Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 818 (2005) 53–59
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification of IgG and insulin on supports grafted by sialic acid developing "thiophilic-like" interactions

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

[a] *Laboratoire Qualité, Santé et Environnement, Faculté Polydisciplinaire de Taza, BP 1223 Taza-Gare, Université Sidi Mohamed Ben Abdellah, Taza, Marocco*
[b] *Département de Chimie, Université Paris-XIII, Institut Galilée, 99 Avenue J.B. Clément, 93430 Villetaneuse, France*

Received 10 June 2004; accepted 26 October 2004
Available online 10 December 2004

Abstract

We developed the synthesis of new supports for the purification of insulin and IgG by affinity chromatography. The preparation of such an affinity support is performed in two steps. First, silica beads are coated with dextran polymers carrying a calculated amount of positively charged diethylaminoethyl groups in order to mask negative charges at its surface. Second, ligand is immobilized using a coupling agent. This support combines the advantages of polysaccharide phases with the excellent mechanical characteristics of silica. The existence of *N*-acetylneuraminic acid (sialic acid) in insulin receptor and in the antigenic determinant of IgG suggests that such an acid may develop specific interactions usable in affinity chromatography. Therefore, *N*-acetylneuraminic acid was used as an active ligand. The immobilization of sialic acid can be carried out by using the conventional coupling agent: the carbonyldiimidazole. The performances of these supports grafted by sialic acid were studied by high-performance liquid affinity chromatography (HPAC). The optimization of the chromatographic conditions (support characteristics and mobile phase) enabled us to observe a behavior of the type "thiophilic" of the support, which does not contain sulfone group. This new affinity support allowed a one-step separation of the IgG from mouse ascitic fluids and also allowed the insulin purification from a pancreatic extract with a good purification yields.

Keywords: Silica; HPAC; Thiophilic interactions; *N*-Acetylneuraminic acid; Insulin; IgG

1. Introduction

The applications requiring obtaining pure proteins are numerous and much of supports are now commercially available. In the case of insulin, the purification is done in several stages on essentially supports of ions-exchange [1,2], gel filtration [3] and reverse phase [4]. In the case of IgG, the supports are essentially of two types: the supports of ions-exchange [5] and the supports grafted by protein A [6], or protein G [7]. However, these supports present some disadvantages, particularly the low specificity of the supports of ions-exchange. The supports grafted by bacterial proteins have a high cost and a low stability under extreme conditions used during regenerations of the supports. Therefore, the development of supports capable of adsorbing IgG and insulin, presenting better chromatographic performances is of a great interest

High-performance affinity chromatography (HPAC) is a technique of separation broadly used for the purification of biological molecules. This method requires, on the one hand, a support that resists high pressures and can be chemically modifiable and, on the other hand, a biospecific ligand to be coupled to the support.

The ligand used in the present study, *N*-acetylneuraminic acid (NANA) (Fig. 1) belongs to the family of "general ligand". In fact, it binds IgG [8] as well as sialidases [9] and it develops an affinity for insulin too [10,11]. At first sight, one can consider that there is no specificity; nevertheless, the particular and selective conditions of adsorption and desorp-

* Corresponding author. Tel.: +33 1 4940 3362; fax: +33 1 4823 2801.
E-mail address: dmuller@galilee.univ-parisl3.fr (D. Muller).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.10.060

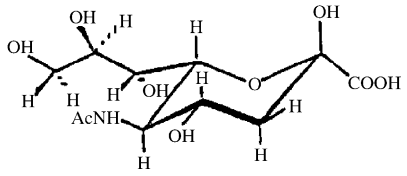

Fig. 1. Structure of *N*-acetylneuraminic acid.

tion 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 immobilized sialic acid. Moreover, the conditions previously used [11] to adsorb insulin on the support are little selective. So, we have to find new selective elution conditions to absorb insulin.

In addition, the elution of insulin on the supports bearing coupling agents shows the contribution of such activation agents in the interaction mechanism between insulin and chromatographic supports. The importance of these interactions varies with the nature of the coupling agent used [12]. Therefore, we felt the need to find another method to confirm or not the specificity of our support for insulin and IgG.

With this intention, the divinylsulfone, used in previous experiments [13], directed the choice of new elution conditions. Indeed, the divinylsulfone is a coupling agent largely used for synthesis of the thiophilic supports [14,15]. The characteristic of this so-called "thiophilic adsorption" is that the proteins are adsorbed on the support with high water-structuring salts (such as potassium sulfate or sodium sulfate) concentration, and selectively desorbed at low salt concentration [16]. This adsorption is based on the formation of an electron-donating (thioether group) and electron-accepting (sulfone group) complex between the ligand and the protein.

In the present study, we synthesized new chromatographic supports based on silica coated with dextran substituted by a calculated amount of diethylaminoethyl (DEAE) groups. *N*-Acetylneuraminic acid was coupled to the activated support by carbonyldiimidazole (CDI). A part of the activated support (support bearing inactivated coupling agent) was used to perform controls and to compare the efficiency of functionalized support (support with ligand) and the support without ligand. The performance of these supports was tested towards both mouse IgG and insulin.

Mouse IgG contains four subclasses, named IgG1, IgG2a, IgG2b and IgG3. IgG3 differs from the others by the presence of a longer hinge region conferring a sensitivity to enzymatic digestion and also a higher molecular mass on these antibodies. Insulin is a 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 interactions between these supports and mouse monoclonal IgG subclasses and insulin was studied, in the similar conditions of thiophilic adsorption, by high-performance affinity chromatography. The influence of some support characteristics on the affinity of IgG subclasses and insulin was also analyzed. The support was then used to attempt the purification of insulin and IgG subclasses from a pancreatic extracts and mouse ascites, respectively.

2. Materials and methods

2.1. Synthesis of SID–NANA chromatographic support

The synthesis of coated silica supports functionalized by *N*-acetylneuraminic acid was carried out as reported previously [11]. The preparation of the affinity support was performed in two steps. First, silica beads were coated with dextran substituted by a calculated amount of diethylaminoethyl (DEAE) functions to hide negative charges at its surface. Second, ligands were immobilized using a coupling agent.

The substitution of dextran T70 (68,000 g/mol) (Pharmacia, Bois d'Arcy, France) by 2-chloro-*N*,*N*-diethylaminoethane (Janssen Chemica, Noisy Le Grand, France) was 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 for dextran modifications for an optimal passivation were previously determined [18,19] 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), were impregnated with a modified dextran solution (8 g of Dx-DEAE in 100-ml) adjusted to pH 11. Dextran coated silica was cross-linked with 1,4-butanedioldiglycidyl ether (BDGE) (Sigma, La Verpilliere, France). The amount of Dx-DEAE covering the silica (SID) beads was 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 was evaluated by testing the elution of standard proteins on the support under high-performance size-exclusion chromatographic (HPSEC) conditions [12].

The immobilization of *N*-acetylneuraminic acid (NANA) extracted from edible birds nests [11], on SID required the use of a coupling agent to create covalent bonding between the ligand and the support (SID–NANA). In this study, NANA was coupled to the activated support by carbonyldiimidazole (CDI). The activation of the basic support by CDI was achieved according to the following protocol: a 3 g quantity of CDI (Sigma) was dissolved in 10 ml of dried 1,4-dioxane. A 6 g quantity of dextran-coated silica was added to the solution and the suspension was gently stirred for 2 h at room temperature. The activated support was then collected by filtration and quickly washed with 200 ml of l,4-dioxane and 250 ml of 0.1 M carbonate buffer, pH 9.

NANA was coupled with this activated support according to the following protocol: a 5 g quantity of activated support were suspended in 25 ml of 0.1 M carbonate buffer, pH

8.7 containing 100 mg of NANA. The suspension was gently stirred for 48 h at room temperature. The supernatant was kept for a further determination of the non-coupled ligand. The support was then filtered and washed with 200 ml of 0.1 M Tris–HCl buffer, pH 8.7. The excess reagent groups were deactivated in 25 ml of the latter buffer, under agitation for 3 h at room temperature. The support was successively washed with solutions of 0.05 M Tris–HCl, pH 7.4, 1 M NaCl and with 200 ml of the same buffer containing 0.05 M NaCl. The functionalized (SID–CDI–NANA) support was conditioned in this buffer at 4 °C.

A part of the activated support (SID–CDI) was used to perform controls and to compare the efficiency of SID–CDI–NANA and SID–CDI. An inactivation was operated on support carrying coupling agent to eliminate its reactive chemical functions and, thus, to avoid the covalent binding of proteins through the column. For this reason, activated support was suspended in a solution of 0.1 M carbonate buffer at pH 8.7, which contained 1.5 ml of ethanolamine (Janssen Chemica, France). The mixture was slowly stirred for 48 h at room temperature and finally washed with a solution of 0.1 M Tris–HCl buffer at pH 8.7. The amount of the ligand fixed on the support was determined by a spectrophotometric assay of the coupling solution supernatant using a periodate–resorcinol method [20].

2.2. HPAC of insulin and lgG

The HPAC 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 supports (SID–CDI–NANA and SID–CDI) were introduced into 12.5 cm × 0.4 cm i.d. stainless-steel column (Merck, France) by dried packing method. The column was generally eluted with 0.05 M phosphate buffer–0.15 M NaCl (pH 7.4) buffer solution.

The insulin used in HPAC, kindly provided by Diosynth (AKZO, Eragny-Sur-Epte, France), was a mixture of porcine insulin with 6% bovine insulin. A 100 μl amount of the insulin was injected onto the column containing the support at a flow-rate of 0.5 ml/min. The fractions corresponding to elution and desorption peaks were collected and the amount of insulin contained in both the injected samples and the eluted fractions was determined by RadioImmunoAssay (RIA, Cis-Biointernational, Gif Sur Yvette, France).

The ascitic fluids used in this study were provided by Cis-Bioindustries (LAPAM, Bagnols sur Cèze, France). The ascitic fluids were diluted in the initial chromatographic buffer and filtered through a 0.2 μm filter before injection onto column. The quantity of mouse monoclonal antibody was determined by an enzyme-linked immunoassay (ELISA) from Boehringer Mannheim (Meylan, France).

3. Results and discussion

3.1. Synthesis of SID–NANA chromatographic 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 [21]; then, mineral phases were modified by coating of hydrophilic polymers [22,23]. In our laboratory, we are currently using silica-based supports, which are coated with a calculated amount of diethylaminoethyl groups.

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

In order to ascertain that the stationary phase used for coupling of the ligand would not undergo non-specific interactions with standard proteins, these proteins were eluted on the SID support under high-performance size-exclusion chromatographic conditions [12]. The results showed that no interaction occurs in the selected elution conditions. The SID support was neutral enough to be coupled to a biospecific ligand and to avoid non-specific interactions with proteins in solution.

Coupling of sialic acid with SID support was performed using a coupling agent: carbonyldiimidazole (CDI). Activation with CDI occurs by condensation of a hydroxyl group on the polysaccharidic support and various functions on the ligand. The amount of NANA coupled with SID was determined by a colorimetric assay using a periodate–resorcinol method. The amount of fixed NANA on SID support was 10 mg/g silica and the coupling yield amounts was 50%. The structure of SID–CDI–NANA support and SID–CDI support is presented in Fig. 2.

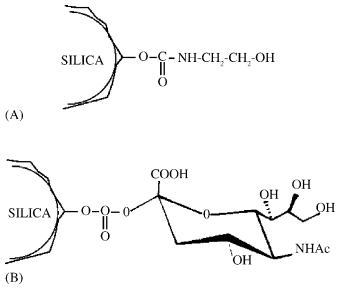

Fig. 2. Structure of affinity supports: (A) SID–CDI; (B) SID–CDI–NANA.

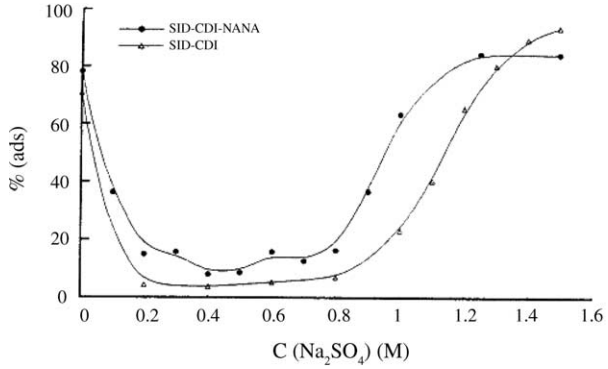

Fig. 3. Influence of sodium sulfate concentration on insulin adsorption on SID–CDI and SID–CDI–NANA supports. % (ads): % adsorbed proteins; *C* (Na_2SO_4) (M): concentration of sodium sulfate.

3.2. Effect of Na_2SO_4

The elution of insulin was carried out at the same time on the support grafted by the sialic acid and on the support carrying only the coupling agent (carbonyldiimidazole), in the presence of Tris–HCl (50 mM) buffer, with various concentration out of Na_2SO_4. The fractions corresponding to elution and desorption peaks were collected and the protein concentration in the different fractions was determined by the Bradford assay. Fig. 3 shows a plot of the amount of adsorbed protein versus sodium sulfate concentration.

Fig. 3 demonstrates that the insulin was adsorbed to the supports in a manner, which can be both independent as well as dependent upon the presence and concentration of sodium sulfate. Under these experimental conditions, the support only modified by grafting of the coupling agent adsorbs insulin in the identical manner that the support carrying sialic acid.

In order to seek the experimental factors affecting the selectivity and the capacity of insulin adsorption on each support, proteins of different structure, size and composition were eluted on the two supports under the same conditions as insulin. These proteins are immunoglobulins G (IgG), the lysozyme and trypsin.

Fig. 4 shows the effect of the sodium sulfate concentration on the adsorption of these proteins on SID–CDI support in comparison with insulin. On the support carrying only the coupling agent and beyond a certain $NaSO_4$ concentration (0.6 M), these proteins were adsorbed to a significant degree. With increasing salt concentration (Na_2SO_4) in the adsorption buffer, the quantity of adsorbed trypsin and lysozyme on the support carrying sialic acid decreased.

The grafting of sialic acid (Fig. 5) on support activated by the carbonyldiimidazole thus prevented the adsorption of trypsin and the lysozyme on the support and allowed a better adsorption of insulin and IgG. This seems to indicate a specificity of the support for these two proteins.

For the continuation of work, the concentration of sodium sulfate required to promote adsorption of insulin and IgG on SID–CDI–NANA was 1.5 M.

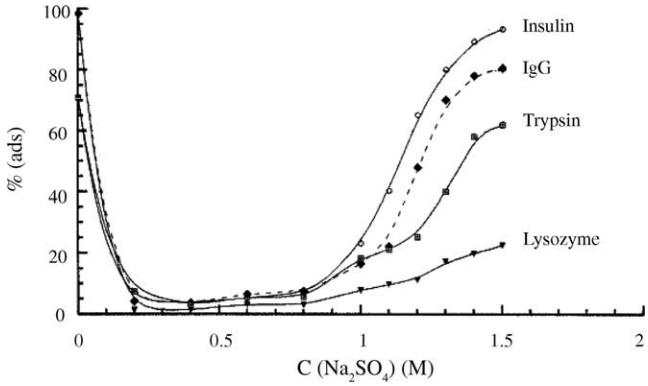

Fig. 4. Influence of sodium sulfate concentration on proteins adsorption on SID–CDI support. % (ads): % adsorbed proteins; *C* (Na_2SO_4) (M): concentration of sodium sulfate.

3.3. Nature of the interaction taking place between insulin, IgG and the functionalized support

The effect of ionic strength on insulin and IgG adsorption was studied using different elution buffers (Na_2SO_4) with NaCl concentration varying from 0 to 1 M. As shown in Fig. 6, the inclusion of 0.3 M sodium chloride did not reduce the adsorption of insulin and IgG promoted by sodium sulfate. However, with increasing sodium chloride concentrations in the adsorption buffer, the quantity of adsorbed proteins diminished gradually.

An increase in ionic force improves the decrease of the electrostatic interactions and the increase of the hydrophobic interactions. These two interactions were probably not implied in the adsorption of insulin and IgG on this support. As for sodium chloride, the effect of methanol was studied in the same way. Fig. 7 shows the percentage of proteins adsorbed according to the concentration of methanol in the adsorption buffer. The results showed that no methanol concentration effects were obvious during adsorption of insulin and IgG on the support.

The absence of hydrophobic interactions was confirmed by the study of the influence of methanol on the adsorption

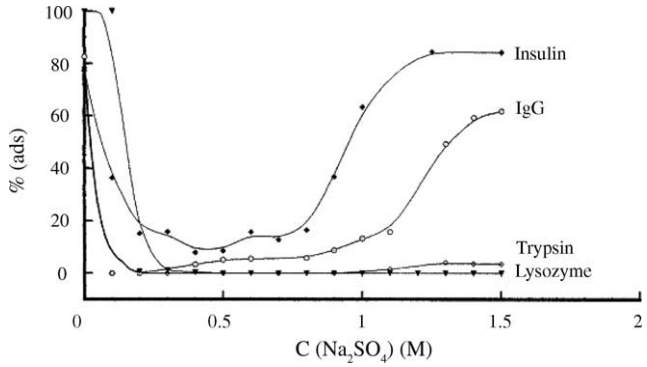

Fig. 5. Influence of sodium sulfate concentration on proteins adsorption on SID–CDI–NANA support. % (ads): % adsorbed proteins; *C* (Na_2SO_4) (M): concentration of sodium sulfate.

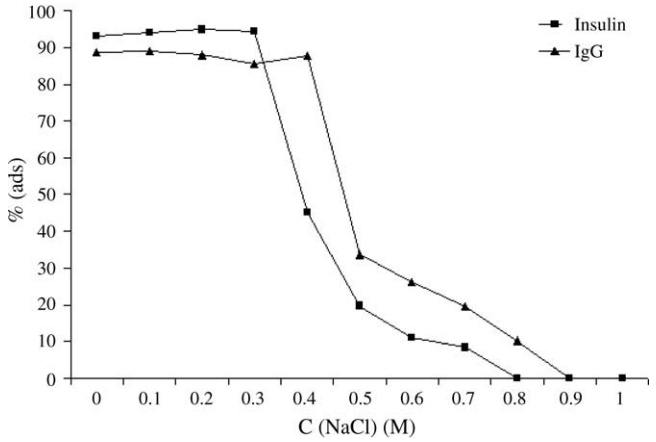

Fig. 6. Effect of sodium chloride concentration on insulin and IgG adsorption on SID–CDI–NANA support in presence of sodium sulfate. % (ads): % adsorbed proteins; *C* (NaCl) (M): concentration of sodium chloride.

of these two proteins on the support functionalized by sialic acid.

As previously described for the effect of NaCl, we proceeded to the elution of insulin and IgG on SID–CDI–NANA at different pH values. The fractions corresponding to the different adsorption and desorption peaks were collected and analyzed by Bradford assay to determine the protein yield. Fig. 8 shows a plot of the amount of adsorbed insulin and IgG versus pH. In the presence of sodium sulfate concentrations normally required to promote adsorption, no pH effects were obvious during adsorption of insulin and IgG on the support. The surface ionization of insulin and IgG as that of the support did not affect the adsorption capacity of these proteins on this support. The electrostatic interactions were thus not responsible for the adsorption observed under these conditions.

So far, the effect of NaCl and methanol as well as for the influence of pH on insulin and IgG adsorption have been discussed. For this discussion, we can conclude that the hydrophobic interactions and the electrostatic interactions were

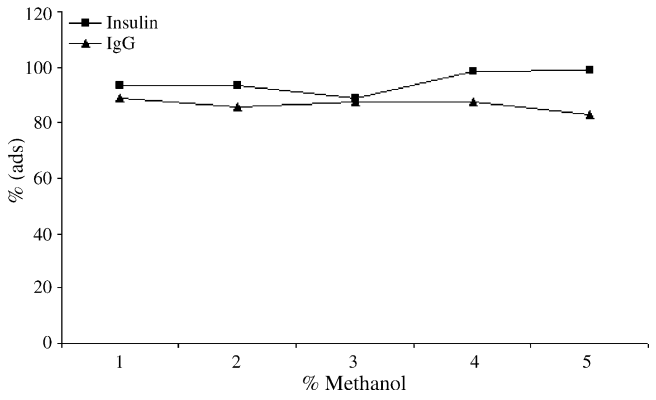

Fig. 7. Effect of methanol on insulin and IgG adsorption on SID–CDI–NANA support in presence of sodium sulfate. % (ads): % adsorbed proteins; % methanol in adsorption buffer.

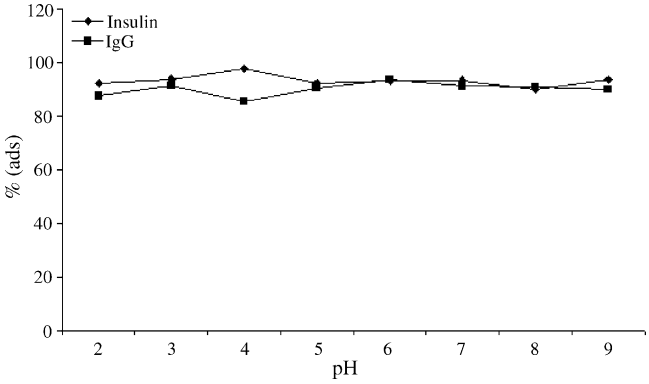

Fig. 8. Effect of pH on insulin and IgG adsorption on SID–CDI–NANA support in presence of sodium sulfate. % (ads): % adsorbed proteins.

thus not responsible for the adsorption observed under these conditions.

3.4. Purification of IgG from mouse ascites

A 100 μl quantity of mouse ascites was injected onto the column at a flow-rate of 0.5 ml/min. The conditions for adsorption of IgG (four IgG subclasses: IgG1, IgG2a, IgG2b and IgG3) on the support are described in Fig. 9. The fractions corresponding to elution and desorption peaks were collected and the quantity of antibodies was determined by an enzyme-linked immunoassay (Table 1). Under these conditions, the amount of IgG retained on the SID–CDI–NANA column was 41, 34, 37 and 64%, respectively for IgG1, IgG2a, IgG2b and IgG3. The results showed that the support SID–CDI–NANA tended to develop a higher affinity for the IgG3 subclass rather than for the other subclasses. The support functionalized with

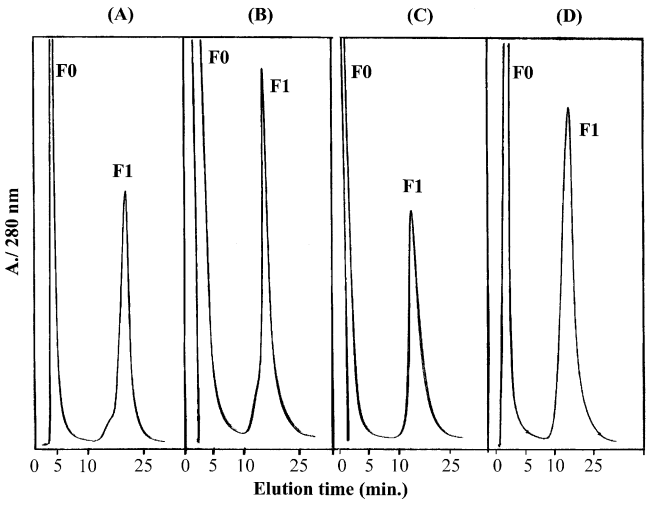

Fig. 9. Elution profile of mouse ascites (100 μl) on SID–CDI–NANA. (A) IgG1; (B) IgG2a; (C) IgG2b; (D) IgG3. Column: 12.5 cm × 0.4 cm i.d. Flow-rate: 0.5 ml/min. Eluents: Ta: Tris–HCl 0.05 M, 1.5 M Na_2SO_4, pH 9; Tb: Tris–HCl 0.05 M, 1 M ammonium acetate, pH 9. Ta: adsorption buffer; Tb: desorption buffer; F0: non-retained fraction; F1: retained fraction.

Table 1
Purification yield of IgG and antibody quantities injected on SID–CDI–NANA support and collected in the eluted fractions (assay numbers, $n = 5$)

Ascites	Injected quantity (μg)	F0 (μg)	F1 (μg)	Yield (%)
IgG1	1350 ± 67.5	702.32 ± 28.09	553.18 ± 22.12	41
IgG2a	2900 ± 174	1169.05 ± 81.83	976.95 ± 68.38	34
IgG2b	3390 ± 135.6	1153.70 ± 57.68	1253.20 ± 62.66	37
IgG3	480 ± 14.4	159.12 ± 9.54	306.48 ± 12.25	64

sialic acid allowed the IgG purification from mouse ascites and particularly the IgG3 subclass.

3.5. Purification of insulin from pancreatic extract

The present study evidence that the support bearing sialic acid allows a good resolution of pure insulin. In order to confirm the affinity 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).

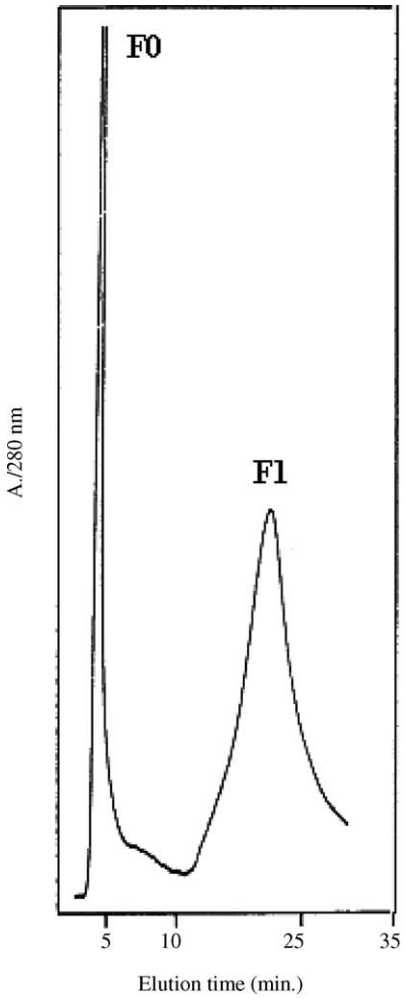

Fig. 10. Elution profile of a pancreatic extract (100 μl) on SID–CDI–NANA. Column: 12.5 cm × 0.4 cm i.d. Flow-rate: 0.5 ml/min. Eluents: Ta: Tris–HCl 0.05 M, 1.5 M Na_2SO_4, pH 9; Tb: Tris–HCl 0.05 M, 1 M ammonium acetate, pH 9. Ta: adsorption buffer; Tb: desorption buffer; F0: non-retained fraction (numerous proteins including insulin); F1: retained fraction (insulin).

Table 2
Purification yield of insulin and insulin quantities injected on SID–CDI–NANA support and collected in the eluted fractions (assay numbers, $n = 5$)

Support	Injected quantity (μg)	F0 (μg)	F1 (μg)	Yield (%)
SID–CDI–NANA	189 ± 9.45	4.5 ± 0.27	109 ± 7.63	58

The chromatogram of Fig. 10 was obtained by injection of a 100 μl amount of a pancreatic extract under the same conditions as those used previously. The fractions corresponding to elution and desorption peaks were collected and analyzed. The amount of insulin contained in the injected samples and the eluted fractions was determined by RadioImmunoAssay (RIA, Cisbiointernational, Gif Sur Yvette, France). The results presented in Table 2 show a purification yield of insulin of 58% from a pancreatic extract. Finally, the silica based support coated with dextran–DEAE functionalized by sialic acid allowed insulin purification from protein mixture. These results evidence the affinity and the specificity of sialic acid for insulin.

4. Conclusion

The affinity of the insulin and mouse monoclonal IgG subclasses for dextran-coated silica supports grafted by NANA was studied. These affinity supports allowed a one-step separation of the insulin and IgG sublclasses from a pancreatic extract and mouse ascitic fluids, respectively, by HPAC. The adsorption of IgG1, IgG2a and IgG2b was weaker than IgG3 under our experimental conditions.

This study also demonstrated the influence of some support characteristics and mobile phase on the performance of the affinity support functionalized by sialic acid. The support carrying only the coupling agent (CDI) adsorbed, in a non-selective way, four proteins tested (insulin, IgG, trypsin and lysozyme). The grafting of sialic acid on support activated by the carbonyldiimidazole thus prevented the adsorption of trypsin and the lysozyme on the support and allowed a better adsorption of insulin and IgG. This tended to show the specificity of the support for these two proteins. This behavior is probably related to the nature of the functional groups carried at the same time by proteins and the supports.

The influence of mobile phase (Na_2SO_4, pH, NaCl, methanol) on the performance of the support bearing sialic acid in affinity chromatography was studied. No pH and methanol concentration effects were obvious during the adsorption of insulin and IgG. The inclusion of 0.3 M sodium

chloride in the adsorption buffer did not reduce the adsorption of insulin and IgG. However, with increasing sodium chloride concentrations in the adsorption buffer, the quantity of adsorbed proteins diminished.

The proteins (insulin and IgG) were adsorbed on the support with high water-structuring salts (such sodium sulfate) concentration, and selectively desorbed at low salt concentration, which indicated interactions of hydrophobic type or thiophilic type. However, the inclusion of high concentrations of sodium chloride (0.5 M) decreased the adsorption capacity of the support. Under such conditions, the support SID–CDI–NANA showed similar adsorption characteristics with thiophilic support. The support SID–CDI–NANA developed "thiophilic-like" interactions.

The use of these new elution conditions thus enabled us to exploit this support in affinity chromatography and to develop a new method of purification of insulin and IgG.

References

[1] F. Horwitz, H. Alp, L. Recant, J. Lab. Clin. Med. 641 (1964) 942.
[2] N.K. Boardman, Biochim. Biophys. Acta 18 (2) (1955) 290.
[3] C.J. Epstein, C.B. Anfinsen, Biochemistry 131 (1963) 461.
[4] I. Ismail Salem, M.C. Bedmar, M.M. Medina, A. Cerezo, J. Liq. Chromatogr. 16 (5) (1993) 1183.
[5] M. Page, R. Thorpe, in: J.M. Walker (Ed.), The Protein Protocols Handbook, Humana Press, Totowa, NJ, 1996, p. 721.
[6] R. Hahn, R. Schlegel, A. Jungbauer, J. Chromatogr. B, Anal. Technol. Biomed. Life Sci. 790 (1–2) (2003) 35.
[7] E. Kitsiouli, M.E. Lekka, G. Nakos, C. Cassagne, L. Maneta-Peyret, J. Immunol. Methods 271 (1–2) (2002) 107.
[8] A. Serres, E. Legendre, J. Jozefonvicz, D. Muller, J. Chromatogr. B 681 (2) (1996) 219.
[9] J. Suzuki, K. Murakami, Y. Nishimura, J. Carbohydr. Chem. 12 (1993) 201.
[10] H. Lakhiari, E. Legendre, D. Muller, J. Jozefonvicz, J. Chromatogr. B 664 (1995) 163.
[11] H. Lakhiari, D. Muller, J. Jozefonvicz, J. Chromatogr. A 711 (1995) 93.
[12] H. Lakhiari, J. Jozefonvicz, D. Muller, J. Chromatogr. B 706 (1998) 33.
[13] H. Lakhiari, Thesis, University Paris XIII, 1996.
[14] L.M. Sandberg, A. Bjurling, P. Busson, J. Vasi, R. Lemmens, J. Biotechnol. 109 (1–2) (2004) 193.
[15] J. Porath, M. Belew, TIBTECH 5 (1987) 225.
[16] J. Porath, F. Maisano, M. Belew, FEBS Lett. 185 (2) (1985) 306.
[17] F.P. Steiner, Diabetes 26 (4) (1977) 322.
[18] X. Santarelli, D. Muller, J. Jozefonvicz, J. Chromatogr. 443 (1988) 55.
[19] F.L. Zhou, D. Muller, X. Santarelli, J. Jozefonvicz, J. Chromatogr. 476 (1989) 195.
[20] G.W. Jourdian, L. Dean, S. Roseman, J. Biol. Chem. 246 (1971) 430.
[21] S. Hjersten, J. Chromatogr. 87 (1973) 325.
[22] R.J. Blagrove, M.J. Frenkel, J. Chromatogr. 132 (1977) 399.
[23] L. Letot, J. Lesec, C. Quivoron, J. Liq. Chromatogr. 4 (1982) 1311.