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High-performance affinity chromatography of insulin on coated silica grafted with sialic acid

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Abstract

High-performance liquid affinity chromatography (HPLAC) is a powerful method for the purification of biological compounds, owing to its specificity, speed and high resolution. We developed new chromatographic supports based on porous silica beads. In order to minimize non-specific interaction between the silanol groups at the silica surface and biological molecules, the beads are coated with dextran carrying a calculated amount of positively charged functions. Such supports have the mechanical properties of the starting inorganic material. Moreover, they can be easily activated and functionalized by active ligands using conventional coupling methods. In the present study, N-acetylneuraminic acid (NANA), a member of the sialic acid family, is coupled to dextran coated silica beads to obtain affinity supports. This class of compounds seems to play an important role in the cell recognition mechanism. In particular, sialic acids are present in the structure of the cellular receptors for insulin. By HPLAC, we can study the interactions between coated silica grafted with NANA and insulin. It is also possible to use these active supports to purify the compounds by affinity chromatography. However, it is important to determine and optimize the conditions for adsorption and desorption of insulin on supports grafted with sialic acid and to estimate the chromatographic performances of these active phases.

1. Introduction

High-performance liquid affinity chromatography (HPLAC) combines the specificity of affinity chromatography with the speed and efficiency of HPLC techniques. It requires the use and design of high pressure-resistant supports with the excellent chromatographic properties of the specific ligands present. Porous silica exhibits excellent mechanical properties, and has a well controlled granulometry and defined porosity. However, negative surface charges caused by the presence of acidic silanol functions in aqueous media make it hardly usable in its native state. Thus, the silica surface has to be modified in order to obtain a support which would be neutral enough to avoid non-specific interactions with proteins in solution [1]. Various methods of inactivation can be used to eliminate those non-specific interactions. This can be done by chemical modification or by coating the silica with a layer of hydrophilic polymer [2]. For this purpose, silica beads are coated with dextran polymers carrying a calculated amount of positively charged diethylaminoethyl groups (DEAE) [2]. After adsorption of the polymer at silica surface, the dextran layer is

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crosslinked in order to obtain a solid polymer coating.

The degree of inactivation of dextran-coated silica (SID) is determined by high performance size-exclusion chromatography (HPSEC) of standard proteins [3]. Then, the support can be grafted with a biospecific ligand and be used for HPLAC.

The choice of a ligand for affinity chromatography depends on two factors: (i) it must exhibit a specific and reversible binding affinity towards the product to be purified and (ii) it must possess modifiable chemical groups allowing its coupling to the support without loss of activity. N-Acetylneuraminic acid (NANA), used here as a ligand, belongs to the family of sialic acids. These molecules usually occupy the non-reducing end of oligosaccharide chains and are involved in intercellular recognition phenomena and in host–parasite interactions [4,5]. The existence of sialic acid-like sugars in the insulin receptor [6-8] suggests that such compounds could exhibit specific interactions (mimicking insulin-receptor interactions) usable in affinity chromatography. The performance of a SID support functionalized with N-acetylneuraminic acid (SID-NANA) to develop specific interactions for insulin derivatives in solution and to separate insulin are studied by affinity chromatography.

2. Experimental

2.1. Synthesis of SID-NANA chromatographic support

Preparation of the affinity support is performed in two steps: first, coating of silica with DEAE-substituted dextran; second, ligand immobilisation (Fig. 1).

Coating of silica beads

Substitution of dextran with diethylaminoethane (DEAE)

Dextrans (T40: 35 600 Da; T70: 68 000 Da; T500: 488 000 Da; Pharmacia, Bois d'Arcy, France) are substituted with positively charged

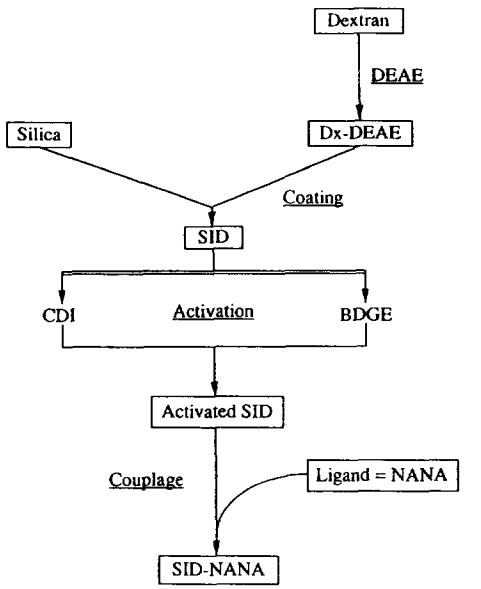

Fig. 1. Synthesis of chromatographic support.

DEAE groups. This substitution reaction takes place through the formation of an ether bond between the DEAE group and an hydroxyl function of the glucose unit, probably in position 2 [3]. The synthesis of the DEAE-dextrans is achieved by a controlled reaction of the hydrochloride, 2-chloro-N,N-diethylaminoethane hydrochloride (Janssen Chimica, Noisy le Grand, France) with the native polymer in a very alkaline medium using the following procedure: 40 g of dextran and 79 g of sodium hydroxide each separately dissolved in 20 ml of doubly distilled water at 22°C are cooled and mixed at 4°C. The mixture is stirred for 20 min and 42.7 g of 2-chloro-N,N-diethylaminoethane hydrochloride are added. The temperature of the mixture is raised to 55°C. The reaction is allowed to continue for various times to achieve the desired substitution ratio. After reaction, the mixture is rapidly cooled in an ice bath and the pH of the mixture is adjusted to 9.0 using concentrated hydrochloric acid. The DEAE-dextran polymer is precipitated with methanol, filtered, washed several times with ethanol and dried under

vacuum at 40°C overnight. The modified polymer is characterized by potentiometric titration and by elemental analysis of nitrogen.

Coating of silica beads with DEAE-dextran

A batch method followed by a crosslinking reaction are used to coat silica beads with DEAE-dextran. A 10-g quantity of DEAE-dextran is dissolved in 100 ml of doubly distilled water at 22°C. The pH of the solution is adjusted to 11.5 by addition of 1 *M* sodium hydroxide. A 5-g quantity of silica beads (15–25 μm diameter, 1250 Å porosity, Biosepra, Villeneuve-la-Garenne, France) degassed under vacuum for 1 h, is gently added to the solution. After a 30-min impregnation period, the slurry is filtered and dried for 15 h at 80°C and the resulting powder sieved. Then, 10 g of impregnated silica are added to 20 ml of a solution of diethyl ether containing 0.3% (v/v) 1,4-butanedioldiglycidyl ether (BDGE). The mixture is stirred for 30 min at 40°C. After evaporation of the solvent, the silica powder is dried for 15 h at 80°C and the product sieved. The amount of Dx-DEAE (dextran substituted by DEAE groups) covering the silica beads is determined by a colorimetric assay of the sugar units after acid hydrolysis and by elemental analysis of carbon.

Functionalization of the coated silica by sialic acid

Coupling of the ligand, NANA, is achieved in two different ways, using either carbonyldiimidazole (CDI) or BDGE, according to the two following protocols:

–CDI reacts with the hydroxyl functions of the dextran coating of the silica, transforming these functions into active groups [9]. Those active groups react mainly with the primary amine functions of active ligands but may possibly react with other chemical functions. The coupling reaction is performed as follows: 5 g of SID are suspended in 25 ml of 1,4-dioxan containing 1 g CDI (Sigma, La Verpillière, France). The mixture is gently stirred for 2 h at room temperature. The support is then washed with 200 ml of dioxan and with 300 ml of carbonate buffer (NaH_2CO_3, 0.1 *M*, pH 8.7). The activated support is resuspended in 25 ml of carbonate buffer containing 100 mg of NANA extracted from edible bird's nest [10]. The mixture is stirred for 48 h at room temperature, washed with Tris-HCl buffer (Tris, 0.1 *M*, pH 8.7) and stirred gently for 3 h in 25 ml of Tris-HCl buffer to deactivate the excess coupling reagent. After reaction the support is successively washed with Tris-HCl buffer, doubly distilled water, 2 *M* NaCl, doubly distilled water, and finally equilibrated in the buffer initially used in the chromatographic separation.

–One of the epoxide groups of the diepoxide compound BDGE reacts with the hydroxyl functions of the dextran polymers covering the silica beads, forming an ether bond. The other epoxide group of BDGE remains free to react with the hydroxyl function of NANA. The coupling reaction is performed as follows: 5 g of SID are suspended in 50 ml of diethyl ether containing 20 μl of BDGE. The mixture is gently stirred for 30 min at room temperature. The solid phase is filtered and washed with diethyl ether. After evaporation of the diethyl ether at 40°C, the support is dried for 30 min at 80°C under vacuum and washed with carbonate buffer (NaH_2CO_3, 0.1 *M*, pH 8.7). The coupling of NANA is performed as described above.

2.2. Insulin affinity under static (batch) conditions

For the determination of adsorption isotherms, a variable amount of insulin is incubated with a fixed amount of support. After a pre-set time, the supernatant is removed and the amount of remaining protein measured by radioimmunoassay (RIA). The amount of insulin adsorbed on the support can than be calculated as a function of its initial concentration.

The insulin concentration was varied from 5 to 100 μg/ml; 100 μl of support suspension (50–100 mg/ml) are incubated with 200 μl of insulin solution at various concentrations in a polystyrene tube for 30 min at room temperature. After phase separation, the amount of residual insulin in the supernatant is determined by RIA. The affinity constant under static conditions can

be calculated from the adsorption isotherms using the Langmuir model.

2.3. Insulin affinity for SID-NANA under dynamic conditions (HPLC)

The HPLC apparatus used in this study consisted of a pump (L-6210 Intelligent Pump, Merck) monitored by a programmer and equipped with an injection valve (Rheodyne 9126, Merck), connected to a UV-Vis spectrophotometer (L-4000 UV detector, Merck), an integrator (D-2520 GPC Integrator, Merck) and a fraction collector (Model 203, Gilson).

Standard proteins used in HPSEC were from Sigma (Saint-Quentin Fallavier, France) and eluted with phosphate buffer (0.05 *M* NaH_2PO_4, 0.15 *M* NaCl, pH 7.4).

Insulin used in HPLAC, kindly provided by Diosynth S.A. (AKZO, Eragny-Sur-Epte, France), was a mixture of porcine insulin with 6% bovine insulin. The insulin was dissolved in various buffers at a concentration of 1 mg/ml and 100-μl volumes were injected.

Chromatographic fractions were analysed by electrophoresis (SDS-PAGE) (Fast System, Pharmacia, Saint Quentin-Yvelines, France) and by Bradford assay.

3. Results and discussion

3.1. Synthesis of SID-NANA

DEAE groups are attached to the saccharidic units of dextran in order to confer a weak anion-exchange capacity to the polymer and to make it usable for silica coating. In the first step DEAE-dextran is adsorbed to the silica. In a second step, the polysaccharide chains are crosslinked in order to increase the stability of the final support. The results of substitution and coating for different silica materials, as determined by elemental analysis are presented in Table 1.

Interactions between the positively charged polymer (Dx-DEAE) and silica are studied by determining the adsorption isotherms of modified dextrans of variable molecular mass (T40: 35 600 Da, T70: 68 000 Da, T500: 488 000 Da) on the silica surface (Fig. 2).

The amount of adsorbed polymer increases with the amount of polymer added until it reaches a saturation plateau. The maximum amount of adsorbed dextran increases with the molecular mass of the starting polymer; thus, the maximal adsorbable amount is obtained with T500 dextran. However, the quantity of adsorbed polymer is only one of the parameters that characterize the interfacial macromolecular layer, and it is necessary to take into account the substitution ratio of the dextran and the ratio of crosslinkage of the coating polymer. It would also be interesting to determine the thickness of the polymeric layer and the dynamics of its macromolecular chains.

In order to ascertain that the stationary phase which is used for coupling of the ligand, will not develop non-specific interactions with standard proteins, these proteins (Table 2) are eluted on the SID support under HPSEC conditions (Fig. 3). The elution of these proteins according to their molecular mass shows that the interactions

Table 1
Characteristics of the coated silica supports

	Silica		Polymer		
	Porosity (Å)	Diameter (mm)	Type	DEAE (%)	Coating (mg Dx-DEAE/g silica)
SID1	1250	15–25	T40	7	73
SID2	1250	15–25	T70	5	35
SID3	1250	15–25	T500	6	37

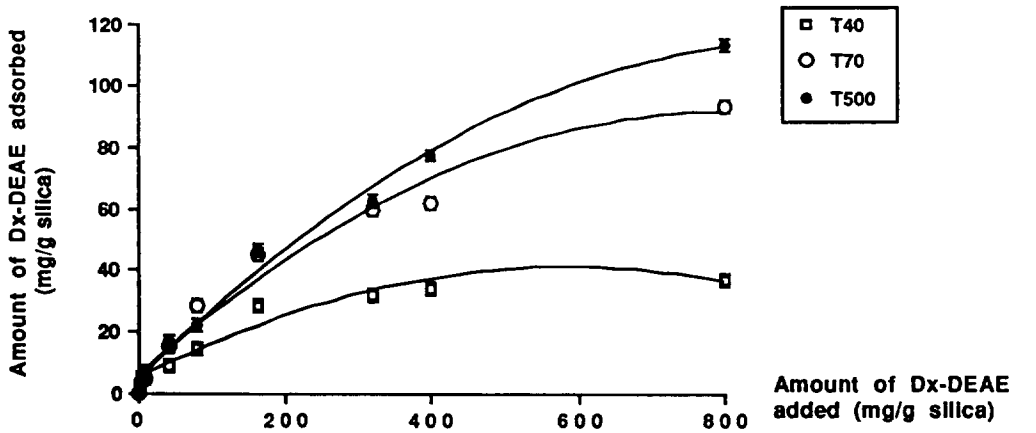

Fig. 2. Adsorption isotherms of dextrans-DEAE on silica.

between the support and these proteins are minimal. According to these results, it seems that Dx-DEAE covers all accessible protein areas of the support, which prevents non-specific interactions and increases the importance of the specific interactions in the affinity process after ligand coupling.

Ligand (NANA) immobilisation on the stationary phase requires the use of a coupling agent to create covalent bonding between the ligand and the support. The coupling agents used in the present study have been carefully chosen, so that we know the chemical functions of the sialic acid that may be involved in the covalent bond. In this study, CDI and BDGE were used. Activation with BDGE occurs by condensation of a hydroxyl group on the polysaccharidic support and various functions on the ligand, especially hydroxyl groups [11], leading to the formation of an ether-type bonding. Activation with CDI also involves ligand attachment via hydroxyl functions, as for BDGE.

The amount of NANA coupled with SID is determined by a colorimetric assay of the coupling solution supernatant using a periodate–resorcinol method [12] and by determining, by substraction, the amount of sialic acid fixed on the support. The quantity of NANA fixed on SID-NANA support using either CDI or BDGE as coupling agent is 10 mg/g silica.

Table 2
Protein standards

Name	M_r (g/mol)	log M_r	Elution time (min)
DL-Serin	105.09	2.02	2.90
L-Threonin	119.12	2.07	2.88
Asp-Ala-His	341.40	2.53	2.82
Cytochrome c	12 384	4.09	2.56
Lysozyme	14 400	4.15	2.46
Myoglobulin	17 800	4.25	2.05
Egg albumin	45 000	4.63	1.72
Bovin albumin	66 000	4.82	1.69
Bovin thyroglobulin	669 000	5.82	1.47
Dextran blue	2 000 000	6.30	1.41

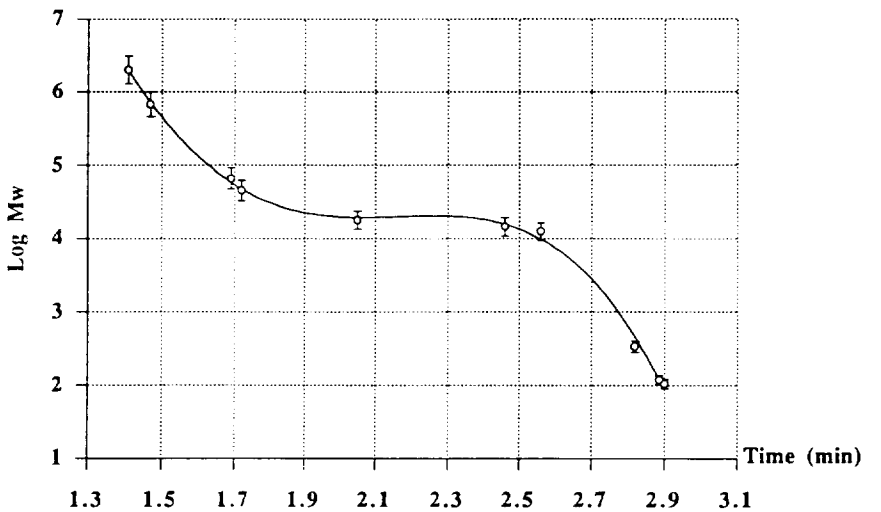

Fig. 3. Molecular mass calibration graph for proteins. Column 12.5 × 0.4 cm I.D., flow-rate 0.5 ml/min, buffer 0.05 *M* phosphate, 0.15 *M* NaCl (pH 7.4).

3.2. Affinity of insulin for SID-NANA under static (batch) conditions

Prior to studying the affinity by HPLAC, adsorption isotherms for insulin on the support are established in order to define the potential selectivity of the ligand and to estimate the strength of the ligand–insulin interactions by measurement of the affinity constants. The basic support is made of silica coated with dextran (T70 dextran, 4.9% DEAE groups; 35 mg of Dx-DEAE/g silica). The ligand (NANA) is coupled to this support in two different ways: by CDI (SID-CDI-NANA) or by BDGE (SID-BDGE-NANA). The performance of these supports is first tested under static conditions, and then under dynamic (high-performance liquid affinity chromatography) conditions. Adsorption isotherms are shown in Fig. 4. The amount of insulin adsorbed increases with the amount of insulin initially present until it reaches a saturation plateau corresponding to the capacity of the active support which is about 10^{-9} mol/l/g (independent of the coupling agent used). From these adsorption isotherms, affinity constants of insulin for both supports have been determined, according to the Langmuir model [13]. The Langmuir isotherms are shown in Fig. 5. Affinity constants (Table 3) are similar (10^{-8} M^{-1}) for the two types of supports under the experimental conditions used.

These results indicate the existence of strong interactions between insulin and the two inactivated silica supports grafted with NANA.

3.3. Affinity of insulin for SID-NANA under dynamic (HPLAC) conditions

Elution of insulin is achieved on both supports, activated either with CDI (SID-CDI-NANA) or with BDGE (SID-BDGE-NANA) under the same experimental conditions.

A 100-μl quantity of insulin (porcine + 6% bovine, dissolved in various adsorption buffers at a concentration of 1 mg/ml) is injected onto the column at a flow-rate of 0.5 ml/min. The conditions for adsorption of insulin on the support are described in Fig. 6. The fractions corresponding to elution and desorption peaks are collected, lyophilized and analysed by SDS-PAGE. As determined by electrophoresis, insulin is present in both eluted and retained fractions. The protein concentration in the different fractions is determined by the Bradford assay (Table 4). Under these conditions, the amounts of insulin retained on the SID-CDI-

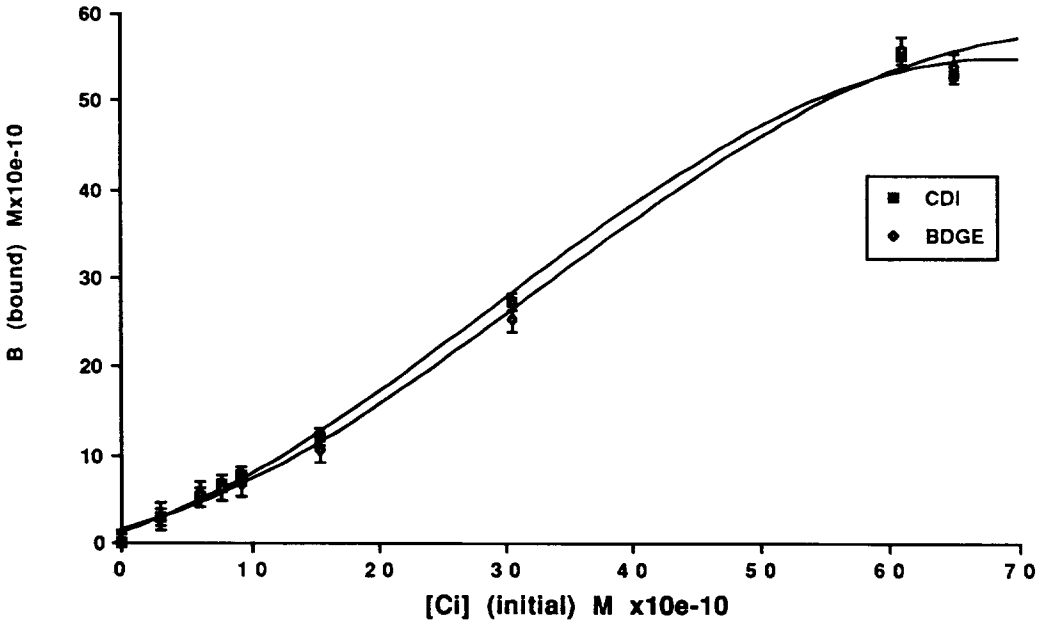

Fig. 4. Adsorption isotherms of insulin on SID-NANA.

NANA and SID-BDGE-NANA columns are 80 and 90%, respectively.

The effect of ionic strength on insulin adsorption is studied using elution buffers with NaCl concentrations varying from 10 to 50 m*M*. Elution profiles are obtained (Fig. 7) and the

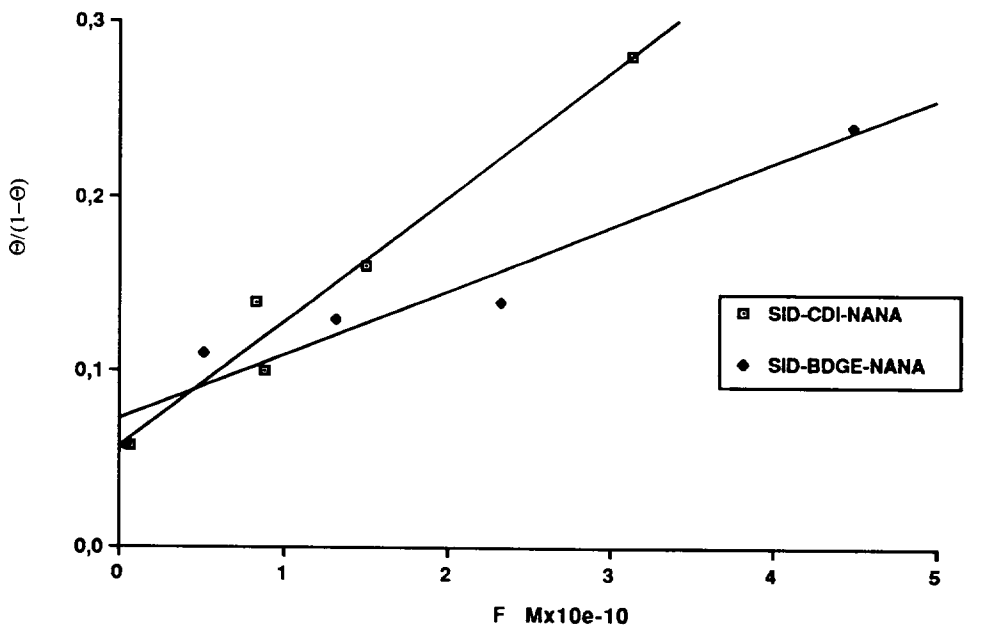

Fig. 5. Representation of Langmuir. ($\Theta = B/B_{max}$, B = bound insulin on SID-NANA, B_{max} = maximal capacity of adsorption of SID-NANA, F = free insulin).

Table 3
Affinity constants and binding capacities of SID-NANA

Supports	Affinity constant (l/mol)	Binding capacity (mol/l/g)
SID-CDI-NANA	$7 \cdot 10^8$	$2.2 \cdot 10^{-9}$
SID-BDGE-NANA	$3 \cdot 10^8$	$2.2 \cdot 10^{-9}$

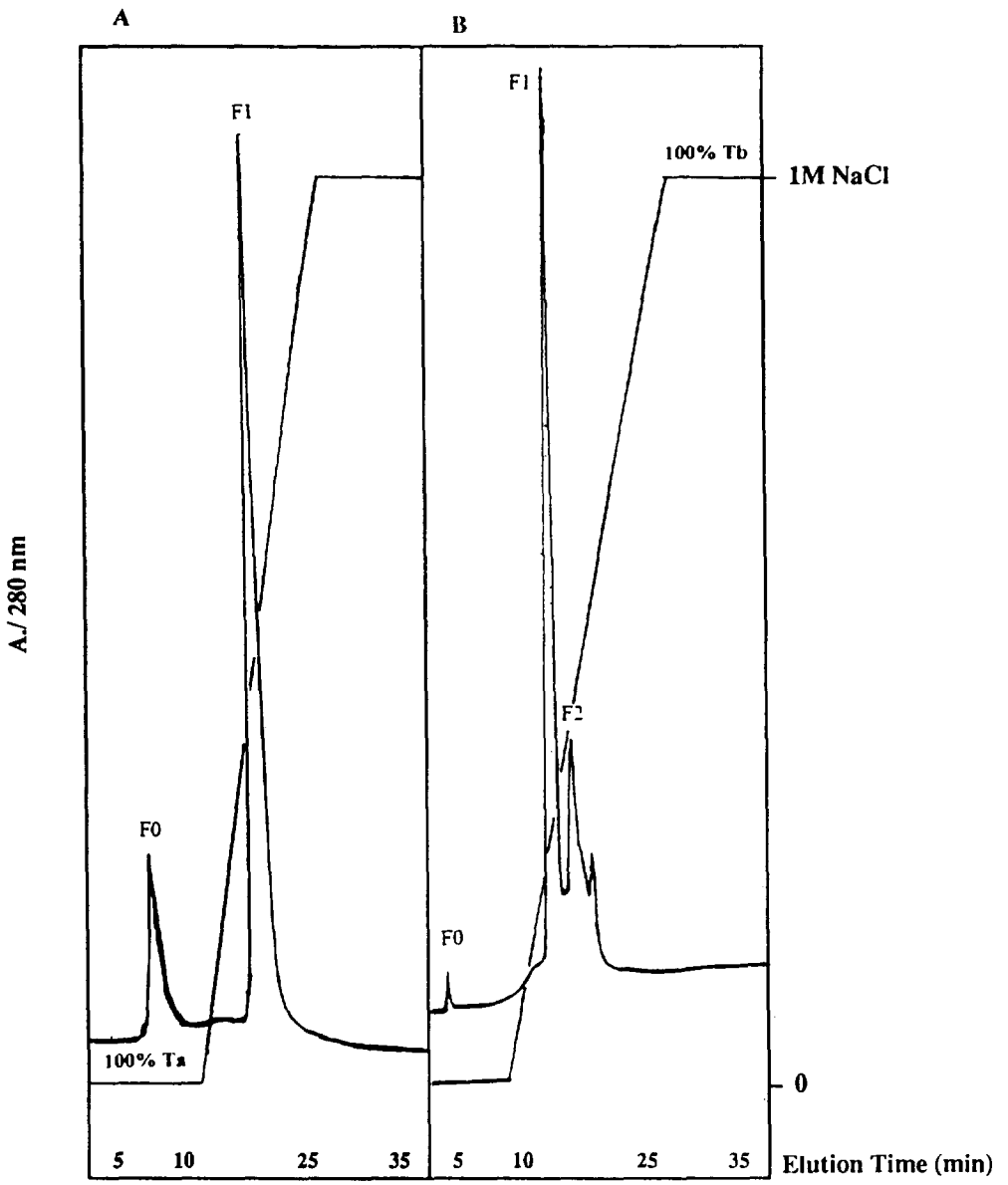

Fig. 6. Elution of 100 μl of insulin (1 mg/ml) on SID-CDI-NANA. Column 12.5 × 0.4 cm I.D., flow-rate 0.5 ml/min; eluents: Ta: bidistilled water pH 6.8. Tb: 0.05 *M* phosphate buffer, 1 *M* NaCl (pH 7.4). A: SID-CDI-NANA. B: SID-BDGE-NANA.

Table 4
Protein concentration in the different fractions

Supports	Eluents	Injected quantity (μg)	F0 (μg)	F1 (μg)	Yield (%)
SID-CDI-NANA	Water	79	14	64	80
	5% Methanol	82	16	67	82
SID-BDGE-NANA	Water	93	–	90	97
	5% Methanol	91	–	72	80

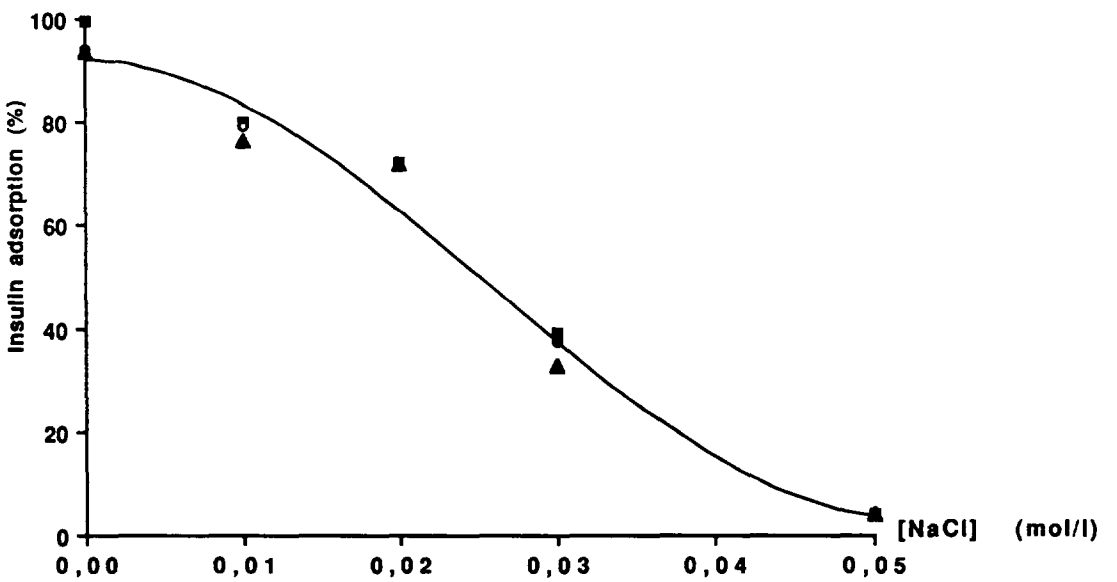

Fig. 7. Adsorption of insulin on SID-NANA support.

protein concentration in the different fractions is measured (Table 5). With increasing salt concentration in the adsorption buffer, the quantity of adsorbed insulin decreases. This shows that insulin interacts with SID-NANA at very low ionic strength. However, insulin can only be desorbed under high-salt elution conditions. These results seem to demonstrate that the

Table 5
Yields of insulin on SID-NANA

[NaCl] (M)	Injected quantity (μg)	F0 (μg)	F1 (μg)	Yield (%)
0.01	66	26	32	50
0.02	73	46	25	35
0.03	79	57	19	25
0.04	79	62	15	20
0.05	79	69	10	14

interactions involved in the adsorption and desorption mechanisms are different.

The inactivated silica supports used are grafted with sialic acid derivatives whose free carboxylic function endows them with cation-exchange capacity under suitable experimental conditions. The chromatographic separations can be described by different types of interactions (ionic, hydrophobic, etc.): all elution conditions used in the chromatographic experiments of this study are presented in Table 6. Two kinds of buffers were used, some of which favour insulin adsorption on the support, and others do not. Salts (Na_2HPO_4, NaCl, $NaOOCH_3$) present in the mobile phase may interact with the free carboxylic functions on the stationary phase, leading to non-retention of insulin. Non-retention of insulin in acidic medium can be explained by the acidic nature of this protein, causing under such conditions repulsion between the protein and the free acidic carboxylic functions present on the surface of the stationary phase, and thus inhibiting adsorption.

Conditions favouring retention of insulin on the stationary phase, e.g. bidistilled water, methanol, or a low NaCl concentration, give some indications about the type of interactions existing between insulin and the active support. In fact, the affinity of insulin for NANA is probably very complex and is influenced by the three-dimensional conformation of the ligand, its steric accessibility, the different chemical functions involved and the nature of the coupling agent. This last parameter can be very complex in itself, involving hydrophilic, hydrophobic and ionic interactions. The cooperative effect of all these interactions determines the specificity of the adsorption process.

3.4. Comparison of the two supports

Quantitative comparison of the two supports is very difficult because they are not substituted with the same amount of ligand. However, qualitatively, the BDGE-activated support (SID-BDGE-NANA) appears significantly more selective than the CDI-activated support (SID-CDI-NANA support), as can be deduced from the presence of two desorption peaks which means that the two forms of insulin are successfully separated. In fact, elution profiles obtained with SID-BDGE-NANA are narrower and slightly more symmetrical than those obtained with the other support, and its purification yield is better. According to these results, BDGE activation seems to be more efficient than CDI activation for the coupling of sialic acid derivatives. This difference has two main causes: on the one hand,

Table 6
List of elution conditions used

Buffer adsorption (Ta)	Adsorption of insulin
0.05 *M* Phosphate, 0.1 *M* NaCl pH 7.4	−
0.04 *M* Phosphate pH 7.4	−
0.03 *M* Phosphate pH 7.4	−
0.02 *M* Phosphate pH 7.4	−
0.01 *M* Phosphate pH 7.4	−
0.05 *M* Sodium acetate pH 7.4	−
0.04 *M* Sodium acetate pH 7.4	−
"pH acid"	−
0.05 *M* NaCl pH 7.4	−
0.04 *M* NaCl pH 7.4	±
0.03 *M* NaCl pH 7.4	±
0.02 *M* NaCl pH 7.4	+
0.01 *M* NaCl pH 7.4	+
Distilled water pH 8	+
5% Methanol pH 8	+

the fixation mechanism of the ligand depends on the coupling agent used and can lead to conformational changes and loss of activity for the adsorbed protein. On the other hand, the coupling agent itself can modify the steric accessibility of the active site. In fact, BDGE creates a spacer arm between the support and the ligand thus improving the steric accessibility of the ligand and therefore its selectivity.

Among other characteristics of the prepared stationary phases, the coating quality is certainly the most important parameter because it can strongly influence the coupling capacity of the supports. As previously demonstrated by Touhami et al. [14], silica-coating polysaccharide chains can be visualized as a succession of "loops", "trains" and "tails" on the inorganic surface with different hydrodynamic behaviours. These structures may also influence the steric accessibility of the active site, depending on whether the ligand is fixed upon a "loop" or upon a "train".

4. Conclusions

The behaviour of insulin towards active chromatographic supports grafted with sialic acid derivatives has been studied and the elution conditions have been determined. The ligand–insulin complex is formed by many attractive forces of various nature, the sum of which determines the stability of this complex and results in an affinity interaction. However, because of protein complexity, multiple polar and non-polar groups may be involved in insulin–support interactions and forces of various nature act simultaneously. The type of elution buffer is an important parameter in so far as it gives ideas about possible existing interactions. The results imply that the affinity of insulin for N-acetylneuraminic acid is complex and involves hydrophilic, hydrophobic and ionic interactions. The cooperative effect of all these interactions determines the specificity.

The dynamic (HPLAC) study of the support performances has allowed, firstly, the elaboration of new methods for the purification of insulin, and secondly, the determination of the interactions which lead to the affinity of this protein for the ligand immobilised on the supports. These results may contribute to the understanding of the interactions between insulin and its cellular receptor.

References

[1] V.P. Zubov, A.E. Ivanov and V.V. Saburov, *Adv. Polym. Sci.*, 104 (1992) 135–172.
[2] F.L. Zhou, D. Muller, X. Santarelli and J. Jozefonvicz, *J. Chromatogr.*, 476 (1989) 195–203.
[3] X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr.*, 443 (1988) 55–62.
[4] R. Schauer, A.P. Corfield, in R. Schauer (Editor), *Sialic Acids, Chemistry, Metabolism and Function*, Springer Verlag, New York, NY, 1982.
[5] W. Wang, K. Erlausson, F. Lindh, T. Lundgren and D. Zopf, *Anal. Biochem.*, 122 (1990) 182–187.
[6] A. William and J.R. Brennau, *Endocrinolgy*, 122 (1988) 2364–2370.
[7] G.R. Hayes and D.H. Lockwood, *J. Biol. Chem.*, 261 (1986) 2791–2798.
[8] A.I. Salhanick and J.M. Amatruda, *Am. J. Physiol.*, 255 (1988) 173–179.
[9] Ultrogel, Magnogel and Trysacryl: Pratical guide for use in affinity chromatography and related techniques, Réactifs IBF, Villeneuve-la-Garenne, 1983.
[10] J. Le Gallic, Thesis, University Paris-Sud, 1990.
[11] J. Suzuki, K. Murakami and Y. Nishimura, *J. Carbohydr. Chem.*, 12 (1993) 201–208.
[12] G.W. Jourdian, L. Dean and S. Roseman, *J. Biol. Chem.*, 246 (1971) 430–435.
[13] S.J. Thomson and G. Weeb, in O. Boyd (Editor), *Heterogeneous Catalysis*, Wiley, New York, NY, 1968, pp. 22–27.
[14] A. Touhami, H. Hommel, A.P. Legrand, A. Serres, D. Muller and J. Jozefonvicz, *J. Colloid Surfaces B, Biointerfaces*, 1 (1993) 189–195.