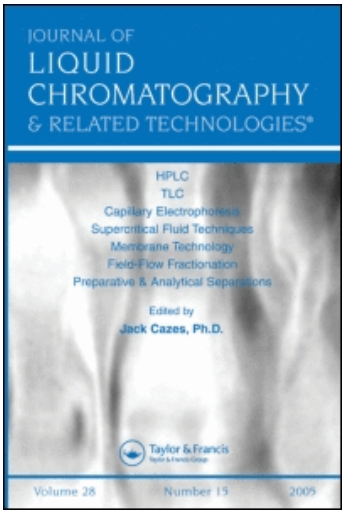

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Separation and Purification of Insulins on Coated Silica Support Functionalized with Sialic Acid by Affinity Chromatography

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SEPARATION AND PURIFICATION OF INSULINS ON COATED SILICA SUPPORT FUNCTIONALIZED WITH SIALIC ACID BY AFFINITY CHROMATOGRAPHY

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ABSTRACT

High performance liquid affinity chromatography (HPLAC) is a powerful method for purification and analysis of biological compounds. It combines the speed and the efficiency of high performance chromatography with the selectivity of affinity chromatography methods. The selectivity depends, first, on the nature of the ligand used; this latter must exhibit a specific binding affinity towards the product to purify; second, it depends on adsorption and desorption processus that improve, or not, the protein affinity for the ligand.

The presence of N-acetyl neuraminic acid surrounding the insulin receptor structure shows that this acid may develop specific interactions with insulin.

We performed the grafting of sialic acid on coated silica supports. The performances of these supports towards insulin were studied by HPLAC. The ligand specificity was tested in presence of two forms of insulin, porcine and bovine insulins, which, differ from each other by only two amino acids in their structure. The support carrying sialic acid exhibits both affinity and specificity for insulin in solution, since it allows the separation of the two forms of insulin. This result is confirmed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) assay. RP-HPLC is a widely used method for the separation of several compounds that exhibit structural homologies, such as, for instance and in particular, the different forms of insulin.

In order to confirm the specificity and the affinity of the support bearing sialic acid for insulin, the elution of a pancreatic extract, consisting of numerous proteins including insulin, is performed. The support allows the insulin purification from a pancreatic extract with high purification yields.

INTRODUCTION

In high performance liquid affinity chromatography (HPLAC), a ligand is a component that may interact with the product to separate in a specific and reversible way. The original concept of ligand-product interaction in HPLC implies the notion of specificity. One ligand is specific for one given protein. The notion of strickness regarding ligand specificity has progressively evolved from very conservative, to a much wider concept, with the appearance of very broad specificity ligands. These latter, are called general or pseudo-specific ligands such as, for instance, enzyme cofactors, calmoduline[1] or lectines[2] or non biologic molecules as dye[3] and metallic ions.[4] Because of their broad specificity, a same affinity support can be used to purify a series of proteins.

The ligand used in the present study, N-acetyl neuraminic acid (NANA) belongs to the family of "general ligands". It binds IgG[5] just as well as sialidases[6] and it develops an affinity for insulin too.[7,8] At first sight, one can consider that there is no specificity; nevertheless, the particular and selective conditions of adsorption and desorption of each of these proteins create the specificity. Indeed, the conditions that allow the binding of IgG or sialidase on the support do not permit the fixation of insulin on immibilized sialic acid. Moreover, the conditions used to adsorb insulin on the support (water 5% methanol)[7] are little selective. Therefore, we felt the need to find another method to confirm, or not, the specificity of our support for insulin.

Table 1

Interspecies Variations of Insulin Amino Acid Sequence (18)

Species	A-8	A-9	A-10	B-30
Human	Thr	Ser	Ile	Thr
Bovine	Ala	Ser	Val	Ala
Porcine	Thr	Ser	Ile	Ala
Sheep	Ala	Gly	Val	Ala
Horse	Thr	Gly	Ile	Ala
Sperm Whale	Thr	Ser	Ile	Ser

In recent years, a large number of reports of studies of the reversed phase chromatography of insulin have been published. In most of them, insulin has been used as one among several polypeptides or proteins in order to caracterize the separation capacity of the system.[9-16] The separation of insulin and its derivatives represents a typical challenge to the separation capacity of reverse phase supports.

Insulin is a 6000 g/mol polypeptide constituted of 51 amino acids contained within two peptide chains: an A chain with 21 amino acids and a B chain with 30 amino acids.[17] The chains are connected by two disulfide bridges. In addition, there is an interchain disulfide bridge that links positions 6 and 11 in the A chain. The cleavage of disulfide bond implies a loss of insulin activity. This dimeric structure is conserved in the animal kingdom but slight variations are observed according to the species. Porcine insulin differs from human by only one amino acid, alanine instead of threonine at the carboxyl terminus of the B chain (position B30), and from bovine by two amino acids A^8 and A.[10] (see Table 1).[18]

The performance of our support was tested towards both bovine and porcine insulin, in order, to evidence the specificity of our ligand for insulin. Such work could also contribute to a better understanding of interaction mechanisms between insulin in solution and the ligand immobilized in HPLAC. The support was used, then, to attempt the purification of insulin from a pancreatic extract.

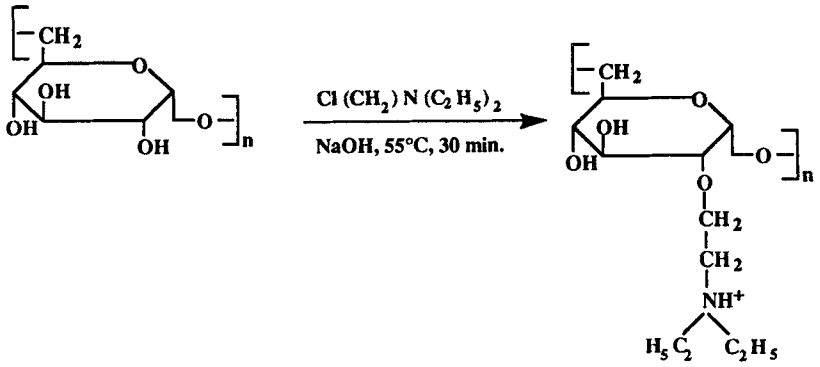


Figure 1. Reaction of substitution of dextran by 2-chloro-N,N-diethylaminoethan.

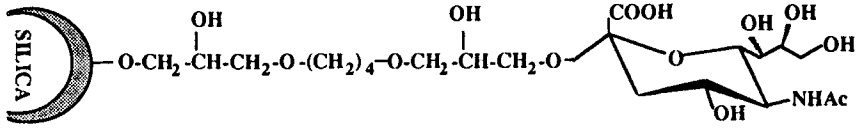


Figure 2. Structure of affinity support (SID-NANA).

MATERIALS AND METHODS

Synthesis of the Affinity Support

The synthesis of coated silica supports functionalized by N-acetyl neuraminic acid was carried out as reported previously.[7] The preparation of the affinity support is performed in two steps. First, silica beads are coated with dextran substituted by a calculated amount of diethyl-aminoethyl (DEAE) functions to hide negative charges at its surface. Second, ligands are immobilized using a coupling agent. The substitution of dextran T70 (68 000 g/mol) (Pharmacia, Bois d'Arcy, France) by 2-Chloro-N,N-diethyl-aminoethane (Janssen Chemica, Noisy Le Grand, France) is performed in a very alkaline medium at 55°C for 30 min. (Fig. 1).

The substitution rate of dextran with DEAE is determined by elemental analysis of nitrogen. The conditions for dextran modifications for an optimal passivation were previously determined[19,20] to obtain a proportion of dextran units carrying DEAE groups (Dx-DEAE) varying from 4 to 13%.

Silica beads (particle size 15-25 μm, porosity 1000 Å), kindly provided by Biosepra (Villeneuve la Garenne, France), are impregnated with a modified dextran solution (8 g of Dx-DEAE in 100-mL) adjusted to pH 11. Dextan coated silica is cross-linked with 1,4-butanedioldiglycidyl ether (BDGE) (Sigma, La Verpillière, France). The amount of Dx-DEAE covering the silica (SID) beads is determined by spectrophotometric assay of the sugar units after acid hydrolysis and by elemental analysis of carbon. Prior to the ligand coupling, the quality of the Dx-DEAE coverage of the silica support is evaluated by testing the elution of standard proteins on the support under high performance size-exclusion chromatographic (HPSEC) conditions.

The immobilisation of N-acetyl neuraminic acid on SID requires the use of a coupling agent to create covalent bonding between the ligand and the support (SID-NANA). In this study, NANA is coupled to the activated support by 1,4-butanediol diglycidyl ether (BDGE), a diepoxide agent, as previously described.[7]

Activation of dextran coated silica is carried out with BDGE in diethylether. NANA is coupled to the activated support in carbonate buffer for 48h. The amount of ligand fixed on the support is determined by spectrophotometric assay on the coupling solution supernatant using a periodate-resorcinol method.[21]

Elution of Insulin on SID-NANA in HPLAC

The HPLAC system consists of a pump (Spectra P100, Thermo Separation Products, Les Ulis, France) monitored by a programmer and equipped with an injection valve (Model 9126, Rheodyne, Merck, Nogent-Sur-Marne, France), connected to an UV-visible spectrophotometric detector (L-4000; Merck), an integrator (D-2520 GPC integrator; Merck) and a fraction collector (Model 203, Gilson, Sarcelles, France). The insulin used in HPLAC, is kindly provided by Diosynth S.A. (Akzo, Eragny-sur-Epte, France).

A 100 μL amount of insulin (porcine or bovine insulin or both porcine and bovine insulins), is injected on to the column (12.5 x 0.4 cm I.D.) containing the SID-BDGE-NANA support (the use of an injection vanne), at a flow-rate of 0.5 mL/min. The fractions corresponding to elution and desorption peaks are collected and analysed, first by SDS-PAGE electrophoresis (Phast system,

Pharmacia, Saint Quentin en Yvelines) and, second by reverse phase HPLC using a RP-18 column (5 µm) LiCrospher 100 (MERCK). The optimisation of the elution conditions used were determined previously.

RESULTS AND DISCUSSION

Synthesis of an Affinity Support

The first attempts to separate proteins in HPLC on unmodified mineral phases (silica or glass with a controlled porosity), have shown the presence of non specific interactions[22] then, mineral phases were modified by coating of hydrophilic polymers.[23,24] In our laboratory, we are currently using silica based supports which are coated with a calculated amount of diethylaminoethyl groups.

The dextran substitution is performed in order to confer a weak anion exchange capacity by DEAE groups into glycosidic units. The substitution rate of T70 dextran polymers by DEAE is 5% in our experimental conditions. The adsorption of Dx-DEAE on silica beads, results from interactions between anionic groups on the silica surface and DEAE groups carrying positive charges.

The coverage is further strengthened by cross-linking dextran chains with BDGE around the silica particles, that leads to the formation of ether-type bonds with polysaccharide hydroxyl groups. The polymeric coverage on the silica support, determined by elemental analysis, is 35 mg of Dx-DEAE per gram of silica.

In order to ascertain that the stationary phase used for coupling of the ligand will not undergo non-specific interactions with standard proteins, these proteins are eluted on the SID support under high performance size-exclusion chromatographic (HPSEC) conditions.[7]

The results show that no interaction occurs in the selected elution conditions. The SID support is neutral enough to be coupled to a biospecific ligand and to avoid non specific interactions with proteins in solution.

The BDGE activation is obtained by an hydroxyl function of the support with hydroxyl groups of the ligand (Fig. 2). The amount of fixed NANA on SID support is 10 mg/g silica and the coupling yield amounts 50%.

Table 2

Elution Conditions Used

	Ta	Tb	Gradient
a	Water 5% meethanol pH 8	0.05M phosphate buffer, 1M NaCl	0-10 min: 100% Ta 10-25 min: Ta→Tb 25-35 min: 100%Tb
b	Water 5% methanol pH 8	0.05M phosphate buffer, 1M NaCl	0-10min: 100% Ta 10-25 min: Ta→Tb 25-35 min: 100% Tb
c	Water 5% methanol pH 8	(0.05 M phosphate buffer, 0.2M NaCl): 90% ACN: 10%	0-10 min: 100% Ta 10-25 min: Ta→ Tb 25-35 min: 100% Tb
d	Water 5% methanol pH8	(0.05M phosphate buffer, 0.2M NaCl): 90% ACN: 10% Tc: distilled water	0-10 min: 100% Ta 10-25 min: Tc→ Tb 25-35 min: 100% Tb
e	Water 5% methanol pH 8	(0.05M phosphate buffer, 0.2M NaCl): 90% 48%Tc ACN: 10% Tc: distilled water	0-10 min: 100% Ta 10-18 min: Tc→52% Tb- 18-25 min: 52% Tb-48%Tc 25-35 min: 52%Tb-48%Tc →100%Tb
f	Water 10% methanol pH 8	0.05M phosphate buffer: 90% ACN: 10%	0-10 min: 100%Ta 10-16 min: Ta→50% Tb-50%Ta 16-25 min: 50%Tb-50%Ta 25-30 min: 50%Tb-50%Ta →100%Tb 30-35 min: 100%Tb

Ta: Adsorption buffer
Tb: Desorption buffer

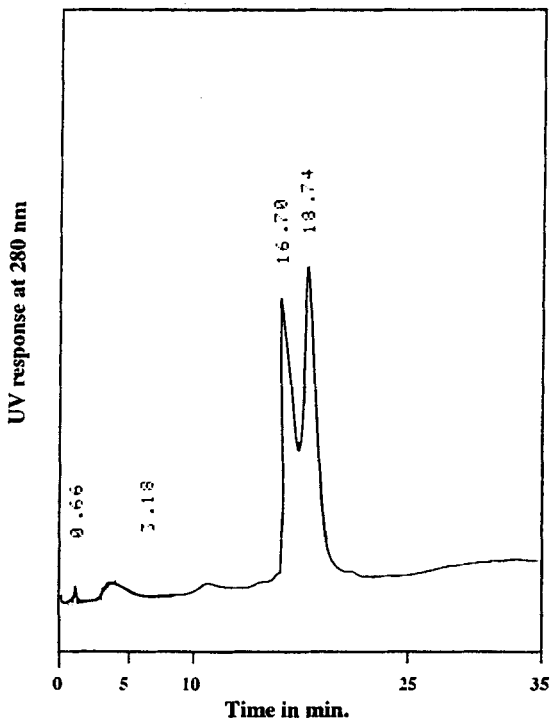


Figure 3. Elution of 100 µL of 50% porcine insulin with 50% bovine insulin mixture (1mg/mL) on SID-NANA. Column: 12.5 x 0.4 cm I.D.; Flow-rate: 0.5 mL/min; Eluents and elution program: See Table 2, f.
Note the fractions are called F1 and F2 (F1: Fraction of the first desorption peak; F2: Fraction of the second desorption peak).

Table 3

Purification Yield of Insulin on SID-NANA

Injection Quantity (µg)	F0 (µg)	F1 (µg)	Purification Yield (%)
85.3	11.2	74.1	87

Elution of Insulin on SID-NANA in HPLC

The elution profiles of a commercial mixture of insulin (porcine + 6% bovine) on the affinity support carrying sialic acid[7] show two desorption peaks. The Bradford assay[25] and SDS-PAGE electrophoresis show that the ratio between the two peaks corresponds to the mixture initially introduced on to the column. We, then, performed the elution of a 100µl of 50% porcine insulin with 50% bovine insulin mixture in the same conditions as described previously (Table2, a). A single peak of desorption is obtained; the two forms of insulin are not separated. We assumed that the second peak obtained from the elution of the mixture of insulin (porcine + 6% bovine) is due to the presence of desamidoinsulin in our sample.

Among the two main steps of HPLC i.e adsorption and desorption, the importance of the latter to increase the specificity throughout the elution step is well-known. We studied the influence of the desorption conditions on insulin elution, in order, to selectively unbind the two forms of insulin. These latter differ from each other by their hydrophobicity. For this purpose, we used mobile phases identical to those applied in RP-HPLC on C_{18} phase. Table 2 represents the different desorption conditions used in our study.

The use of a mixture, consisting of 70 volumes of 0.05M monosodic phosphate with 0.2M NaCl and about 30 volumes of acetonitrile (70:30, v/v), did not allow the separation of the two types of insulin (Table 2, b). Acetonitrile (ACN) high concentration could lead to a cleavage of interactions between both insulins and the chromatographic support. The ACN concentration is then decreased up to 10%; this did not affect the resolution of the two proteins (Table 2, c).

In order to optimize the conditions of desorption, we used a linear gradient of eluting buffer and TB (Table 2, d). Elution of bovine:porcine insulin mixture results were unsatisfactory, since a very poor second peak shape is obtained. To improve the two peaks resolution, we used a slightly concave gradient (Table 2, e) of same composition; two desorption peaks are obtained with similar retention times. The use of such a gradient seems to promise some improvement in the resolution of the different components of the sample.

The elution of the mixture is performed with some modifications of the mobile phase and the elution gradient. On one hand, NaCl, favouring hydrophobic interactions, is removed from the desorption buffer; on the other hand, the adsorption buffer is increased in methanol concentration to improve the binding yield.[7] The use of a broad concave gradient (Table 2, f), allows us to obtain a good separation of the two types of insulin with a difference of 2 min in retention times (Fig. 3). In order to identifly the two peaks, the elution of each

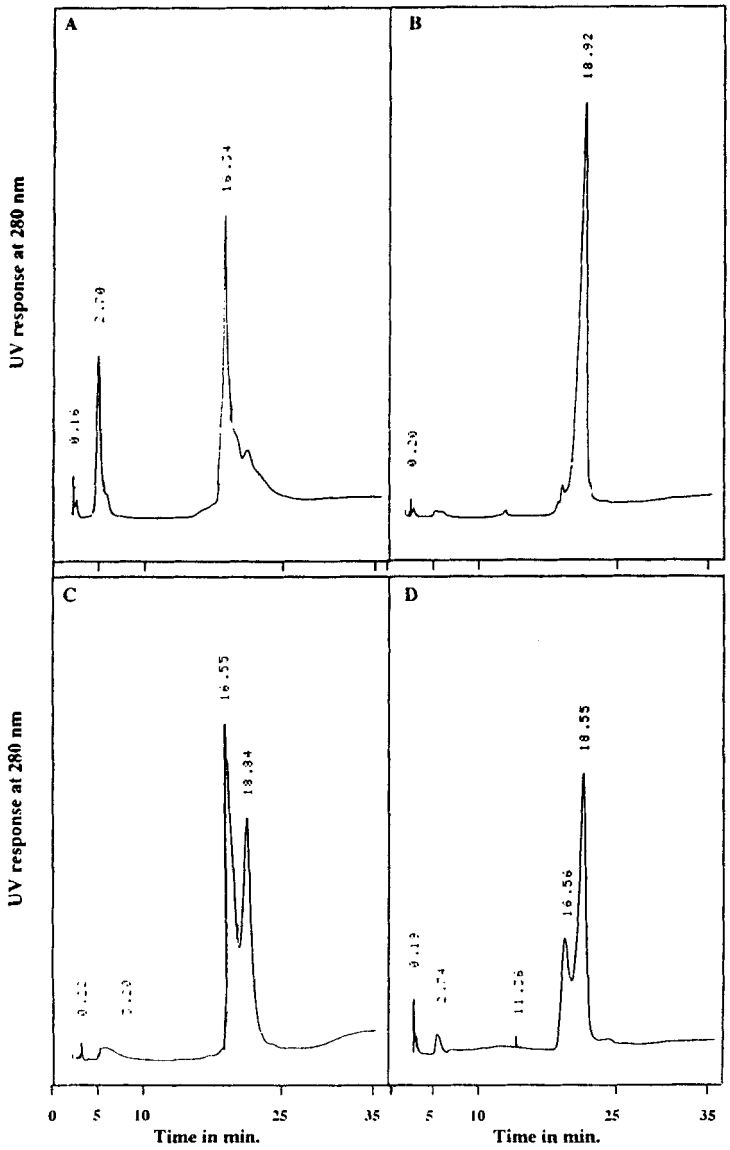


Figure 4. Elution of 100 µL amount of insulin (1 mg/mL) on SID-NANA (the same conditions as described in Figure 3). A: Porcine insulin; B: Bovin insulin; C: Porcine insulin with 10% bovine; D: Bovine insulin with 10% procine.

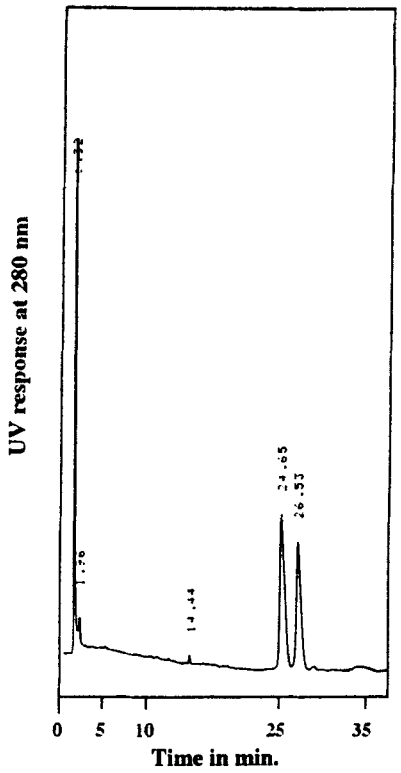


Figure 5. Elution of 100 µL of 50% procine insulin with 50% bovine insulin mixutre (1 mg/mL) on C_{18} (5 µm) LiChrospher 100. Column: 125 x 0.4 cm I.D.; Flow-rate: 1 mL/min; Eluants: Ta: Water 0.1 % TFA; Tb: Acetonitrile.

type of insulin, as well as, a mixture of porcine insulin with 10% bovine insulin and a mixture of bovine insulin with 10% porcine is performed. The elution profiles are presented in Figure 4. The individual elution of porcine insulin and bovine insulin is obtained; the size ratio of the peaks is consistent with the mixture initially introduced on the support. Moreover, bovine insulin develops a higher affinity for sialic acid than porcine insulin since it is eluted the last.

It is observed, that the same sample analyzed several times throughout the day, shows an increasing retention time, with a linear relationship existing between the retention times of the different components and the duration of the analysis due to the evaporation of the ACN. The fractions corresponding to the desorption peaks were collected, concentrated by means of micro-concentrator

of porosity 1000 and analyzed by SDS-PAGE electrophoresis. The presence of insulin is caracterized by a migration band of 6000 g/mol. Insulin is present in all fractions; insulin retained on the column is in a monomeric state since no migration band of 12000 g/mol occurs. The selective elution of porcine and bovine insulins on SID-NANA show evidence that this support develop distinct affinities for these two proteins. To confirm these results, collected fractions are analysed by RP-HPLC. RP-HPLC is a powerful method to detect small differences in the structure of different compounds and particularly in their hydrophobicity.

First of all, we undertook the optimization of elution conditions that will allow the separation of the two types of insulin with a significative resolution; then we analyzed the collected fractions from the two proteins elution in affinity chromatography on SID-NANA. We performed the elution of a mixture (50%/50%) of porcine and bovine insulins by RP-HPLC whose base principle is a very polar mobile phase and an hydrophobic stationary phase. Several modifications of the mobile phase are selected to achieve a satisfactory resolution.

First, the concentration of the organic component is fine tuned, checking how the percentages of ACN used could produce drastic alteration in the retention times. Then, a low concentration of trifluoroacetic acid (TFA) (0.1%, v/v) is added to improve the resolution by increasing the polarity of the mobile phase. A 100 µL amount of porcine or bovine insulin or a mixture of 50%-50% is injected on to column (C_{18}) using a concave gradient from water 0.1% TFA to 40% ACN. The composition of solvents and the elution program are presented in Figure 5. Elution profiles show that the two types of insulin are eluted selectively.

The analysis of collected fractions from the insulin samples elution on SID-NANA by RP-HPLC, reveal (Fig. 6) that the fractions corresponding to the first desorption peaks and the second desorption peaks are eluted at the same time as, respectively, porcine insulin and bovine insulin. This result confirms that porcine insulin is eluted before bovine insulin on the support carrying sialic acid and that the reverse order is obtained compared to their elution on C_{18} support.

Comparaison of Two Methods: HPLAC and RP-HPLC

In order to understand the interaction mechanisms that govern the separation of the two forms of insulin on the support functionalized with sialic acid, we performed the elution of insulins (porcine + bovine), first on the support carrying NANA under the elution conditions used in RP-HPLC and

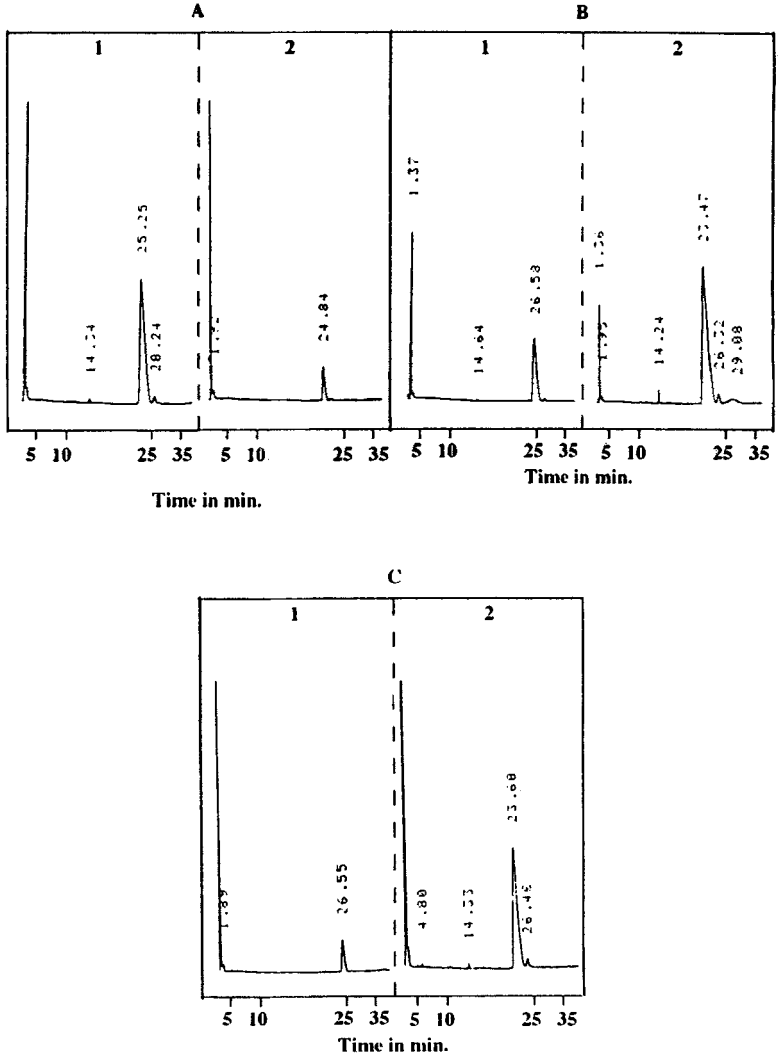
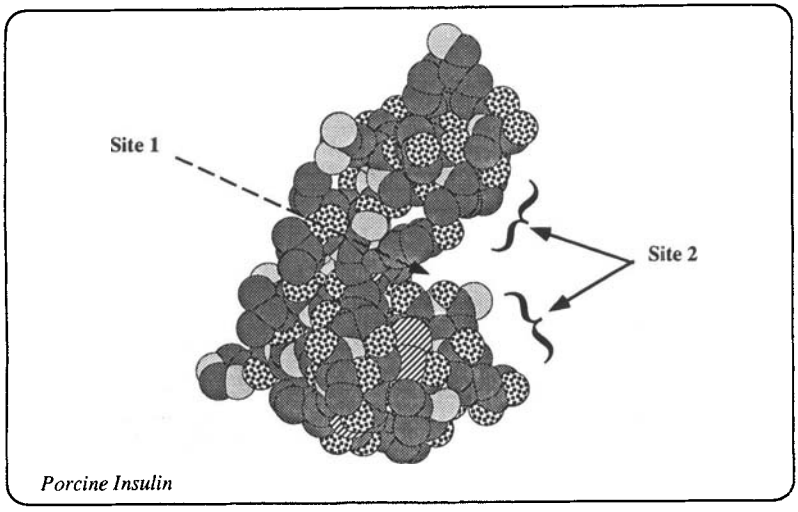


Figure 6. Elution of a 100 µL amount of collected fractions on C_{18} (5 µm) same conditions as described in Figure 5. A: 1-F1 of the insulins mixture 90% porcine + 10% bovine; 2-F2 of the insulins mixture 90% procine + 10% bovine; B: 1-F1 of the insulins mixture 50% porcine + 50% bovine; 2-F2 of the insulins mixture 10% procine + 90% bovine; C: 1-F1 of the insulins mixture 10% procine + 90% bovine; 2-F2 of the insulins mixture 10% porcine + 90% bovine.



Site 1= A (Thr8-Ser9-Ile10) for Porcine Insulin
A (Ala8-Ser9-Val10) for Bovine Insulin

Site 2= A (Gly1-Val3-Glu4)
B (Arg22-Gly23-Phe24-Phe25)

Figure 7. Putative interaction sites between insulin and NANA grafted on SID.

second, on a column C_{18} by using the mobile phase applied in HPLAC. Most of insulin mixture eluted on SID-NANA under RP-HPLC conditions are not retained on the support. A low amount of insulin is adsorbed and desorbed from the column within a single peak. This result demonstrates that the two forms of insulin are eluted similarly. The conditions used in RP-HPLC allow the protein to develop hydrophobic interactions with the support. The hydrophobic interactions alone do not allow neither insulins retention on the support nor their separation.

DISCUSSION

We have previously demonstrated[7] that the interactions between insulin and the support carrying sialic acid, are complex and involve simultaneously hydrophilic, hydrophobic and ionic interactions. The cooperative effect of all these different interactions determines the affinity and the specificity.

The predominance of one type of interaction, depends on the nature of the elution solvent used. According to the solvent nature, different conformational changes of insulin occur so that the region of insulin structure implied in the insulin-ligand interaction is different. This interpretation infers the existence of different active sites on the insulin molecule.

In order to have an understanding of these interaction mechanisms, one must modelize the insulin molecule as an hydrophilic surface carrying negative charges and surrounding an hydrophobic pocket. A conformational modification occurs and regulates the expression of different apolar regions at the protein surface modifying the insulin-supports interactions. Porcine insulin differs from bovine insulin by only two amino acids A^8 and A^{10} (Table 1), the SID-NANA support allows the distinct resolution of each protein under well defined conditions. This performance suggests that residues A^8 and A^{10} are somehow involved in the interactions between the support and the peptide.

The conditions which allow the separation of the two different forms of insulin on the support, probably favour the appearance of a different active site for each protein at the protein surface. The difference in the affinity of the two types of insulin let us assume, that the active site (site 1) is constituted of the amino-acid sequences from A^8 to $A,^{10}$ Thr 8-Ser 9-Ile 10 for porcine insulin, and Ala 8-Ser 9-Val 10 for bovine insulin (Fig. 7). When the conditions used do not permit the separation of the two forms of insulin, it may suggest the occurence of a same binding site for both proteins at the surface. This site (site 2) is constituted of the amino-acid sequences from B 24 to B27 (Phe B24-Phe B25-Tyr B26-Thr B27) and from A1 to A4 (Gly 1-Val 3-Glu 4)[25] (Fig. 7). The interaction between insulin and the immobilized ligand occurs through the sequence (site 1) which provides the specificity to the complex and other regions (site 2) which ensure its stability.

Purification of Insulin from Pancreatic Extract

The present study evidences that the support bearing sialic acid allows a good resolution of the two types of insulin. The separation of both insulins was performed in purified medium.

In order to confirm the selectivity of the support for insulin, we proceeded, then, to the elution of a pancreatic extract consisting of numerous proteins including insulin. The pancreatic extract was kindly provided by J. OLIVIE from societe Diosynth S.A. (Akzo, Eragny-Sur-Epte, France).

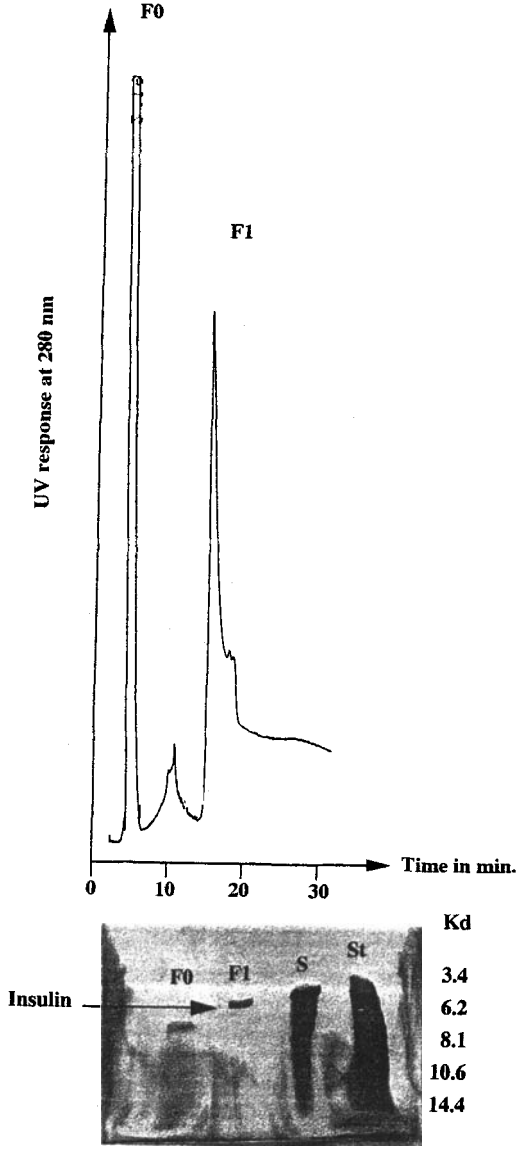


Figure 8. Elution profile of extract (100μL) on SID-NANA. For experimental details, see legend to Figure 3. SID-PAGE of pooled fractions F0 and F1 shown in chromatogram, unfractioned pancreatic extract (S) and standard proteins (St).

The chromatogram of the Figure 8 was obtained by injection of a 100 µl amount of a pancreatic extract, under the same conditions as those used for the resolution of the two types of insulin. The fractions corresponding to elution and desorption peaks are collected and analyzed by SDS-PAGE. The presence of insulin is revealed by a migration band of 6000 g/mol. The results described in figure 8 show that only insulin is retained and evidence the selectivity of the support towards insulin. Furthermore, the amount of insulin contained in the injected samples and the eluted fractions, is determined by RadioImmunoAssay (RIA, Cis biointernational, Gif Sur Yvette, France). The results presented in Table 3 show a purification yield of insulin of 87% from a pancreatic extract.

Finally, the silica based support coated with dextran-DEAE functionalized by sialic acid, allows a good resolution of the two types of insulin and the insulin purification from protein mixture. These results evidence the affinity and the specificity of sialic acid for insulin.

CONCLUSION

The present study demonstrates the influence of the mobile phase, especially the desorption phase on the performance of the affinity support functionalized with sialic acid residus. The interactions developed between insulin and the chromatographic support depend strongly on the nature of the eluting solution used. Insulin presents two binding sites, site 1 and site 2, which are not simultaneously expressed at the protein surface. In contrast to site 2, the expression of site 1, at insulin surface, allows the distinct resolution of each form of insulin. The expression of each site is regulated by reversible conformational changements that depend on the nature of the mobile phase used. The insulin-immobilized ligand interaction occurs through site 1, which ensures both specificity and selectivity of the complex and through site 2, which maintains the stability of the complex.

The support functionalized with sialic acid, exhibits an affinity for insulin with a high binding yield. It allows the separation of the two forms of insulin, i.e porcine and bovine insulins. The reverse elution order of the two forms of insulin obtained in HPLAC, in comparison with RP-HPLC, shows that the interaction mechanisms which permit the separation of the two insulins differ from those involved in RP-HPLC.

The support also allows the insulin purification from a pancreatic extract with high purification yields. The use of such a support could be broadened out to the separation of other forms of insulin, as well as, insulin analogues. These results may also contribute to a better understanding of interaction mechanisms between insulin with its receptor.

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