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Angiotensin I retention behavior on Butyl-Sepharose under linear loading chromatographic conditions

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ABSTRACT

Adsorption behavior of angiotensin I on a commercial Butyl-Sepharose support has been studied in function of temperature and ammonium sulphate concentration. Under isocratic elution conditions and at the higher salt concentrations, a characteristic of the chromatographic performance of angiotensin I was the broadness of the corresponding peak and in most of the cases the appearance of two peaks. These results have been interpreted in terms of on-column *cis–trans* isomerization of angiotensin I (a proline containing polypeptide) followed by its "re-conformation" after the interaction with the support. It has been proposed that the peak splitting phenomenon, a combined effect between temperature, salt concentration in the mobile phase and the ligand, is caused by slow kinetics of isomerization that is on the same time scale as the chromatographic separation. Salt concentration and temperature promote the conversion of the *trans* form of angiotensin I into its *cis* form, which has a bigger hydrophobic surface area, in the presence of Butyl-Sepharose. The retention of the *cis* form of angiotensin I increases with the increase in salt concentration in the mobile phase and seems to be little affected by temperature.

1. Introduction

Many of the recent advancements in biotechnology research were based on the development of techniques and methods for the separation and purification of biomolecules. Due to its high resolving capacity, chromatography has become a dominant biomolecule purification technique [1].

Over the last three decades, hydrophobic interaction chromatography (HIC) received much attention in the separation science literature [1–3]. The selectivity in this type of chromatography derives from the hydrophobic interactions between the hydrophobic ligand and non-polar hydrophobic patches on the solute surface. In HIC, the nature of biomolecule–adsorbent interaction is broadly understood to involve hydrophobic interactions [4]; however at a molecular level the adsorption process is complex and other processes may be present, influencing biomolecule retention. There is, therefore, considerable practical interest in developing a better understanding of the mechanisms underlying the adsorption of biomolecules in HIC. Many attempts have been made to study the parameters influencing biomolecule retention in HIC, the major part of them being focused on retention obtained under linear loading conditions [5]. Hydrophobic interactions are affected by different experimental variables, like salt type and concentration, biomolecule characteristics, temperature, ligand type and density, pH and additives [1]. The most widely used models typically account only for the salt type and concentration (solvophobic theory and preferential interaction theory) and not for the other factors [6]. Structural properties of the protein were considered by Lienqueo et al. [7] in a mathematical model developed to predict retention based on protein average hydrophobicity, this methodology was able to correlate adequately the retention data for monomeric proteins, however like the other methods outlined above it does not consider the other variables. Extensive experimental studies have been conducted to investigate the effects of ligand type [8–10] and density [4,11], pH [12] and temperature [12–14] on protein retention in HIC, suggesting that the performance of HIC can be optimized by using appropriate combination of these factors. However a prediction of optimal conditions is not yet possible [5]. An additional complicating factor that can be significant in HIC is that a biomolecule native conformation can change during or/and upon adsorption onto a hydrophobic surface [5]. Biomolecules are flexible polymers, and it is well known that conformational changes can occur whenever the environment of the molecules differs from that provided by physiological conditions. These variations can alter retention profiles and may affect production yield. In 1989, Karger and Blanco [15] provided strong evidence that the conformation of a protein may change in the adsorbed state or upon adsorption. Goheen and Gibbins [16] haves postulated that media surface act like catalysts for protein unfolding. Jones and Fernandez [17] and Wu et al. [18] showed that the adsorption of α-lactalbumin onto HIC surfaces could cause the protein to unfold.

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Jungbauer et al. [19] demonstrated that interaction of protein with ligands of HIC media might promote partial conformational changes of proteins upon adsorption and further also hypothesize that the HIC media surface acts as a catalyst for partial unfolding. Purcell et al. [20] used bandwidth measurements to study the effect of secondary structure of peptidic solutes on their reversed-phase high-performance liquid chromatography (HPLC) retention behavior. However, despite the large number of studies on the effect of biomolecule structure on their chromatographic retention behavior, only few studies have addressed biomolecules conformational dynamics in hydrophobic interaction chromatography.

In our research on the development of a better understanding of the mechanism underlying hydrophobic interaction chromatography of biomolecules we used, a biomolecule structurally simpler than a protein, the peptide human angiotensin I. This peptide is generated in the circulation by the action of rennin, secreted by the kidneys, on angiotensinogen produced by the liver [21]. Although angiotensin I does not exhibit strong biological effects, it is the precursor of angiotensin II known by its multiplicity of biological actions. Angiotensin II is a potent vasoactive agent having a vital role in the regulation of blood pressure, in the conservation of total blood volume and salt homeostasis [21]; it is also involved in the release of alcohol dehydrogenase (ADH), cell growth and the stimulation of the sympathetic system. In the hydrophobic interaction chromatography of pure human angiotensin I using a commercial Butyl-Sepharose column under linear loading conditions, splitted peaks were observed. In this paper, the causes of this behavior were analyzed by changing temperature, modulator concentration, flow rate and peptide concentration.

2. Experimental

2.1. Materials

Human angiotensin I, with purity ≥95% (HPLC), was chosen as the probe peptide. It was purchased from Sigma (St. Louis, MO, USA) and used without further purification. Human angiotensin I has a molecular weight of 1296 Da and an amino acid sequence (Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu).

The HIC support, Butyl-Sepharose High Performance was purchased from GE Healthcare (Uppsala, Sweden) and packed in the column (1.9 cm × 1.0 cm I.D.).

A 10 mM sodium phosphate buffer (pH 7), which consisted of a mixture of dibasic and monobasic sodium phosphate from Aldrich (Steinheim, Germany) was used for all the experiments. Ammonium sulphate from Aldrich was used as modulator.

2.2. Apparatus

The retention factor measurements were carried out in a biocompatible high-resolution liquid chromatographic system [HPLC/fast protein liquid chromatography (FPLC)] AKTA Purifier 10, from GE Healthcare consisting of a separation unit and a personal computer running the UNICORN 5.11 control system. The temperature was controlled by a bath from Amersham Bioscience (Uppsala, Sweden), the Multi-Temp III, which circulates constant temperature water into the thermostatic jacket with a precision of ±0.1 °C.

2.3. Isocratic elutions

The retention factor measurements were made in the temperature range of 288–308 K. The column was initially equilibrated with the desired mobile phase (with different concentrations of modulator) at a flow rate varying from 0.5 to 5.0 ml min^{-1}. Solute elution times (t_r) were obtained by injecting 100 μl of different concentrations of human angiotensin I (using as solvent the phosphate buffer, pH 7, with modulator). Elution time for the "inert" tracer (t_0) was determined by injecting at the same flow rate the same quantity and concentration of human angiotensin I prepared in phosphate buffer, pH 7, without modulator. The elution profile was obtained by continuous measurements of the absorbance with the UV detector fixed at 220 nm. Two or three replicated studies were done for each elution profile. Following the elution, the column was washed with 10 mM phosphate buffer (pH 7).

3. Results and discussion

The retention behavior of angiotensin I on the Butyl-Sepharose column was investigated as a function of salt concentration (Fig. 1) and temperature (Fig. 2). Isocratic elutions of angiotensin I on the Butyl-Sepharose support carried out with different ammonium sulphate concentrations at 298 K (Fig. 1) indicate that no significant retention is obtained at lower concentrations of this salt (below 0.9 M). Under isocratic elution conditions and at the higher salt concentrations, a characteristic of the chromatographic performance of angiotensin I is the broadness of the corresponding peak and in most of the cases the appearance of two peaks (Figs. 1 and 2). The appearance of more peaks in a chromatogram than expected from the number of sample components is usually attributed to impurities. To test this hypothesis, peaks I and II from the separation depicted in Fig. 3A were collected and re-chromatographed under the same conditions. Interestingly, both fractions, despite their initial homogeneity, elute also as two peaks as shown in Fig. 3B. From these data it appears that no impurities are present which is to be expected since the angiotensin I used was HPLC grade with a purity ≥95%.

It is known, however, that for a variety of physical reasons a given pure substance can yield multiple peaks. Poor sample introduction, heterogeneity of column packing [22], maldistribution of eluent flow [23] and non-uniform radial temperature profile in the column [24] are among these reasons. The sample introduction and the eluent flow control were done by the chromatographic system used, the AKTA Purifier 10. To check the packed bed, the asymmetry factor (A_f) was calculated to be 1.5, a reasonable value for a short HIC column [25]. Finally, the temperature profile in the column was ensured by a thermostatic jacket, with a precision of ±0.1 °C, provided by GE Healthcare. So, none of the physical reasons enumerated before seems to be in the origin of the chromatographic profiles observed.

Another interpretation of the data could be the presence of a non-homogenous surface [19]. There could be sites with high and low affinity for angiotensin I. In this case, the ratio of both peaks should be constant and should not depend on ammonium sulphate concentration in the mobile phase (Fig. 1) and on temperature (Fig. 2).

Mass transfer kinetics and column overloading could also be in the origin of the chromatographic profile observed. The kinetic processes that dictate the band-broadening behavior of low molecular weight and rigid organic molecules is influenced by axial dispersion in the bulk mobile phase, dispersion due to slow mass transfer in the intraparticulate spaces and dispersion due to resistance to mass transfer in the mobile phase and in the stationary phase [26]. For compounds where the molecular surface area and the respective diffusivity of an individual solute remain essentially constant throughout the chromatographic analysis, each of the band-broadening contributions enumerated above can be satisfactorily accounted for by current theoretical treatment [26], namely the Van Deemter equation. Two main points may distinguish the mass transfer kinetic origin of peak broadening from other effects, particularly nonlinear ones (resulting from column overloading): it is known that nonlinear broadening decreases as the sample con-