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Protein adsorption on histidyl-aminohexyl-Sepharose 4B I. Study of the mechanistic aspects of adsorption for the separation of human serum albumin from its non-enzymatic glycated isoforms (advanced glycosylated end products)

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Abstract

The characteristics of albumin adsorption on histidyl-aminohexyl-Sepharose 4B were investigated. In particular, the adsorption capacity of the gel was studied as a function of conductivity and pH of the running buffer. The adsorption was maximum at low salt concentration around neutral pH, involving electrostatic and hydrophobic interactions. Kinetic aspects were also investigated. Dissociation constant (K_D) and maximum capacity (Q_x) were, respectively, estimated to be 4.5×10^{-5} *M* (medium affinity) and 93.3 mg (high capacity) of human serum albumin per ml of adsorbent. According to these preliminary results, separation of HSA and its non-enzymatically glycated isoforms (conventionally named advanced glycated end products: AGEs) was achieved. Chromatographic potential of this separation tool is discussed.

Keywords: Proteins; Histidyl-aminohexyl-Sepharose 4B; Human serum albumin; Advanced glycated end products

1. Introduction

L-Histidine has been reported to be a fairly selective and efficient immobilized ligand for the purification of a variety of bio-molecules [1,2]. Previous studies on histidine coupled matrices showed that support (soft gel, hollow fiber), spacer arm and orientation of the ligand through its COOH or α-NH_2 groups were important to confer the specificity/capacity towards the molecule to be separated [3,4]. In fact, due to the residual charge of the remaining free charged group and the properties of imidazole side chain (mild hydrophobicity, hydrogen bonding, charge transfer, van der Waals interactions), adsorption and elution conditions could be finely adjusted to purify molecules from crude extracts as well as to discriminate between subspecies/isoforms of that protein [5,6].

Among the different proteins purified on immobilized histidine ligand, particularly interesting results were obtained with immunoglobulins G (IgGs) adsorption on histidyl-aminohexyl-Sepharose 4B (His-AH-Seph). El kak and Vijayalakshmi have thus shown that this support was efficient to purify IgGs

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from mouse ascites fluid and to separate IgG_1 and IgG_2 isoforms from prepurified Cohn II fractions [7,8]. However, due to the free remaining cationic α-NH_3^+ group of histidine, other proteins of serum could be also adsorbed, especially human serum albumin [9].

Human and bovine serum albumins (HSA and BSA) are often considered as model proteins. Nonetheless, the 'serum carrier' presents a great heterogenicity from a molecular point of view [10], since it is associated in serum with drugs, amino acids (notably cysteine or tryptophan), metals, steroid hormones. Furthermore, due to its hydrophobicity and its low isoelectric point, this molecule appears as a dominant contaminant in many purification systems. As a consequence, albumin is a very difficult protein to study and to isolate notably in the case of pathologies such as diabetes or renal failure, where albumin heterogenicity is even more increased by randomized AGEs (advanced glycated end products) addition via Maillard reaction [11,12]. In order to study this very important biological molecule, investigators need efficient methods in terms of adsorption capacity and separation efficiency. An optimal method could be a compromise between low specificity but high capacity adsorbent (such as ion exchanger) and very specific sorbent (such as anti-AGEs antibodies); compromise is offered by pseudobiospecific ligands such as dyes, histidine, metal chelates or boronic acids.

In the present work, we investigated the parameters (salt concentration, pH, buffer composition) of albumin adsorption on histidyl-aminohexyl-Sepharose 4B. Mechanistic and kinetic aspects were taken up to appreciate the potential of proteins adsorption capacity of this gel as a function of chromatographic conditions. In addition, we showed that HSA and its non-enzymatic glycated AGEs products could be separated in one gentle chromatographic step, designing a new simple technique for the adsorption/separation of AGEs products.

2. Experimental

2.1. Materials

Aminohexyl-Sepharose 4B and Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Acetic acid, acrylamide, bovine and human serum albumins (BSA and HSA), ammonium persulfate, 1,4-butanedioldiglycidyl ether (bisoxirane), D-glucose, glycine, hydroxyethylpiperazine propane sulfonic acid (EPPS), methylenbisacrylamide, morpholino ethane sulfonic acid (MES), morpholino propane sulfonic acid (MOPS), sodium acetate, sodium chloride, sodium dodecyl sulphate (SDS), sodium phosphate, tetramethyl ethylene diamine (TEMED) and Tris[hydroxymethyl]-aminomethane (Tris–HCl) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals used were of analytical-reagent grade. Ultra-pure water, obtained with the Milli-RO–Milli-Q+ System (Millipore, Bedford, MA, USA), was used throughout.

2.2. Histidine immobilization on Sepharose 4B

Histidine was coupled onto aminohexyl-Sepharose 4B via its COOH group using water-soluble carbodiimide at pH 4.5–6.0 with lateral stirring for 6 h at room temperature as previously described [7]. Histidyl-bisoxirane-Sepharose 4B (His-B-Seph) was synthetised as described by Kanoun et al. [13]. In this way, a reactive oxirane group was introduced into the matrix and subsequently coupled with the α-amino group of histidine. The proposed structures of the gels are shown in Fig. 1.

2.3. Sample preparation and protein quantification

Glycation of commercial HSA (50 mg ml^{-1}) was performed in 100 m*M* phosphate buffer, pH 7.4, by the addition of 100 m*M* D-Glucose. Solutions were sterilized by microfiltration (0.22-μm filters, Amicon, Beverly, MA, USA) and incubated in the dark during 90 days at 37°C. Proteins were then purified and washed with water on Centricon concentrators (cut-off, 10 kDa; Amicon, Beverly, MA, USA). Protein concentrations were determined by the Bradford method [14], using crystalline bovine serum albumin as standard.

2.4. Chromatographic procedures

Gels suspended in ultra-pure water were degassed and packed into a column (10 cm×1 cm I.D.; Pharmacia, Uppsala, Sweden) to provide bed vol-

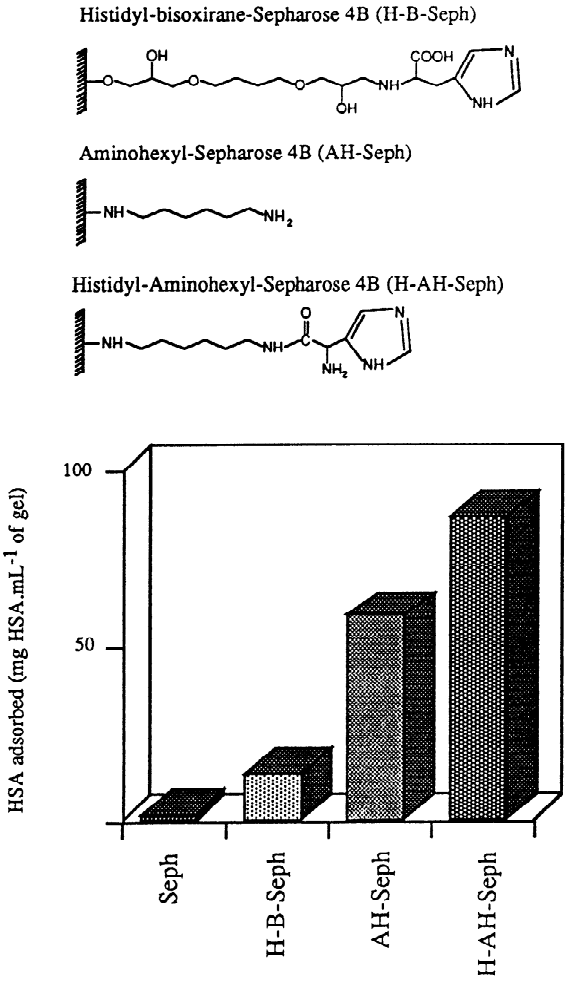

Fig. 1. Schematic structures and HSA adsorption capacities of different Sepharose gels. Seph, Sepharose 4B; H, histidine; B, bisoxirane = 1,4-butanedioldiglycidyl ether; AH, aminohexyl.

umes of ca. 1 or 2 ml of gel depending on the experiments. All chromatographic procedures were carried out at room temperature (unless other unspecified) with a linear velocity of 1.27 cm min^{-1} (flow-rate of 1 ml min^{-1}) generated by a peristaltic pump (Millipore). Equilibration of the column was performed by passing three column volumes of adsorption buffer before injection of the protein solutions. Protein concentration was monitored during chromatography by 280 nm absorbance (LKB Bromma monitor, Sweden). Adsorbed proteins were then eluted by increasing NaCl concentrations in the adsorption buffer. After each use, the column was washed with three column volumes of 50 m*M* NaOH, followed by water and finally by the equilibration buffer.

The determination of maximal HSA capacity of the gels was performed in closed-loop, until the absorbance of the effluent reached a plateau (approximately after 15 min). In order to minimize the proteins losses, the close-circuit was applied when absorbance was relayed on the chromatogram (this time corresponding to the dead volume of the column: e.g., ~1.5 min for a column of 1 ml of gel). The same delay was observed during the washing step with adsorption buffer before diverting to waste. The column was thus washed until the measured absorbance reached the base-line. Elution of proteins was then performed by 1 *M* NaCl added to the adsorption buffer. Proteins concentrations were determined in the remaining non-retained fraction (tank) and in the eluted fraction in order to control the mass balance. This protocol ensured a minimum of protein loss without any dilution, since the initial volume of the tank was kept constant.

Elution of proteins by linear gradient of NaCl was performed on a BioLogic low pressure chromatographic apparatus (Bio-Rad, Richmond, CA, USA), including UV (280 nm) and conductivity monitors at the outlet of the column.

2.5. Conductivity and pH

Conductivity and pH of solutions were adjusted using a Consort C831 apparatus (Consort, Turnhout, Belgium). The conductivity of sodium chloride (0–5.0 *M*), dissolved in 25 m*M* MOPS buffer, pH 7.0, was determined. The linear part of the curve (0–0.5 *M* [NaCl]) was used to adjust solutions conductivity by salt addition (sodium chloride dissolved in water), when the same conductivity for different buffers was necessary. This plot was also used for the determination of NaCl concentration during elution by linear salt gradient (measured by conductivity of the column effluent).

2.6. Determination of dissociation constant and maximum binding capacity

A column of 1 ml of gel was filled with His-AH-Seph. Different concentrations of HSA, ranging from 2.5 to 50.0 mg ml^{-1}, were fed in 10 ml of 25 m*M* MOPS buffer, pH 7.0. Injection was carried out

under frontal mode circuit, until the adsorption reached a plateau. Column was then washed with the equilibrium buffer until the UV optical density returned to the base-line. The adsorbed HSA was eluted with the same MOPS buffer containing 1 *M* NaCl and protein concentrations were determined by Bradford method.

For each injected concentration, the capacity of the gel (Q_a) was determined as the amount of protein retained in milligrams per milliliter of the His-AH-Seph gel. The data obtained were fitted to the Langmuir model.

$$Q_a = \frac{Q_x[C]}{K_D + [C]} \quad (1)$$

where $[C]$ is the concentration of starting protein solution (mg ml^{-1} or *M*), K_D is the equilibrium dissociation constant (mg ml^{-1} or *M*), Q_a is the amount of adsorbed protein (mg ml^{-1} of gel), Q_x the maximum adsorption capacity of the gel (mg ml^{-1} of gel).

The dissociation constant, K_D, was calculated from the linearized plot of the equation:

$$\frac{[C]}{Q_a} = \frac{[C]}{Q_x} + \frac{K_D}{Q_x} \quad (2)$$

Data were fitted with Minim 3.0 non-linear parameter estimation computer program (R.D. Purves, University of Otago, New Zealand).

2.7. Electrospray ionization mass spectrometry (ESI-MS)

Human serum albumin and non-enzymatic glycated HSA (AGEs product) were analysed by a single quadrupole mass spectrometer (Finnigan SSQ 710, San Jose, CA, USA) with a 100°C drying gas Electrospray Ionization source (Analytica source of Brandford, CT, USA) under 2.5–3.5 kV. Each sample volume was diluted in 1 volume of methanol–acetic acid (100:1, v/v). The flow-rate was set at 1 μl min^{-1} by a syringe pump (Harvard Apparatus, South Natick, USA). Multiply charged ions from horse myoglobin were used to calibrate the m/z scale of the mass spectrometer.

2.8. Native and SDS–PAGEs

Electrophoreses were performed in a Miniprotean II system (Bio-Rad). Proteins were separated on polyacrylamide gels with anode at electrode end. Experiments were performed, respectively, under reducing conditions (β-mercaptoethanol) SDS–PAGE, 15% acrylamide, according to Laemmli [15] or under native conditions (5% acrylamide, without SDS and β-mercaptoethanol in buffers), with Tris–HCl 50 m*M*, pH 7.2, as migration buffer. Gels were Coomassie-blue stained.

3. Results and discussion

Previous work on histidyl-aminohexyl-Sepharose 4B (His-AH-Seph) showed its large capacity for proteins. This gel seems thus to be able to adsorb all the proteins from mouse ascetic fluids, including IgG and albumin [6]. Adsorbed proteins could then be separated thanks to different elution conditions. El kak et al. have previously studied human IgG adsorption on this support and have demonstrated the involvement of electrostatic forces and hydrogen bonding in the retention mechanism of IgG [3]. The aim of our present experiments was to investigate the adsorption characteristics of this adsorbent for the albumin, as another model of human serum protein.

3.1. Albumin adsorption on His-AH-Seph

Albumin adsorption was studied on three different aminohexyl or histidine grafted Sepharose 4B gels (proposed structures are shown in Fig. 1) with underivatized Sepharose 4B as control. Adsorptions were performed using 1 ml of gel, using 10 ml (tank) of a solution of 10 mg HSA ml^{-1}, with 25 m*M* MOPS, pH 7.0, as equilibrium buffer. Chromatographic procedures were as described in Section 2.

The quantification of albumin adsorbed reveals that histidine immobilisation on aminohexyl-Sepharose 4B significantly increases (Fig. 1) the capacity for HSA of the gel (90 versus 55 mg HSA per ml of adsorbent). At pH 7.0, adsorption seems to be due to a mechanism of ion-pairing between the cationic NH_3^+ free group presented on both the gels and the anionic net charge of albumin ($pI = 4.9$), as sug-

gested by Anspach et al. [9]. In contrast, histidyl-bisoxirane-Sepharose 4B shows a lower adsorption capacity, which might be due to the electrostatic repulsion of albumin by the negative charge of free remaining COO^- group of histidine, as previously described [3]. Nonetheless, the increased capacity on His-AH-Seph gel as compared with AH-Seph indicates that the unprotonated imidazole group (pK= 6.0) of histidine is also involved in the retention of albumin around neutral pH.

3.2. Influence of salt concentration, pH and buffer on albumin adsorption

In order to evaluate the nature of interactions involved in the albumin retention on His-AH-Seph and to determine the optimal conditions for maximal protein adsorption, the influence of chromatographic conditions was investigated.

The capacity of albumin on His-AH-Seph gel, determined as a function of different NaCl concentrations between pH 5.0 and 9.0, seems to be maximal at the lower salt concentration (low conductivity) and around neutral pH.

In fact, HSA capacity is significant under a wide range of pH (>50 mg albumin ml^{-1} of gel between pH 6.0 and 9.0) with a maximum at pH 7.5 and decreases at lower and higher pH (Fig. 2A). The effect of salt concentration on the adsorption of albumin on immobilised histidine was investigated using 0–5.0 *M* sodium chloride diluted in the starting buffer (25 m*M* MOPS buffer, pH 7.0). Fig. 2B shows that the capacity decreases with increasing salt concentration above 10 m*M* NaCl to reach a minimum at 1.0 *M* NaCl. However, a low adsorption (around 10 mg BSA ml^{-1} of adsorbent) is also observed at 3.0 and 5.0 *M* NaCl. Finally, adsorption experiment in presence of 1.0 *M* of Na_2SO_4 (anti-chaotropic salt that stimulates hydrophobic interactions) restored 60% of the initial albumin capacity (data not shown).

The influence of buffer conductivity was investigated in order to determine the effect of the nature of buffer (zwitterionic or non-zwitterionic), on the maximal capacity of albumin on His-AH-Seph support. Previous studies on histidine coupled onto hollow fiber membranes [4] have demonstrated that non-zwitterionic buffers, in spite of a weaker ionic strength compared to that of zwitterionic buffers, presented a lower protein adsorption capacity. This was explained by a competitive inhibition, probably by masking the binding site of adsorption by net charge of non-zwitterionic buffers. Here, we studied the adsorption capacity of HSA on His-AH-Seph in

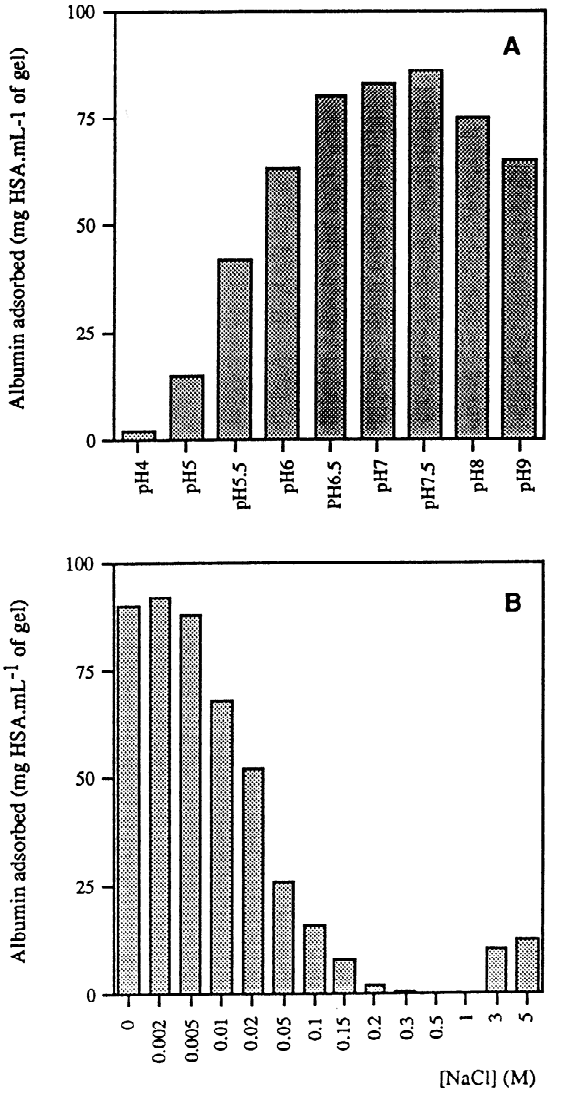

Fig. 2. HSA adsorption capacity of His-AH-Seph in function of pH or sodium chloride concentration. (A) Buffers used (25 m*M* each one) were, respectively, sodium acetate (pH 4.0 and 5.0), MES (pH 6.0 and 6.5) MOPS (pH 7.0, 7.5 and 8.0) and EPPS (pH 9.0), pH equilibrated with HCl or NaOH, without any salt adduct. (B) Sodium chloride dilutions were performed in 25 m*M* MOPS buffer, pH 7.0.

the presence of two representative zwitterionic and non-zwitterionic buffers: MOPS and Tris–HCl, respectively. At the same molarity, the conductivity of Tris–HCl buffer is higher than MOPS. Moreover, Tris–HCl conductivity is increased rapidly with molarity, while that of MOPS buffer did not (Fig. 3A). At the same molarity (25 m*M*) MOPS allowed a larger HSA adsorption capacity than Tris–HCl, while MOPS buffer adjusted to the same conductivity (using sodium chloride) as Tris–HCl buffer presented the same capacity (Fig. 3B). This indicates that the adsorption capacity of albumin on His-AH-Seph was correlated more with the buffer conductivity than to its nature. These data confirm the hypothesis of Haupt et al., assuming that free net charged entities could weaken the interactions between histidine ligand and proteins. Zwitterionic buffers are consequently more appropriate to ensure a maximum adsorption on histidine coupled matrices, because of the neutralisation between positive and negative charges of buffer molecule.

3.3. Mechanistic and kinetic aspects of Albumin adsorption

As previously described [3,4], retention on histidine ligand is mediated by ion-pairing mechanism (charge–charge or charge–dipole), with a predominance of electrostatic forces (elution by salts) in cooperation with other interactions, such as hydrophobic, van der Waals or hydrogen bonding of imidazole group. Furthermore, these relative forces can change with pH depending on the p*K* values of the different chemical groups involved. In addition, specific adsorption on histidine was described to be observed around the isoelectric point of the molecules. The residual charge of the ligand was supposed to form ion pairs with some localised charges of the protein, rather than its net charge [2,3].

In the case of albumin adsorption on His-AH-Seph, we cannot claim the strict specificity of albumin for the histidine ligand. In fact, electrostatic interactions on aminohexyl-Sepharose gel were predominant and this gel is able to adsorb albumin as an ion exchanger as previously reported [9]. The low conductivity and the adsorption pH values determined on His-AH-Seph, in accordance to the net

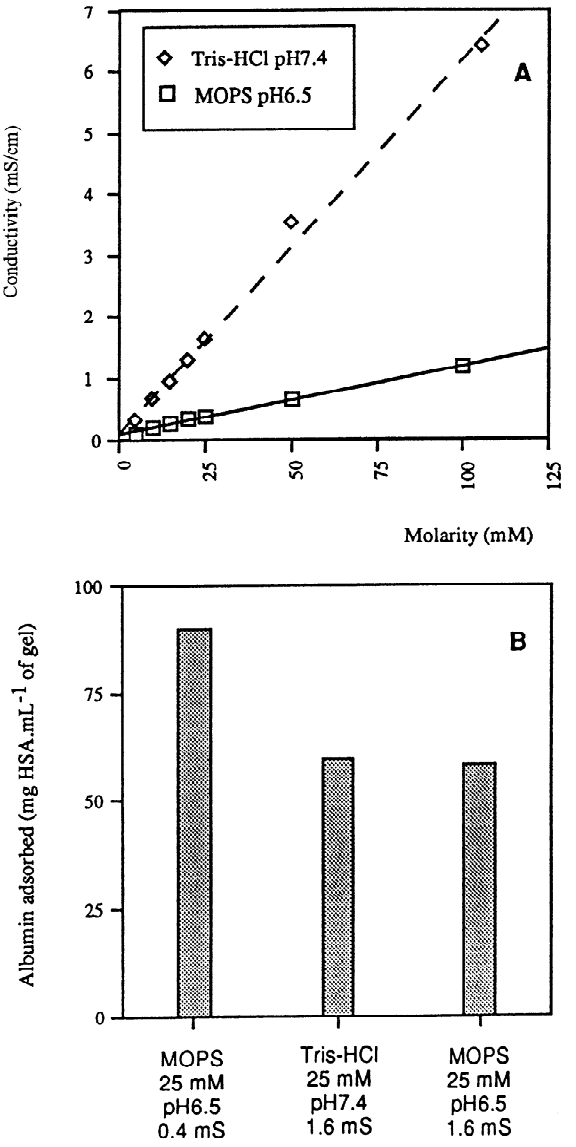

Fig. 3. Influence of conductivity of zwitterionic and non-zwitterionic buffers on HSA adsorption capacity. (A) Conductivity of MOPS (zwitterionic buffer) and Tris–HCl (non-zwitterionic buffer). (B) HSA adsorption capacity in function of conductivity of MOPS and Tris–HCl buffers.

cationic charge of albumin (Fig. 2) seem to show the same ionic involvement.

However, histidine coupled on AH-Seph seems to ensure a greater capacity for serum proteins adsorption, including albumin [7] (Fig. 1). Albumin

adsorption under high salt concentration conditions (3.0 and 5.0 *M* [NaCl], Fig. 2B or in presence of 1 *M* Na_2SO_4), where no electrostatic interactions are possible, indicates that other interactions (such as hydrophobic interactions) are involved in the retention of albumin on His-AH-Seph. Complementary experiments, using water structure-modifying additives (such as urea and ethylene glycol) in the adsorption buffer, according to the conditions described by El kak et al. [3], showed that chaotropic agents did not disturb the albumin adsorption (data not shown). This indicates the weak involvement of hydrogen bonding in the interaction between albumin and the support, as shown in IgGs adsorption study [3].

The temperature on the adsorption capacity of albumin on His-AH-Seph was maximal at room temperature and decreased at 4°C and 37°C. This reflects the well-balanced involvements between ionic and hydrophobic interactions as a function of temperature, with the predominance of electrostatic forces at low temperature and hydrophobic interactions at high temperature (hydrophobic interaction is the only one that increase with temperature [16]) and effects combines at room temperature. This was also described by Haupt and Vijayalakshmi [17] and Minobe et al. [18] for two hydrophobic molecules: catechol-2,3-dioxygenase and endotoxins on histidine/histamine coupled Sepharose. Moreover, structural analysis of albumin, well known to carry in serum hydrophobic ligates (such as steroid hormones or fatty acids), reveals hydrophobic cavities in subdomains IIA and IIIA [19]. Nevertheless, these hydrophobic contribution on His-AH-Seph seem to be relatively weak through secondarily interaction points, since low salt concentration ensures albumin elution.

Based on these above experiments, we determined the kinetic adsorption parameters: constant of dissociation (K_D) and maximal binding capacity (Q_x) in the low conductivity zwitterionic 25 m*M* MOPS buffer, at neutral pH 7.0. The values of $K_D = 4.5\ 10^{-5}$ *M* and of $Q_x = 93.3$ mg HSA ml^{-1} of adsorbent show His-AH-Seph as a low-affinity media presenting a high protein capacity equivalent to DEAE cellulose (89.5±2.3 mg BSA ml^{-1} of gel [9]).

3.4. Separation of HSA and its non-enzymatic glycated isoforms (AGEs product)

Elution of HSA from His-AH-Seph gel was investigated as a function of the initial salt concentration (monitored by conductivity) of the running buffer (Fig. 4). Five mg of HSA in 5 ml of 25 m*M* MOPS buffer, pH 7.0, with five different salt concentrations were respectively, injected in a 2-ml gel column. Elution was performed by increased linear sodium chloride concentration (Fig. 4A). Conductivity monitoring at the outlet of the column, reveals that HSA elution was always obtained at 13.1±1.0 mS/cm (Fig. 4B). Furthermore, starting buffer conductivity adjusted to 16.3 mS/cm (corresponding to 150 m*M* [NaCl]) did not allow adsorption, while 11.2 mS/cm in running buffer allowed partial HSA adsorption on His-AH-Seph.

Theoretically, glycated HSA should show stronger retention on His-AH-Seph than native species, since Maillard reaction results in the loss of positively charged amino acid. Nonetheless, the heterogenicity of HSA and of its AGE products could disturb their theoretical separations, as already observed with PBA (phenylboronic acid ligand) chromatography both in open column and in HPLC mode, which could appear as limited separation tools in this case [11,20,21].

Efficiency of HSA glycation (glycation details are given in Section 2) was checked by electrospray ionisation mass spectrometry (ESI-MS), by comparison with non-modified HSA analysis (Fig. 5). Mass determination of deconvoluted spectra revealed that glycated HSA presented up to 5 mol of glucose per mol of albumin, with a majority of 2 and 3 mol glucose populations, probably corresponding to Lys-525, Lys-199, Lys-281, Lys-439 and N-ter-Val AGEs product described in literature [22,23].

HSA and glycated HSA were applied on His-AH-Seph (2 ml of gel) under the same chromatographic conditions, using 5 ml of running buffer 25 m*M* MOPS, pH 7.0. The conductivity was adjusted to 1.33 mS/cm (corresponding to 10 m*M* [NaCl]), with the same linear elution procedure of Fig. 5. Fig. 6A shows that glycated HSA was more retained than HSA and eluted at 18.3±3.0 mS/cm.

Fig. 6B,C, where different concentrations of HSA and artificially glycated HSA mixture were applied,

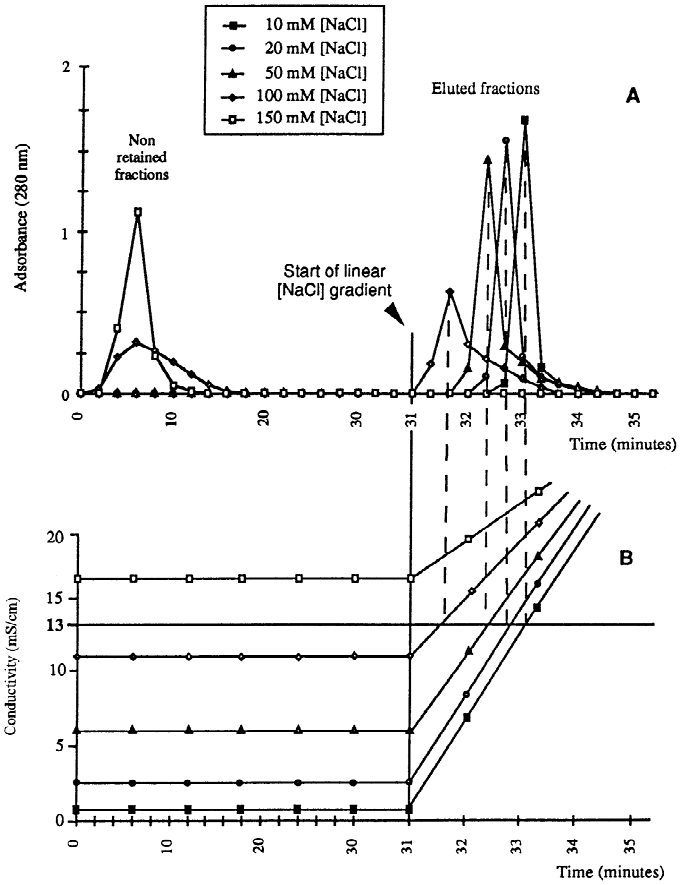

Fig. 4. Influence of the initial salt concentration (10–150 m*M*) in adsorption buffer on the elution of HSA. (A) Elutions, performed by a linear increasing sodium chloride gradient, were monitored at 280 nm. (B) Influence of salt concentration on HSA elution were shown by monitoring the conductivity of the solutions and at the exit of the column.

seemed to prove the efficiency of separation, which was confirmed by electrophoretic control of fractions eluted from chromatography represented in Fig. 6C (Fig. 7). Separation of HSA from its glycated isoforms (Fig. 7A) under native electrophoretic conditions (while SDS–PAGE failed, Fig. 7B), indicates the electrostatic mode of interaction involved in their adsorption on His-AH-Seph, as discussed above. The higher isoelectric points of the glycated protein might be responsible for this increase in affinity. Nonetheless, the same experiment on AH-Seph (without histidine ligand grafted) totally failed to separate the two molecules (data not shown). This indicated that the increase in the isoelectric points of heterogenic glycated HSA was not solely responsible for the efficient separation of glycated HSA from non-glycated HSA on the His-AH-Sepharose column. The addition of histidine ligand seemed to enhance the adsorption of glycated isoforms of HSA, probably through secondarily interactions such as van der Waals, hydrogen bonding or charge transfer interactions with glycated proteins. The glycation of HSA might change the ‘solvation’ of HSA, inducing localised variations in the ‘protonation’, which in turn may result in a stronger binding to histidine containing adsorbents.In fact, it has been previously reported that, despite the fact that the major interactions implied in proteins recognition in immobilized histidine affinity chromatography, it is rather the localised charge changes on specific loci on the

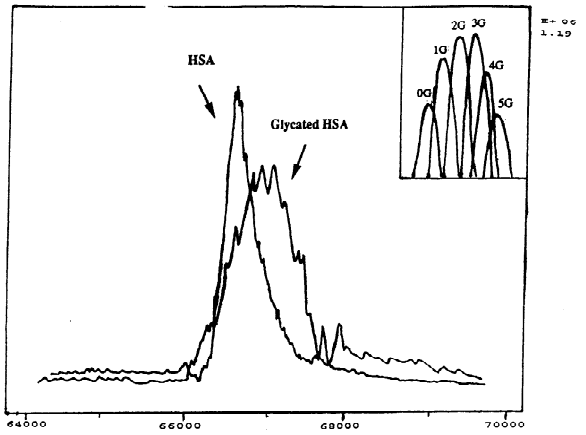

Fig. 5. Schematic deconvoluted spectra of glycated and non-glycated HSA; 0–5G, in the inserted panel, represent the relative populations subspecies of non-enzymatic glucose adducts on HSA.

protein surface, than the net charge of the protein, which is responsible for the protein- (immobilised) histidine interactions.

4. Conclusion

The study of HSA adsorption on histidyl-amino-hexyl-Sepharose 4B, presented in this paper, confirms the high protein adsorption capacity of this low affinity pseudo-biospecific media. Cooperative involvement of ionic, hydrogen bonding and hydrophobic interactions of the histidine coupled support, could explain the observed adsorption properties. The preliminary step of glycated proteins discrimination could thus be achieved by this functionalized support, employed as an alternative tool instead of the fragile and expensive immobilized anti-AGE affinity columns.

The present data are encouraging to pursue further this study for identifying the glycated HSA (advanced glycosylated endproducts) from diabetes patients' sera after a preliminary separation of the total albumins, followed by His-AH-Sepharose affinity chromatographic step.Thus, glycated HSA might be become a better alternative as an indicator of AGEs in patients' sera, compared to glycated haemoglobins, which are sometimes coeluted with the haemoglobin mutant forms.

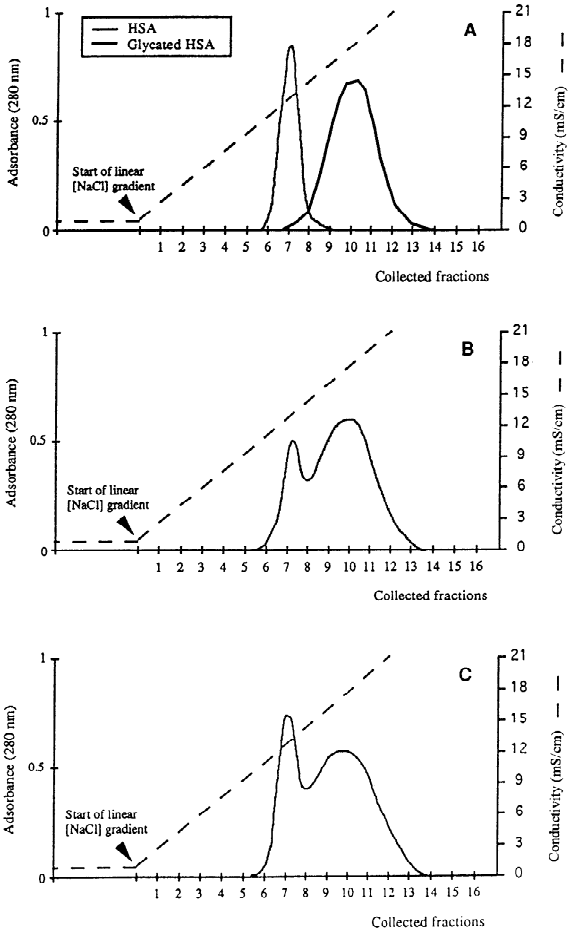

Fig. 6. Chromatography profiles of glycated and non-glycated HSA on His-AH-Seph. (A) Respective elutions of HSA and glycated HSA (2 mg ml^{-1} each one) during linear increasing [NaCl] concentration. (B) Elution of mixed HSA (1 mg ml^{-1}) and glycated HSA (2 mg ml^{-1}). (C) Elution of mixed HSA (2 mg ml^{-1}) and glycated HSA (2 mg ml^{-1}). Salt concentrations were determined by monitoring the conductivity at the exit of the column (dashed lines). Chromatographs were performed in 25 m*M* MOPS buffer, pH 7.0.

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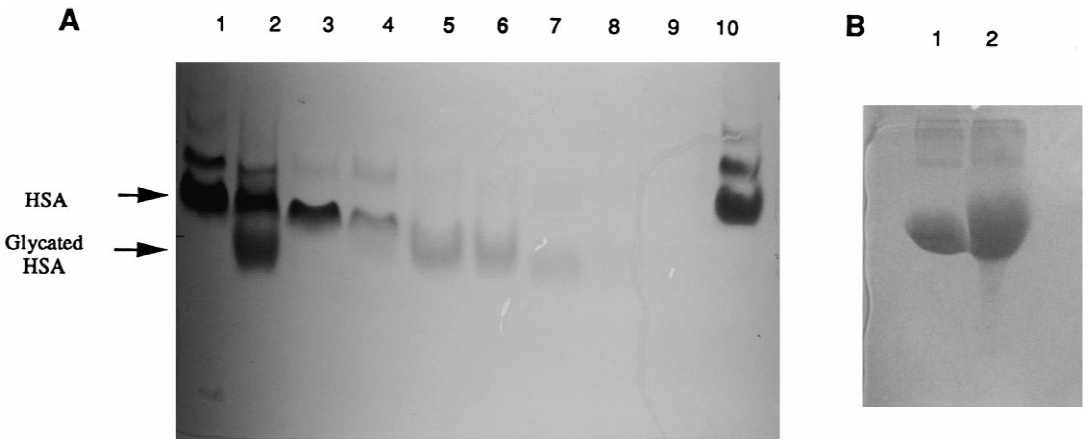

Fig. 7. Electrophoresis analysis of separated fractions on His-AH-Seph. (A) HSA and glycated HSA analysis on native-PAGE after chromatographic separation on His-AH-Seph. (1,10) HSA; (2) mixed HSA and glycated HSA (starting chromatography solution); (3–9) fractions 7–13. (B) Non-separation of HSA and glycated HSA by SDS–PAGE. (1) HSA; (2) mixed HSA and glycated HSA.

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References

[1] M.A. Vijayalakshmi, Trends Biotechnol. 7 (1989) 71.
[2] M.A. Vijayalakshmi, in: T.T. Ngo (Ed.), Molecular Interactions in Bioseparation, Plenum, New York, 1993, p. 257.
[3] A. El kak, S. Manjini, M.A. Vijayalakshmi, J. Chromatogr. 604 (1992) 29.
[4] K. Haupt, S.M.A. Bueno, M.A. Vijayalakshmi, J. Chromatogr. Biomed. Appl. 674 (1995) 13.
[5] M.A. Vijayalakshmi, Mol. Biotechnol. 6 (1996) 347.
[6] O. Pitiot, M.A. Vijayalakshmi, in: M.A. Vijayalakshmi (Ed.), Theory and Practice of Biochromatography, Harwood Academic Publishers, The Netherlands, 2001, Chapter 8 (in press).
[7] A. El kak, M.A. Vijayalakshmi, J. Chromatogr. Biomed. Appl. 570 (1991) 29.
[8] A. El kak, M.A. Vijayalakshmi, Bioseparation 3 (1992) 47.
[9] F.B. Anspach, D. Petsch, W.D. Deckwer, Bioseparation 6 (1996) 165.
[10] J.F. Forster, in: V.M. Rosenoer, M. Oratz, M.A. Rothshild (Eds.), The Albumin Structure, Function and Uses, Pergamon, Oxford, 1977, p. 53.
[11] P. Vidal, T. Deckert, B. Hansen, B.S. Welinder, J. Chromatogr. 476 (1989) 467.
[12] I. Miksik, Z. Deyl, J. Chromatogr. Biomed. Appl. 699 (1997) 311.
[13] S. Kanoun, L. Amourache, S. Krishnan, M.A. Vijayalakshmi, J. Chromatogr. 376 (1986) 259.
[14] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[15] U.K. Laemmli, Nature 227 (1970) 680.
[16] V.V. Mozhaev, K. Martinek, Enzyme Microbiol. Technol. 6 (1984) 50.
[17] K. Haupt, M.A. Vijayalakshmi, J. Chromatogr. 644 (1993) 289.
[18] S. Minobe, T. Sato, T. Tosa, I. Chibata, J. Chromatogr. 262 (1983) 193.
[19] X.M. He, D.C. Carter, Nature 358 (1992) 209.
[20] P. Vidal, E. Nielsen, B.S. Welinder, J. Chromatogr. 573 (1992) 201.
[21] L. Cruschelli, A. Clerico, G. Penno, R. Navalesi, O. Giampietro, Acta Diabetol. Lat. 27 (1990) 349.
[22] R.L. Garlick, J.S. Mazer, J. Biol. Chem. 258 (1983) 6142.
[23] N. Iberg, R. Fluckiger, J. Biol. Chem. 261 (1986) 13542.