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Insulin adsorption on coated silica based supports grafted with *N*-acetylglucosamine by liquid affinity chromatography

Hamid Lakhiari [a], Daniel Muller [b,*]

[a] *Centre d'Etudes Universitaires de Taza, BP 1223 Taza-Gare, Université Sidi Mohamed Ben Abdellah, Taza, Morocco*
[b] *Département de Chimie, Université Paris-Nord, Institut Galilée, Avenue J.B. Clément, 93430 Villetaneuse, France*

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Abstract

Silica beads are coated with dextran carrying a calculated amount of positively charged diethylassminoethyl groups (DEAE) in order to neutralize negative charged silanol groups at the silica surface and in this way to minimize non specific interactions between silica surface and proteins in solution. Dextran-coated silica supports are potentially excellent stationary phases for high-performance liquid chromatography of proteins. These supports combine the advantages of polysaccharide phases with the excellent mechanical characteristics of silica. These supports (silica–dextran–DEAE = SID) are easily functionalized by grafting *N*-acetylglucosamine (GlcNAc) using conventional coupling methods. The performances of the support bearing GlcNAc are studied by high-performance liquid affinity chromatography (HPLAC) of insulin, the hypoglycemic peptide hormone of the human organism. The study shows that these supports exhibit a reversible and specific affinity towards insulin and allow separations with high purification yields. Moreover, the influence of different physico-chemical parameters (pH, NaCl and insulin concentration) on insulin retention on the support was analysed. This allowed us to optimize the conditions of adsorption and to better understand the interaction mechanisms between insulin and GlcNAc as biospecific ligand.

Keywords: *N*-Acetylglucosamine; Insulin

1. Introduction

The adsorption of proteins is a phenomenon which intervenes in the majority of essential biological process. So, the adsorption of proteins at solid–liquid interface is the subject of extensive theoretical [1] and experimental studies [2]. This phenomenon results from interactions between proteins and surface on the one hand, and between proteins and solvents on the other hand. The importance of the latter interaction depends essentially on the chemical nature of the species in the reactional medium.

In the case of insulin and according to the nature of the solvent, structural modifications can occur and modify its biological activity. Insulin is the hypoglycemic peptide hormone of the human organism; it is 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 [3]. The two chains are connected by two disulfide bridges. This 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 threonine at the carboxyl terminus of the B chain (position B30), and from bovine by two amino acids (position A8 and A10) [4]. Endogenous insulin circulates predominantly in monomer state in the blood stream. Nevertheless, insulin has a strong tendency to dimerize in solution with a dimerization constant of $1.5 \times 10^4\ M^{-1}$ [5,6]. Insulin also has a strong tendency to form tetramers and hexamers [7,8] in solution.

The dimeric form is nevertheless, the prevailing species in most systems; the main driving forces for the formation of this dimeric form are hydrophobic interactions. In the C-terminus of the B chain, a high population of non polar amino acids is dominant. By forming an antiparallel association with a neighbouring insulin monomer, the insulin monomers constitute strongly bound dimers (Fig. 1). The dimerization phenomenon depends on numerous parameters [9]. The dimerization constant decreases six-fold over the pH range, from 2 to 11.2. From pH 11.2 to 12.7, this constant decreases another 20-fold. In the latter range, electrostatic repulsion occurs resulting from the ionisation of

* Corresponding author. Tel.: +33-1-4940-3362; fax: +33-1-4823-2801.
E-mail address: dmuller@galilee.univ-paris13.fr (D. Muller).

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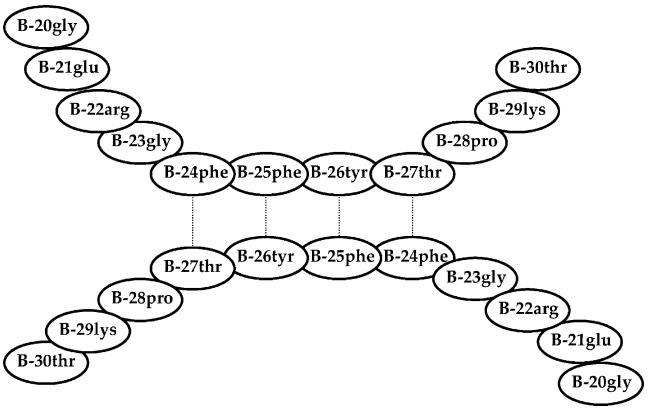


Fig. 1. Schematic representation of insulin–insulin interactions [9].

amino acids residues located in the centre for dimerization and bioactivity.

Insulin aggregation depends also strongly on its concentration. The aggregation phenomena appear more rapidly when the concentration exceeds 10 mg/ml in aqueous solutions. pH has a strong influence on the insulin solubility. In fact, Insulin is relatively insoluble in aqueous medium from pH 4 to 7 (Fig. 2) [4].

Finally, the activity and the stability of insulin in solution depend on its concentration as well as the pH and the temperature of the medium. So, it appears necessary to study the influence of those parameters on insulin behaviour in affinity chromatography.

The influence of different physicochemical parameters on insulin-surface interactions can be investigated by high-performance liquid affinity chromatography (HPLAC). Indeed, HPLAC allows us to study adsorption and desorption phenomena in hydrodynamic conditions. These phenomena are correlated to several conjugated physicochemical effects, the resultant of which determines the specificity and the bioaffinity of the functional supports.

The presence of *N*-acetylglucosamine (GlcNAc) surrounding the insulin receptor structure [10] let us assume that *N*-acetylglucosamine may probably lead to the formation of specific interactions which can be used in a separation procedure by affinity chromatography.

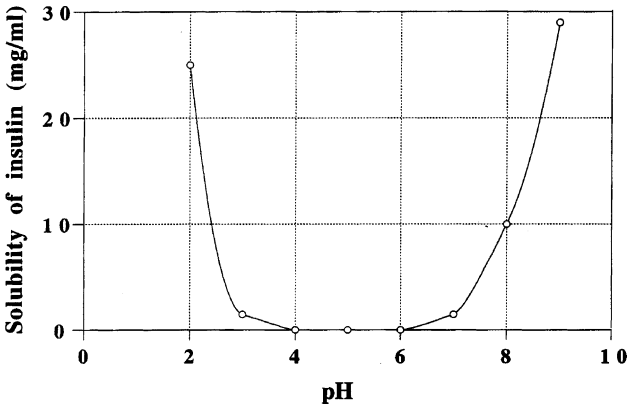


Fig. 2. Insulin solubility vs. pH, in 0.1 M phosphate buffer with 0.3 M ionic strength adjusted with sodium chloride [4].

For this purpose, we have synthesized chromatographic supports based on silica coated with dextran–DEAE and functionalized these solid phases by *N*-acetylglucosamine (SID–GlcNAc) using conventional coupling procedures. The interaction of these active phases and their biospecificity for insulin in solution were studied. Such work could also contribute to better understanding of interaction mechanism between insulin in solution and the ligand immobilized by HPLAC. Then, the support was used to attempt the purification of insulin from a pancreatic extract.

2. Materials and methods

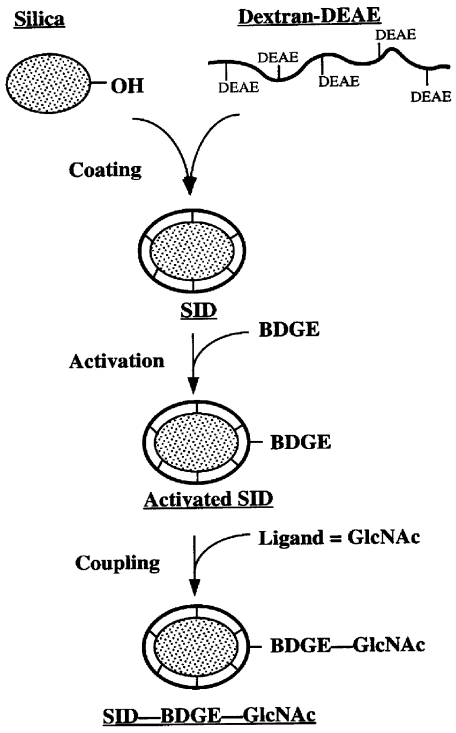
2.1. Synthesis of SID–GlcNAc chromatographic support

The synthesis of coated silica supports is carried out as previously reported [11]. The preparation of affinity support is performed in two steps. First, silica beads are coated with dextran substituted by a calculated amount of DEAE functions to hide negative charges at its surface. Second, ligands are immobilized using a coupling agent. (Fig. 3).

The substitution of dextran T70 (68,000 g/mol, Pharmacia, Bois d'arcy, France) by the 2-chloro *N*,*N*-diethylaminoethane (Janssen Chemica, Noisy le grand, France) is performed in a very alkaline medium at 55 °C for 30 min. The substitution rate of dextran with DEAE is determined by elemental analysis of nitrogen. The conditions of dextran modification were previously determined [12,13] to obtain a percentage of dextran units carrying DEAE groups varying from 4 to 13%.

The 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 dextran–DEAE in 100 ml) adjusted to pH 11. Dextran-coated silica is crosslinked with 1,4-butanedioldiglycidyl ether (BDGE) (Sigma, La Verpillière, France). The amount of dextran–DEAE covering the silica beads is determined by a colorimetric assay of the sugar units after hydrolysis and by elemental analysis of carbon.

The immobilisation of *N*-acetylglucosamine on the stationary phase requires the use of a coupling agent to create covalent bonding between the ligand and the support. In the present study, the coupling agent used is 1,4-butanedioldiglycidyl ether (Sigma, LaVerpillière, France) [14,15]. Activation of dextran coating silica occurs with BDGE in a 1,4-dioxane solution (Carlo Erba, Reuil-Malmaison, France). *N*-Acetylglucosamine is coupled to the activated support in a carbonate buffer for 48 h. The amount of ligand fixed on the support is determined by a colorimetric assay on the coupling solution supernatant using a potassium tetraborate method [16].



DEAE : Diethylaminoethyl
SID : Silica coated by dextran–DEAE
BDGE : 1,4-butanediol diglycidyl ether
GlcNAc:N-acetyl glucosamine

Fig. 3. Schematic representation of the synthesis of a chromatographic support.

2.2. Determination of affinity constants

For the determination of adsorption isotherms, a calculated amount of insulin is incubated with a fixed quantity of support (SID–GlcNAc). The amount of adsorbed insulin on the support can be represented as a function of its initial concentration. Adsorption isotherms were obtained; the affinity constant of insulin for the support and the capacity of the active support were determined using the Langmuir model (Fig. 4) [17].

Adsorption protocol of insulin on different supports is the following [18,19].

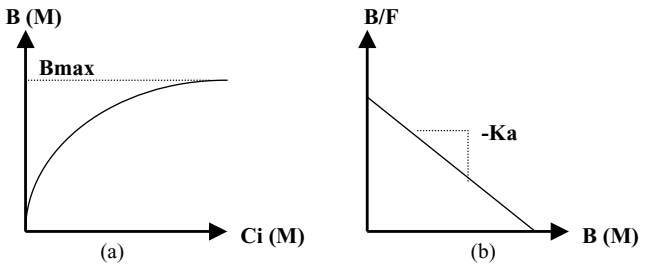


Fig. 4. Chart of adsorption according to the Langmuir model: (a) adsorption isotherm; (b) scatchard representation. *B* (M): concentration of bound protein; Ci (M): initial concentration of protein; *F* (M): concentration of free (non adsorbed) protein.

Ten milligrams of a support suspension in 100 μl phosphate buffer 0.05 M, 0.1 M NaCl, pH 7.4 were incubated with 300 μl of radiolabelled insulin (^{125}I-insulin), at various concentrations (0–200 nM) for 40 min at room temperature under rotative agitation. After five washing steps, the beads are removed and adsorbed insulin is counted by the use of a γ counter (1470 Wizard/W allac).

2.3. HPLAC of insulin

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

Insulin used in HPLAC, kindly provided by Diosynth S.A. (AKZO, Eragny-Sur-Epte, France), is a mixture of porcine insulin with 6% bovine insulin.

A 100 μl quantity of insulin (porcine + 6% bovine) is injected at room temperature, onto the column containing the SID–GlcNAc support at a flow-rate of 0.5 ml/min. The fractions corresponding to elution and desorption peaks are collected, analysed by the Bradford assay to estimate the purification yield.

3. Results and discussion

3.1. Synthesis of SID–GlcNAc

The synthesis of a silica based support coated with polysaccharides permits to combine the advantages of the use of polysaccharides in chromatography, particularly their hydrophylic properties and their ability to be grafted with bioactive ligands, with the silica mechanical resistance properties.

Dextran substitution was made in order to confer a weak anion-exchange capacity by DEAE groups into glycosidic units. T70 dextran substitution rate by DEAE groups amounts to 6%.

The adsorption of dextran–DEAE on silica beads results from interactions between anionic groups on silica surface and DEAE groups carrying positive charges. The coverage is further reinforced by crosslinking dextran chains with BDGE that results in the formation of ether type bonds with polysaccharide hydroxyl groups. Conditions for the optimisation of the silica passivation were already determined [13]. Indeed dextran substitution rate by DEAE groups lower than 4% leaves negative charges to the surface of silica, and a rate higher than 13% creates a surplus of positive charges, which are likely to cause non specific interactions.

The amount of polymeric coverage on the silica supports is 65 mg of dextran–DEAE per g of silica.

In order to ascertain that the stationary phase used for coupling the ligand will not develop non specific interactions with standard proteins, these letters are eluted on the SID support under high-performance size-exclusion chromatography (HPSEC) conditions [11]. The results show that dextran–DEAE covers all accessible protein areas of the support and prevents non specific interactions in the affinity process after ligand coupling.

Activation with BDGE results from condensation of hydroxyl functions on the polysaccharidic support and various functions on the ligand, especially hydroxyl groups. This reaction leads to the formation of an ether-type bonding (Fig. 5). The amount of GlcNAc coupled on SID is 10 mg of GlcNAc per gram of SID; thus, a coupling yield of 50%.

3.2. Insulin adsorption isotherm on support grafted with GlcNAc

Adsorption isotherms for insulin on support are established in order to define the potential selectivity of the ligand and and to estimate the strength of the ligand–insulin interactions by measurement of the affinity constants.

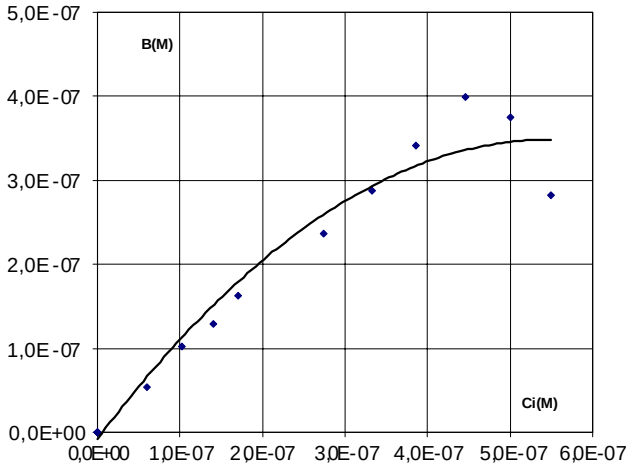


Fig. 6. Adsorption isotherm of insulin on SID–GlcNAc support. *B* (M): concentration of bound insulin; Ci (M): initial concentration of insulin in solution.

Adsorption isotherms are obtained by incubating a variable amount of insulin with a fixed amount of support and they are presented in Fig. 6.

From these adsorption isotherms, the affinity constants of insulin for the support (Ka) and the maximal adsorption capacity (Bmax) were determined according to the Langmuir model. Affinity constant and maximal adsorption capacity amount, respectively to about $10^8\ M^{-1}$ and 10^{-7} M.

These results indicate the existence of strong interactions between insulin and the inactivated silica support with GlcNAc.

3.3. Effect of NaCl

The effect of ionic strength on insulin adsorption was studied using elution buffers with NaCl concentrations varying from 10 to 50 mM. Elution profiles obtained and the protein concentration in the different fractions are reported in Fig. 7. With increasing salt concentrations in the adsorption buffer, the quantity of adsorbed insulin diminishes. This shows that ionic interactions between insulin and SID–GlcNAc are very weak. However, insulin can only be desorbed under high-salt elution conditions. These results seem to demonstrate that the interactions involved in the adsorption and desorption mechanisms are different.

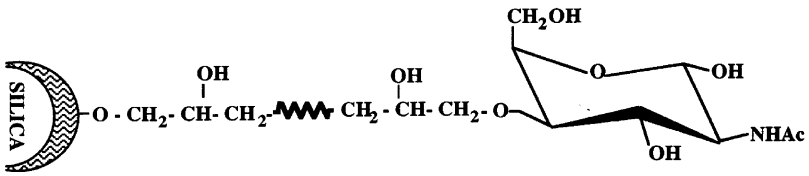


Fig. 5. Structure of an affinity support (SID–GlcNAc).

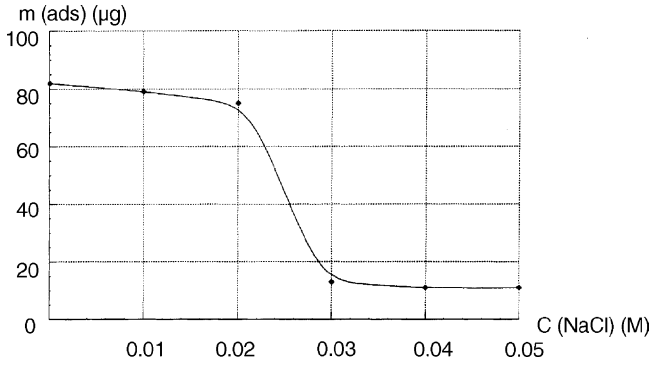


Fig. 7. The effect of ionic strength on insulin adsorption on SID–GlcNAc. *m* (ads) (µg): mass of adsorbed insulin; *C* (NaCl) (M): concentration of sodium chloride.

3.4. Effect of pH

Insulin is a very pH dependant protein. The study of its affinity for the SID–GlcNAc support according to the pH is important to analyse the influence of this pH parameter on the interaction mechanisms of insulin with this support.

The elution of 100 µl of insulin (porcine + 6% bovine) dissolved in an adsorption buffer composed of a 95:5 mixture of water:methanol (v/v) at different pH, is performed. The fractions corresponding to the different adsorption and desorption peaks are collected and analysed by the Bradford assay to determine the protein yield.

Fig. 8 presents the amount of adsorbed protein versus the pH. At acidic pH, no insulin retention occurs, whereas at basic pH the quantity of adsorbed protein increases with the pH.

The non retention of insulin at low pH can be explained by the acidic nature of this protein which provokes, under such conditions, a repulsion between the protein and the positive charges present on the surface of the stationary phase, and thus inhibits insulin adsorption.

Moreover, insulin is relatively insoluble in aqueous medium, in a pH range from 4 to 7, and it has a strong tendency to dimerize. Insulin dimerization prevents its retention on the SID–GlcNAc support.

We suggest that the association of two or several insulin monomers inhibits the interaction between insulin and the support. Furthermore, the region responsible for insulin dimerization is known and sequenced [9] and it can be suggested that insulin interacts with *N*-acetylglucosamine via this region, composed of the following amino acids: Phe B24–Phe B25–Tyr B26–Thr B27.

At basic pH, an increase of insulin retention on the support occurs. Nevertheless, in this pH range, insulin exhibits a negative global charge (pH > pI, insulin pI = 5.3) and; therefore, it should not be retained on the negative charged support.

The interaction between insulin and the support depends on the monomeric state of insulin which governs the accessibility to the active site.

3.5. Effect of insulin concentration

The study of insulin affinity for a given support according to its concentration allows to measure both the maximal adsorption capacity of the support and the affinity constant of insulin for the support under dynamic conditions.

The elution of a 100 µl of insulin at different concentrations is performed as previously described.

The amount of adsorbed insulin increases with the initial insulin concentration until it reaches a saturation plateau corresponding to the maximal adsorption capacity of the active support which is about 9×10^{-5} M/g of silica (Fig. 9). The apparent affinity constant determined under dynamic conditions is about 4×10^{5} M^{-1} and is far weaker than the affinity constant obtained under static conditions. This difference in the affinity constants reflects probably the influence of kinetic parameters on the adsorption or desorption process in chromatographic conditions.

Indeed, under dynamic conditions insulin adsorption comprises four successive steps: the protein transport towards the support (surface), adsorption, structural rearrangement of the protein and finally desorption.

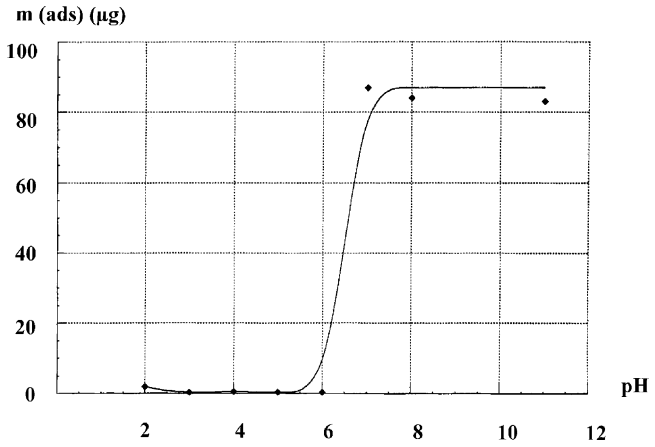


Fig. 8. Influence of the pH eluent on chromatographic adsorption of insulin on SID–GlcNAc support. *m* (ads) (µg): mass of adsorbed insulin.

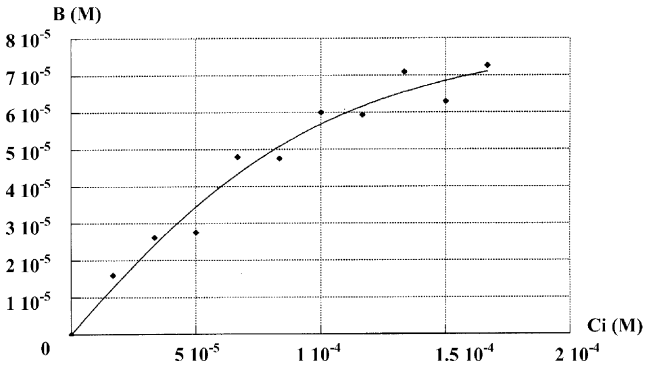


Fig. 9. Influence of insulin concentration on chromatographic adsorption of insulin on SID–GlcNAc support. *B* (M): concentration of bound insulin; Ci (M): initial concentration of insulin in solution.

For a weak insulin concentration, transport towards the support surface mainly depends on convection and diffusion movements of molecules. For a high insulin concentration, interactions between insulin and the support become the limiting factor. Evaluation of the amount of adsorbed insulin is made after insulin desorption.

Under static conditions, insulin is in contact with the support so that less parameter is implicated and the developed surface is greater. The determination of the amount of adsorbed insulin is a direct evaluation.

3.6. Purification of insulin from pancreatic extract

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

The chromatogram (Fig. 10) was obtained by injection of a 100 μl amount of a pancreatic extract. The fractions corresponding to elution and desorption peaks are collected and the amount of insulin contained in both the injected samples and the eluted fractions is detained by RadioImmunoAssay (RIA, CisBiointernational, Gif Sur Yvette, France). The results presented in Table 1 show a purification yield of insulin of 75% from a pancreatic extract.

Finally, the silica based support coated with dextran–DEAE and functionalized by GlcNAc allows a good resolution of insulin and the insulin purification from proteins mixture.

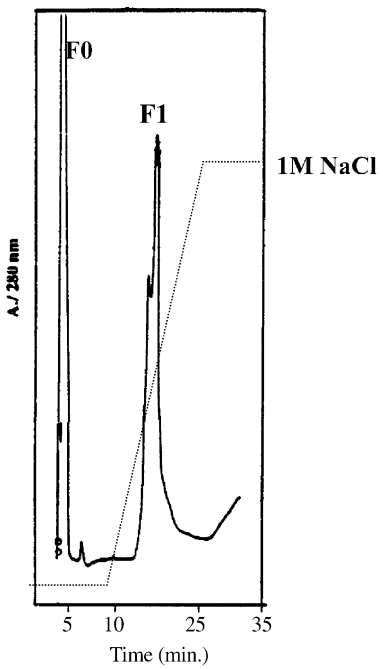


Fig. 10. Elution profile of a pancreatic extract (100 μl) on SID–GlcNAc. Column 12.5 cm × 0.4 cm i.d.; flow-rate 0.5 ml/min; eluents—Ta: 5% methanol (pH 7.4), Tb: phosphate buffer 0.05 M, 1 M NaCl (pH 7.4); F0: non retained fraction; F1: retained fraction.

Table 1
Purification yield of insulin on support functionalized with *N*-acetylglucosamine

Injected quantity (μg)	85.3
F0 (μg)	9.3
F1 (μg)	64.7
Purification yield (%)	75

F0: non retained fraction; F1: retained fraction.

These results evidence the affinity and the specificity of GlcNAc for insulin.

4. Conclusion

The behaviour of insulin towards the support functionalized with *N*-acetylglucosamine was studied by affinity chromatography and the results obtained led us to analyse the influence of several physico-chemical parameters on ligand–insulin interactions.

As previously demonstrated [11], the ligand–insulin complex is formed by different attractive forces of various natures resultant of which determines the stability of this complex and constitutes an affinity interaction. Hydrophilic, hydrophobic and weak ionic interactions are probably involved in the overall interaction mechanism.

Different parameters (pH, NaCl and insulin concentration) influence the mechanisms of the separation in HPLAC and show the complexity of these interactions. These interactions depend both on adsorption and desorption process and affinity mechanisms.

The influence of insulin concentration allowed us to estimate the maximal adsorption capacity of the support and finally to improve the purification yield. pH determines the insulin solubility and the protein ionisation. Both pH and insulin concentration influence the retention of insulin on the functional support.

From the present study, we can assume that insulin interacts with the *N*-acetylglucosamine ligand via the amino acids sequence Phe B24–Phe B25–Tyr B26–Thr B27 of the B chain.

The interactions between insulin and immobilized *N*-acetylglucosamine residues result in an affinity mechanism. This affinity was utilized to purify insulin by affinity chromatography on coated silica based supports grafted by *N*-acetylglucosamine.

Further experiments would be necessary to improve the purification yield of the support as well as its selectivity towards insulin.

This can be carried out by grafting, in addition to *N*-acetylglucosamine, another specific ligand on the support in order to mimic the insulin receptor structure. Finally, the aim would be the performance of an “insulin-receptor like” support.

Such a work could also contribute to better understand the interaction mechanisms of insulin with its receptor.

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