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Conformational effects in reversed-phase high-performance liquid chromatography of polypeptides II. The role of insulin A and B chains in the chromatographic behaviour of insulin[☆]

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Abstract

The contribution of the insulin A- and B-chain to the retention and bandwidth behaviour of bovine insulin has been investigated. The influence of temperature and residence time on the logarithmic capacity factor ($\log \bar{k}$) versus the mole fraction of organic modifier ψ, i.e. the effect of temperature and ligand residency on the S and $\log k_0$ values of the individual peptide chains, were assessed at temperatures between 5 and 85°C and elution times between 30 to 90 min with an *n*-octadecyl (C_{18}) and an *n*-butyl (C_4) sorbent. Analysis of these $\log \bar{k}$ *versus* ψ dependencies revealed that the insulin A-chain exhibits retention behaviour significantly different to the intact insulin molecule whilst the B chain exhibits retention behaviour which is remarkably similar to the parent protein. However, in terms of kinetic processes, the A-chain exhibited a peak-splitting phenomenon at higher temperatures which was similar to the behaviour of the intact insulin molecule, whilst only bandbroadening with no peak splitting was apparent for the B-chain. Overall, the similarity of the retention behaviour of the insulin B-chain and the intact insulin molecule with regard to their temperature and residency dependencies suggests that the insulin B-chain makes a significant contribution to the chromatographic contact region of the insulin molecule when this polypeptide is exposed to hydrocarbonaceous ligands at low to intermediate temperatures due to the progressive unfolding of the molecule and greater accessibility of the previously buried B-chain residues. Under these same conditions, the A-chain contributes a relatively smaller proportion to the contact region but strongly influences the conformational stability of insulin at these lower temperatures. However, at higher temperatures the unfolding of the A-chain helices leads to significant bandspreading and eventually to the emergence of multiple peaks for the insulin molecule in the presence of both C_{18} and C_4 ligands.

1. Introduction

The interaction of insulin with reversed-phase chromatographic matrices has been shown to be dependent on temperature, sorbent and ligand composition as well as column residence time effects, consistent with conformational changes being induced in this polypeptide solute by

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alteration of these chromatographic variables. In a previous study [1], we demonstrated that closely related insulin variants could be resolved using either *n*-octadecyl (C_{18}) or *n*-butyl (C_4) sorbents in a condition-dependent manner which involved the interplay of these chromatographic variables. The separation of these molecules was attributed, in part, to the participation of amino acid residues A8–A10 of the insulin A-chain which contribute to the polypeptide contact region established between the surface of the insulin molecule and the *n*-alkyl ligands. These amino acids form a solvent-accessible loop structure between the two A-chain α-helices. In addition, it was proposed that sequence regions associated with the more flexible N- and C-termini of the B-chain of insulin are also involved in the interaction of insulin with the reversed-phase sorbents. It would appear from these chromatographic results as well as associated electrospray mass spectral data that insulin, when exposed to a hydrocarbonaceous ligand at high temperatures (>65°C) for short periods of time, undergoes bandbroadening and peak splitting behaviour consistent with irreversible conformational interconversions.

The composition and conformation of the chromatographic contact region, which is established between a polypeptide or protein and a chromatographic ligand in the interactive models of chromatography, determine in a global context the retention behaviour [2–8]. However, in order to develop a detailed understanding of the mechanism of polypeptide or protein surface interactions with chromatographic ligands, the extent to which surface and internal amino acid residues of a polypeptide or protein influence the chromatographic behaviour of these biosolutes in RP-HPLC needs to be more adequately characterised. The extent to which polypeptides or proteins can maintain their three-dimensional structure during the chromatographic migration will determine both the physicochemical characteristics of the interactive surface(s) presented by the solute and the adsorption kinetics. The specific aims of this present study were to investigate the contribution of the structural properties of the insulin A- and B-chains to the chromatographic behaviour of the fully folded insulin molecule.

The relative influence of these individual polypeptide chains on the retention and kinetic parameters associated with the reversed-phase migration of the parent molecule was assessed from their comparative behaviour over a wide range of experimental conditions. In particular, chromatographic conditions were adjusted to include variation in column temperatures over the range 5–85°C and elution times between 30 to 90 min with both the C_{18} and C_4 sorbents. The results provide further support for the hypothesis that small sequence segments of both the A-chain and the B-chain contribute to the contact region of insulin when this polypeptide interacts with an *n*-alkyl ligand.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KY, USA) and HPLC grade trifluoroacetic acid (TFA) acquired from Pierce Chemical Co. (Rockford, IL, USA). Water was quartz distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA). Bovine insulin was obtained from Sigma (St. Louis, MO, USA) and was of the highest purity available (>95%). Bovine insulin A- and B-chains were obtained from this insulin preparation by reductive alkylation of the inter-chain disulphide bonds of the intact molecule using standard methods. The method involved dissolving 1 mg of the polypeptide in 250 μl of guanidine–HCl buffer (i.e. 6 *M* guanidine–HCl, 200 m*M* Tris, 2 m*M* EDTA, pH 8.0). This mixture was incubated at 37°C for 1 h. After cooling, 1 mg of dithiothreitol (Boehringer Mannheim, Germany) in 30 μl of guanidine–HCl buffer was added. This solution was immediately flushed with nitrogen and incubated at 37°C for 3 h. The mixture was cooled and 1.9 mg of iodoacetic acid (Fluka, Germany) in 70 μl of 1 *M* Tris–HCl (BDH), pH 8.0 was added. This solution was incubated at room temperature in the absence of light for 15 min.

Finally, 10 μl of 2-mercaptoethanol (Sigma) was added to terminate the alkylation reaction. This reduced and alkylated crude sample was directly injected onto a Bakerbond (J.T. Baker Chemicals, Phillipsburg, NJ, USA) reversed-phase C_{18} Widepore column (250 × 4.6 mm I.D., 30 nm nominal pore size, 5 μm particle size) and chromatographed using a basic solvent system consisting of buffer A, 25 mM aqueous ammonium bicarbonate (NH_4HCO_3) (BDH), and buffer B, 35 mM NH_4HCO_3 containing 65% aqueous acetonitrile.

Linear gradient elution, with a flow-rate of 1 ml/min and a gradient time of 60 min for 0–100% buffer A to buffer B, yielded excellent separation of the parent bovine insulin molecule and the reduced and alkylated A- and B-chain polypeptides. After this RP-HPLC fractionation, Tricine-Polyacrylamide gel electrophoresis [9], amino acid composition analysis and N-terminal sequencing (data not shown) revealed that both the A- and B-chains were of >95% purity and had the anticipated amino acid sequences.

2.2. Chromatographic apparatus and procedures

All chromatographic measurements were performed with a Perkin Elmer (PE) Series 4 liquid chromatograph (Perkin Elmer, Norwalk, CT, USA) using experimental procedures as previously described [1,10]. The various chromatographic parameters log $\bar{k}$, $\bar{\psi}$, σ_v, S and log k_0 required for the characterisation of the structure–retention behaviour dependencies were calculated as reported in an earlier paper [11].

3. Results and discussion

3.1. The retention behaviour of the bovine insulin A chain and insulin B chains

In order to further investigate the chromatographic behaviour of bovine insulin, the retention behaviour of the bovine insulin A- and B-chains was individually characterised with a C_{18} and a C_4 sorbent of similar ligand density, particle size, porosity and silica type. Bovine insulin A- and bovine insulin B-chain polypeptides were prepared by reduction of the disulphide bridges which interconnect the two polypeptide chains followed by a subsequent alkylation of the cysteine residues to prevent re-oxidation.

The experimental plots of log $\bar{k}$ versus $\bar{\psi}$ for the monomeric insulin and the A- and B-chain derivatives when chromatographed on the same C_{18} and C_4 sorbents were linear (r^2 typically ranged between 0.96 and 1.00 for these polypeptides, data not shown). Least squares regression analysis was used to derive the slope and y-intercept values of these log $\bar{k}$ vs. $\bar{\psi}$ plots for the calculation of the chromatographic parameters S and log k_0 respectively. The S values are related to the hydrophobic contact area between the solute and the n-alkyl ligands, whereas log k_0 values are a measure of the affinity of the interaction under aqueous conditions [2–6,10–13]. In Fig. 1 the plot of the S and log k_0 values derived for bovine insulin (BI), bovine insulin A-chain (BIA) and bovine insulin B-chain (BIB) are shown as functions of column temperature. As previously observed [1], BI demonstrated complex changes in these parameters in response to increases in the column temperature from 5 to 85°C. The S values of insulin increased non-uniformly as the column temperature was elevated with accompanying increases observed in the log k_0 values over this temperature range. These data suggest that the changes in contact area were related to increases in the number and/or the accessibility of amino acid residues contributing to the contact region on the surface of the insulin molecule. Thus, a multi-step model [13] of unfolding of the insulin molecule with increasing temperature involving the progressive exposure of the core amino acid residues to the C_{18} ligands, would be consistent with the observed retention data. Moreover, the nonlinear variation in the S and log k_0 values observed for BI suggest that this process may involve a number of different structural intermediates.

From Fig. 1, it can also be seen that BIB demonstrated S and log k_0 values of similar magnitude to BI. However, the pattern of variation in the S and log k_0 values for BIB over the

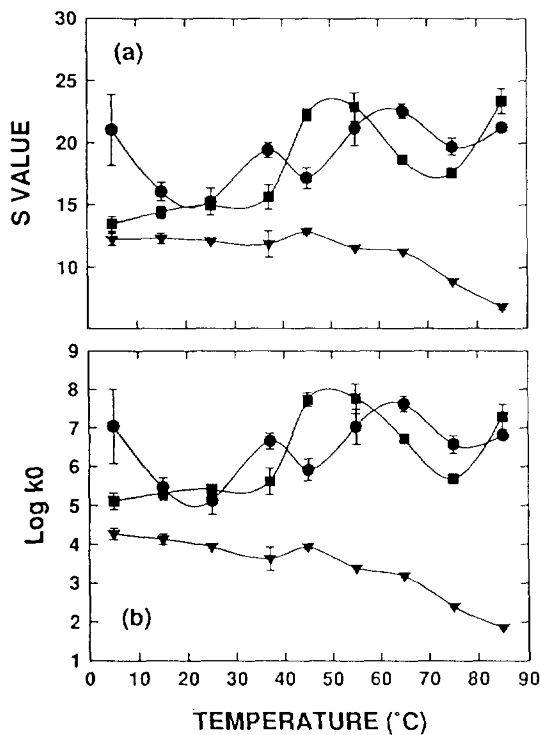

Fig. 1. The temperature dependence of (a) S and (b) log k_0 values derived for bovine insulin (●), bovine insulin A-chain (▼) and bovine insulin B-chain (■) chromatographed with the C_{18} sorbent. See Materials and Methods for other experimental details.

temperature range observed with the C_{18} sorbent appears displaced to lower temperatures compared to that observed for the intact molecule. The retention behaviour of BIB can be divided into two distinct phases. The first phase occurs between 5 and 37°C where the S and log k_0 values remained essentially constant as the temperature was progressively increased over this range. This behaviour is consistent with that of peptides or polypeptides which undergo very little conformational change in their chromatographic contact regions as the temperature is changed over a defined range [2,10,11,15]. The second phase occurs between 37 and 85°C where large changes in the S and log k_0 values were observed. These results suggest that BIB undergoes conformational transitions which involved changes in both the interactive contact area and the number of amino acid residues participating in the contact regions between 37°C and 65°C. The maxima and minima in the S and log k_0 values of BIB which occur between 37 and 85°C arise at lower temperatures than observed for the parent insulin molecule. These results suggest that both insulin and the B-chain undergo a similar type of interactive process with the C_{18} ligand, but that the structural (conformational) intermediates associated with the unfolding of insulin are more stable than the corresponding B-chain intermediates. In contrast, BIA demonstrated a small but discrete transition in the S and log k_0 values around 45°C, which involved a decrease in both the interactive contact area (i.e. to lower S values) and the affinity (i.e. to lower log k_0 values). The behaviour of the A-chain monomer was distinct from the parent molecule (or BIB) with the S and log k_0 values derived for BIA being much smaller than those observed for BI and BIB when chromatographed with the same C_{18} sorbent. These variations in the S and log k_0 values of BIA are reminescent of the unfolding of polypeptides with amphipathic helices where disruption of the secondary structure leads to a reduction in the size of the hydrophobic face of the polypeptide presented to the reversed-phase surface and thus a shorter retention time [11–17]. Moreover, disruption of the A-chain secondary structure would destabilise the entire insulin molecule.

Fig. 2 displays the temperature dependence of the retention parameters S and log k_0 for BI, BIA and BIB chromatographed with the C_4 sorbent. As evident from Fig. 2, bovine insulin demonstrated complex changes in both the S and log k_0 values with the C_4 sorbent as the column temperature was raised from 5°C to 85°C. As was observed for the C_{18} sorbent these results with the C_4 sorbent suggest that BI undergoes a series of conformational transitions which, collectively, represent the unfolding pathway of the molecule via intermediate structures involving the progressive exposure of the core amino acid residues to the probing n-butyl ligands. The bovine insulin B-chain demonstrated relatively constant S and log k_0 values at temperatures below 45°C, but exhibited more complex depen-

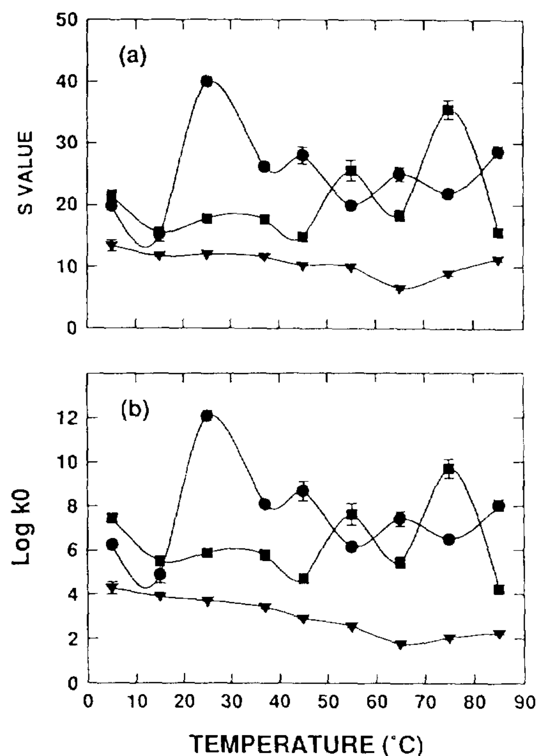

Fig. 2. The temperature dependence of (a) S and (b) log k_0 values derived for bovine insulin (●), bovine insulin A-chain (▼) and bovine insulin B-chain (■) chromatographed with the C_4 sorbent. See Materials and Methods for other experimental details.

dencies in these parameters at the higher column temperatures (i.e. 45–85°C). These results are consistent with the perturbation of a preferred interactive structure(s) of BIB around 45°C during the chromatographic process. In contrast, BIA exhibited more constant S and log k_0 values over the entire temperature range. This observation collectively suggests, as was the case with the C_{18} sorbent, that amino acid residues from both the A- and B-chains comprise the interactive surface of insulin when chromatographed with the C_4 sorbent but that the changes in the chromatographic parameters S and log k_0 values for the intact insulin were more similar to those exhibited by BIB than by BIA.

The experimental data for BIA and BIB with the C_{18} and C_4 sorbents demonstrate that under certain conditions both of these single-chain polypeptides adopt distinctive interactive structures during their adsorption to these hydrophobic sorbents. Since the retention behaviour of the individual B-chain has chromatographic features in common with those of the intact insulin molecule, it can be concluded that the interaction of insulin with the C_{18} or the C_4 ligands may involve a significant contribution from the B-chain amino acid residues whilst amino acid residues within the A-chain, particularly those within the sequence region A8–A10, make a further contribution to the interaction and conformational stabilisation of the parent molecule as it interacts with the C_{18} ligand. However, as the A-chain helices unfold the A-chain amino acid residues make a more significant contribution to the overall interaction which ultimately leads to multiple peak zones. These considerations invoke the possibility that both the isolated A- and B-chains exhibit relatively long-lived secondary structural features in both the aqueous mobile phase and reversed-phase sorbent environments analogous to those found with the intact polypeptide. Alternatively, the differences and similarities in the chromatographic behaviour observed for BI, BIA and BIB could be considered to be due to a serendipitous occurrence which arises because the reduced and alkylated A- and B-chain polypeptides can transiently mimic structural and conformational features of the insulin molecule at the nonpolar sorbent surface but generally assume nonidentical structures (i.e. extended conformations) to that exhibited by the intact insulin molecule in the aqueous mobile phase. As it is known [18–21] from circular dichroism and NMR measurements that these individual polypeptide chains lose their ordered structure in water but regain their helical conformation in nonpolar detergents such as sodium dodecylsulphate or dodecyltrimethylammonium chloride environments this second possibility must be considered at this stage on the basis of the available data to be more feasible. As a consequence, it is likely that in the intact molecule both polypeptide chains contribute to the overall chromatographic behaviour of insulin. In addition, the hydrophobic nature of the C_{18} and C_4

sorbents may confer similar stabilising influences on the single chain polypeptides as those experienced by the intact insulin molecule.

3.2. *The contribution of the insulin A and B chains to peak-splitting behaviour*

Solute bandwidth changes can be used to characterise conformational changes of polypeptides in reversed-phase chromatography. In particular, the influence of temperature and residence time on solute bandwidths provides information on the role of protein conformation in the dynamics of protein–surface interactions [11–15]. The chromatographic profiles for BI, BIA and BIB were determined for temperatures between 5 and 85°C and for column residence times corresponding to 30, 45, 60 and 90 min duration during reversed-phase chromatography with the C_{18} and C_4 *n*-alkylsilica sorbents. As reported earlier [1], the chromatographic profile of insulin at 85°C consisted of two peaks resulting from an irreversible conformational interconversion.

When chromatographed on the C_{18} sorbent, more complex elution profiles were observed for the insulin A- and B-chains compared to those observed for previously studied peptides such as bombesin, β-endorphin and glucagon [10,11]. Although smaller in molecular size than insulin, BIA displayed analogous peak-splitting behaviour (Fig. 3) to that previously observed for the intact bovine insulin [1]. Relatively sharp gaussian peaks were observed for BIA when chromatographed on the C_{18} sorbent at temperatures between 5 and 55°C. However, this peak shape subsequently became distorted and asymmetric between 65 and 75°C whilst at 85°C, two resolvable peaks were observed. Reinjection experiments revealed that this interconversion was irreversible. The bovine insulin B-chain in contrast did not exhibit this peak-splitting phenomenon and chromatographed with essentially constant peak shapes under these experimental conditions over the complete temperature range studied.

The multiple peak formation that was observed for BI and BIA with the C_{18} sorbent was also evident in analogous studies carried out with the C_4 sorbent. Again, the changes in elution

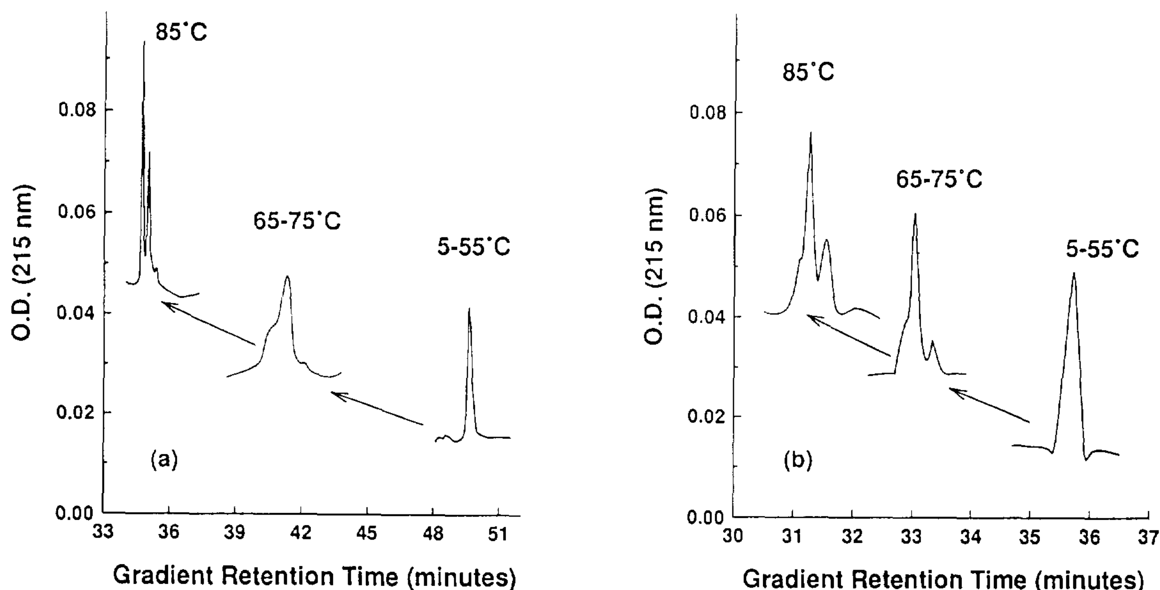

Fig. 3. The elution profiles for bovine insulin A-chain chromatographed with the (a) C_{18} and (b) C_4 sorbents. Representative chromatograms are displayed for 90-min gradient time for the temperature ranges shown.

profiles obtained for BIA were remarkably similar to the changes in the peak shapes of the parent molecule under the same conditions with the characteristic formation of a second peak at 85°C (Fig. 3). Subsequent reinjection experiments indicated that the interconversions responsible for the formation of these second peaks were irreversible. This common behaviour with the C_{18} and the C_4 sorbents associated with the irreversible interconversion to at least two conformational species of the A-chain moiety of insulin was also reflected as similar changes with the intact insulin molecule chromatographed under similar conditions. This observation is also consistent with the observed dependence of insulin retention on the participation of amino acid residues A8–A10 in the interactive contact site. The behaviour of BIB was characterised by the broadening of gaussian peaks but not the emergence of a second resolvable peak.

Fig. 4 displays the 3D mesh surfaces representing the experimental bandwidths determined for BI, BIA and BIB chromatographed with the C_{18} sorbent over the entire range of temperatures and residence times studied. BI exhibited a small rise in experimental bandwidths between 5 and 15°C followed by a decrease between 15 and 25°C. This trend became more pronounced as the column residence time was increased and corresponded to the broadening of relatively gaussian peaks. The near linear increase in bandwidths observed between 25 and 65°C corresponded to further broadening of the peaks and the eventual formation and resolution of a second peak. In contrast, the dependence of experimental bandwidth on column temperature and gradient time for BIA was relatively constant between 5 and 37°C. However, the bandwidths progressively increased between 37 and 75°C and were associated with the broadening of gaussian peaks. The subsequent decrease in experimental bandwidth observed between 75 and 85°C was associated with the resolution of a second peak which represented approximately 30% of the total peak area. The 3D mesh surface for BIB revealed an initial rise in bandwidth between 5 and 15°C followed by a subsequent decrease between 15 and 25°C. This behaviour became more evident as the column residence time increased and was associated with the broadening of gaussian peaks. The bandwidth increased in a near linear fashion between 25 and 75°C and then slightly decreased between 75 and 85°C, corresponding to an initial bandbroadening and then a peak sharpening, respectively, over these two temperature ranges. Overall, the similarity of the results of the bandwidth analysis for

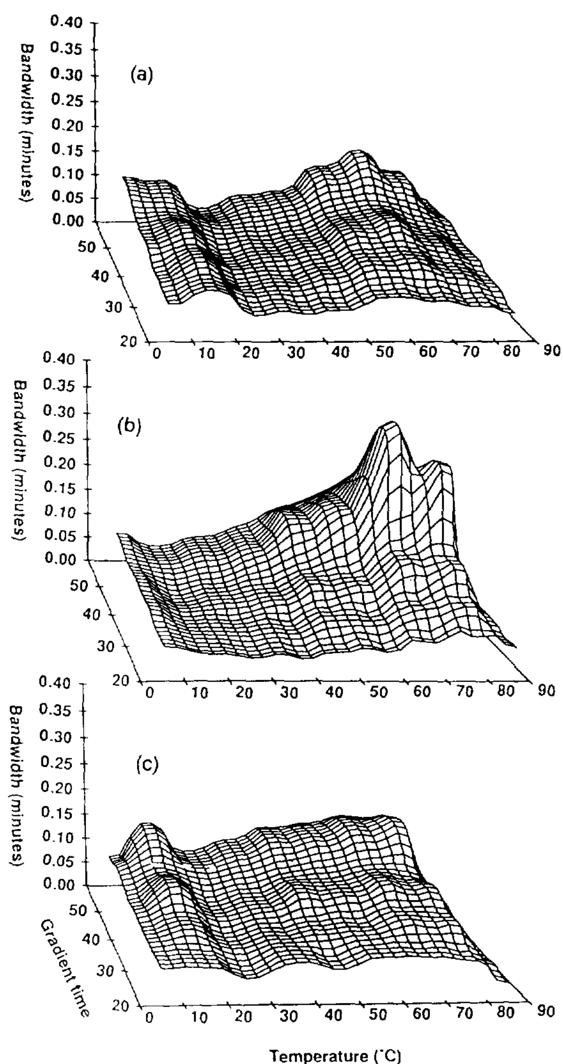

Fig. 4. The 3D mesh surface plot of the dependence of experimental bandwidth (min) on the gradient time (min) and column temperature (°C) for (a) bovine insulin, (b) bovine insulin A-chain and (c) bovine insulin B-chain chromatographed with the C_{18} sorbent.

BI and BIA as a function of temperature and residency time suggest that the peak-shape changes associated with the interaction of insulin with the C_{18} and C_4 sorbents may be predominantly controlled by kinetic processes involving the orientational docking and unfolding of the A-chain helices. It is interesting to note that these changes in bandbroadening behaviour observed with insulin also occurred over the same range of experimental conditions that changes in the retention parameters were evident. For BI, the changes in bandwidth values between 5 and 25°C corresponded to decreases in the S and log k_0 values, while the bandwidth changes at higher temperatures were associated with increases in S and log k_0 values. For BIA, the large changes in bandwidth at 65–75°C were consistent with a secondary structural change associated with relatively slow interconversion rates and the formation of multiple peaks which were however characterised by small differences in their S and log k_0 values. The changes in bandwidth for BIB between 5 and 25°C did not correspond to any major changes in the S and log k_0 values, whilst the more pronounced changes in S and log k_0 values evident between 45 and 65°C for BIB were not associated with any significant changes in bandwidth typical of peptide systems with fast interconversion rates between flexible conformational intermediates [11,13].

Fig. 5 displays the 3D mesh plot for BI, BIA and BIB chromatographed with the C_4 sorbent. BI displayed relatively constant bandwidths below 75°C, while the increases in bandbroadening at temperatures between 75 and 85°C were associated with the formation of a second peak. Bandbroadening changes for BIA were observed over discrete temperature and residency ranges, i.e. at 55°C for 60-min gradients, 65°C for 45-min gradients and 85°C for 30-min gradients. This behaviour may reflect the decreasing stability of the structural intermediates formed during the unfolding of BIA which ultimately become manifested as split peaks. The experimental conditions where significant changes in bandwidth behaviour were observed with the C_4 sorbent also correlated with changes in retention be-

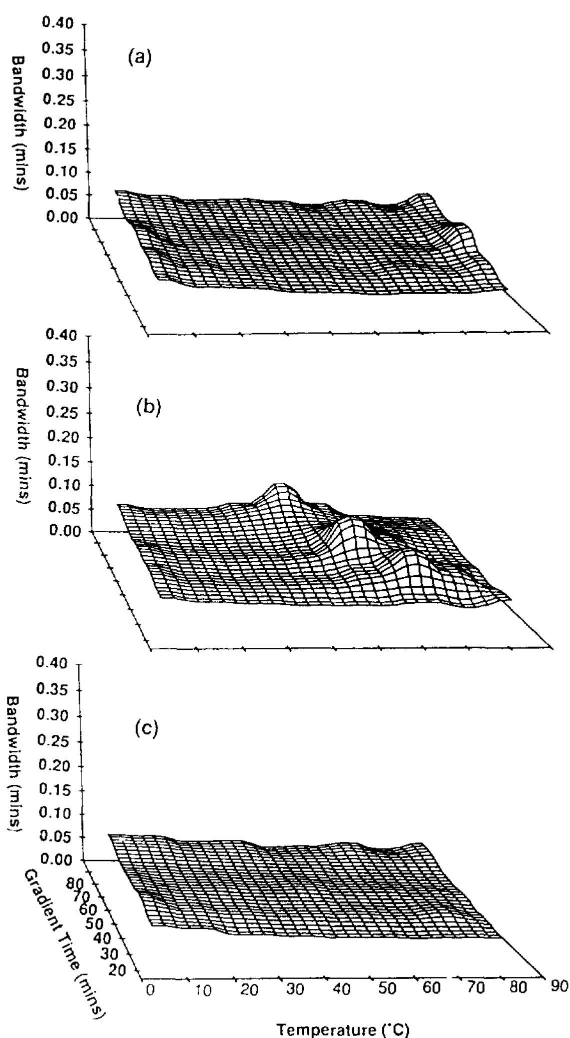

Fig. 5. The 3D mesh surface plot of the dependence of experimental bandwidth (min) on the gradient time (min) and column temperature (°C) for (a) bovine insulin, (b) bovine insulin A-chain and (c) bovine insulin B-chain chromatographed with the C_4 sorbent.

haviour of these polypeptides. For example, the bandbroadening changes observed between 75 and 85°C for BI which involved the formation of a second peak corresponded to a decrease in the S value. Similarly, the changes in bandbroadening evident for BIA between 45 and 85°C corresponded to a small transition in the S and log k_0 values near to 45°C. In contrast, only small changes in bandwidths were observed for BIB over the entire temperature and residency time

range, suggesting that the conformational changes which gave rise to the relatively large changes in S and log k_0 values between 5 and 85°C were associated with flexible intermediates where the rate of interconversion was faster than the chromatographic time scale [14,15].

4. Conclusions

Based on the results presented in this investigation and a previous study [1], the chromatographic behaviour of insulin can be rationalised in terms of the combined effects of the contributions of the A- and B-chains. The results indicate that the more extended B-chain dominates the chromatographic contact region at lower temperatures whilst the more highly folded A-chain with the helix-turn-helix motif partially contributes to the contact region but is also responsible for maintaining the structural stability of the insulin molecule as it interacts with hydrophobic surfaces. The chromatographic behaviour of both BI and BIB was significantly different to that of BIA with both the C_{18} and C_4 sorbents, reflecting the structural and conformational differences in the insulin A- and B-chains in terms of their hydrophobicity and flexibility. As the temperature reached a particular critical value for a fixed residence time, conformational transitions occur, possibly involving the unfolding of the A-chain helix-turn-helix motif near the A8–A10 residues, leading to complex peak shape and retention dependencies for these molecules. This study therefore illustrates the ability of RP-HPLC techniques to characterise the role of secondary structure in the interactive behaviour of polypeptides and proteins with hydrocarbonaceous ligands.

Acknowledgement

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