9)	6.0 mg/ml
Ribonuclease–Sepharose	2.0–2.5 mg/ml
Uridine–dextran–Sepharose (7)	12 μmoles/ml
Uridine–Sepharose	8 μmoles/ml
L-Lysine–amylopectin–hydrazidosuccinyl-Sepharose (13)	2 μequiv./g wet weight
L-Lysine–glutaraldehyde–hydrazidosuccinyl-Sepharose (14)	0.8 μequiv./g wet weight

The use of polysaccharide spacers in affinity chromatography offers an additional possibility for elution of adsorbed biopolymers. The elution procedure is frequently the most complicated step in biospecific purification. The use of high concentrations of denaturating agents, solutions having extreme pH values or organic compounds are sometimes required for successful desorption. In these cases denaturation of biopolymers, especially enzymes, may occur. In the presence of polysaccharide spacers this complication may be overcome by destruction of the spacer by means of the corresponding enzyme (for instance, dextranase or amylase). A similar technique has been applied for elution of cells from adsorbents based on Sephadex[32] or gelatin–Sepharose[33].

Affinity chromatography on adsorbents having polysaccharide spacers is complicated if protein impurities possessing affinity for the spacer itself are present in a

crude enzyme preparation. In this case preliminary chromatography on a column of a "polysaccharide spacer–support" adsorbent is recommended. This adsorbent would selectively bind the biopolymers possessing the affinity for the spacer. The "polysaccharide spacer–support" system may be used as a biospecific adsorbent for purification of proteins with affinity for a spacer (for instance, glycogen–Sepharose for purification of α-amylase[34], glycogen phosphorylase[35], etc.).

In this paper we have described some methods for introducing polysaccharide spacers into various supports. The development of new methods for immobilization of biologically active compounds on polysaccharides provides ample opportunities for the attachment of various organic compounds to polysaccharide spacers. In the case of protein ligands the method can also be used for enzyme immobilization.

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