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Multiple peaks in high-performance liquid chromatography of proteins
β-Lactoglobulins eluted in a hydrophobic interaction chromatography system

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Abstract

The chromatographic behavior of β-lactoglobulins when eluted in hydrophobic interaction chromatography systems is studied. By modifying some factors, such as pH and temperature, the relationship between shape of the chromatographic peak and protein structure is shown. At pH 4.5 and low temperature multiple peaks for β-LG A and β-LG B are observed and assigned to aggregates. The effects of other parameters, besides pH and temperature, such as volume and concentration of injected sample, contact time between protein and stationary and/or mobile phases, and nature and concentration of mobile phase upon aggregation are studied. Comparison of the chromatographic behavior of both variants of β-lactoglobulin is made. © 1997 Elsevier Science B.V.

Keywords: Hydrophobic interaction chromatography; Mobile phase composition; Proteins; Lactoglobulins

1. Introduction

Hydrophobic interaction chromatography (HIC) and reversed-phase liquid chromatography (RPLC) are mainly based on separation of biopolymers according to their hydrophobicity. In HIC interactions between stationary phase and solute are weaker than in RPLC and thus elution of proteins can be achieved by employing a salt gradient of decreasing ionic strength, avoiding the use of organic reagents. In this way the probability of denaturation decreases. However, depending on working conditions, structural changes in a protein can occur even in these mild systems [1].

Some knowledge about the chromatographic behavior and the structural changes of proteins during elution in HIC is necessary in order to choose the best separation conditions and to correctly interpret the chromatograms obtained assigning the different peaks to either different proteins or to diverse states of the same protein. If multiple peaks result from chemical or structural alterations of a chemically pure protein, these changes can take place before its chromatographic analysis, as we have shown for bovine serum albumin aggregation [2], or can be caused by the chromatographic system itself. It is known that the chromatographic behavior of each protein depends on all the parameters which are able to modify the interactions between solute and stationary or mobile phases. The study of the dependence of peak shape on chromatographic conditions can provide valuable information about these mutual influences. A factor that has a major effect on these interactions is protein structure. The influence of

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different conditions (solvent, temperature, etc.) on the structure of β-lactoglobulins has been studied using diverse methods, such as scanning calorimetry, circular dichroism and nuclear magnetic resonance [3].

In this paper the behavior of bovine β-lactoglobulins A and B in hydrophobic interaction chromatography systems is studied. Taking into account the modifications in the structure of β-lactoglobulins which originate from the surrounding environment (e.g. its nature, pH, temperature, etc.) and by the concentration of proteins in the sample, we explore the effect of several chromatographic parameters on the peak shape of β-lactoglobulin A and β-lactoglobulin B. The chromatographic profiles obtained are correlated with the structural changes happening in the different experimental conditions. Comparison between both variants of β-lactoglobulin is made.

2. Experimental

2.1. Instrumentation

HIC separations were performed on a Gradient LKB system (LKB-Produkter, Bromma, Sweden) with two LKB 2150 pumps, an LKB 2152 controller and an LKB 2152-400 mixer chamber. Unless otherwise mentioned, sample was introduced using a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 20-μl loop. The signal was monitored using a Perkin–Elmer LC-95 UV–Vis detector (Perkin–Elmer, Norwalk, CT, USA) and registered by a Perkin–Elmer 56. Sometimes a home made acquisition data system, using a personal computer (Multitech 500, Taiwan) was employed. Column (5 cm×4.6 mm I.D.) was slurry packed using carbon tetrachloride–methanol (9:1, v/v) as slurry solvent and a non-ionic polyether phase bonded to a 5-μm, 300 Å pore spherical silica (generously donated by Beckman Instruments, San Ramon, CA, USA) as packing material. A precolumn (5 cm×4.6 mm) containing Merckosorb SI 60 (Merck, Darmstadt, Germany) as packing was placed before the injection valve to prevent the support of the separation column from dissolving. Temperature was controlled by a Haake F-35 bath (Haake Mess-Technik, Karlsruhe, Germany) in which the precolumn, the column, the injection system, the tubing connecting the injection system to the column and that connecting the column to the detector, and a mobile phase temperature equalizer capillary (2 m× 0.25 mm), were introduced. In those cases where column temperature was below 20°C a cryostat was used to help the water bath to reach the desired temperature.

2.2. Chemicals

Ammonium sulfate, ammonium acetate and acetic acid from Merck and HEPES [N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)] and MES [2-(N-morpholino)ethanesulfonic acid] both from Sigma (St. Louis, MO, USA) were employed. β-Lactoglobulin A (β-LG A) and β-lactoglobulin B (β-LG B) were purchased from Sigma and used without further purification as aqueous solutions unless another solvent is indicated.

2.3. Procedures

HIC separations were performed in a linear gradient 0% B to 100% B. Gradient time was 20 min and mobile phase flow-rate was 1 ml min^{-1} unless otherwise indicated.

Preparation of component B of the mobile phase was dependent on its nature (see Figure caption in each case). For acetic acid–acetate buffer, 0.5 *M* of ammonium acetate was prepared by dissolving in water the weighed necessary amount; acetic acid was added to obtain the desired pH, a small quantity of water was added in the volumetric flask to the mark and pH was verified. If buffer was composed of HEPES and MES (10 or 20 m*M*), the necessary quantities of each acid were weighed and dissolved in water; the pH was also adjusted with acetic acid and the same steps as those performed for the acetate buffer were carried out. Component A of mobile phases was prepared by dissolving in component B the mass of ammonium sulfate necessary to obtain the desired concentration. The pH was adjusted with acetic acid. Oxygen in the mobile phases was eliminated by continuously sparging with helium.

3. Results and discussion

3.1. Effect of pH, nature of mobile phase and temperature on chromatographic behavior of β-lactoglobulins

The shape of the chromatographic peaks of β-lactoglobulins (β-LAs) eluted in the HIC system changes with separation conditions.

Gradient elutions (as indicated at Procedures) of 7.5 μl (10 mg ml^{-1}) of β-LG A, β-LG B, and their mixture (β-LG A+B) in pH 6 acetate buffer using ammonium sulfate 3 *M* (phase B) at temperatures ranging from 4 to 40°C were carried out. It was observed that both individual proteins and their mixture are eluted as a single peak in the whole temperature range considered. It was seen that the higher the temperature, the longer the protein retention time as a consequence of the increase of hydrophobic interactions at higher temperatures [4]. The plot of retention time of β-lactoglobulins vs. temperature showed a change in the slope at 30°C. The peak width of these proteins decreases from 4 to 30°C and increases at higher temperatures.

These changes in the trend of the plots observed around 30°C suggest the possibility that modifications in the structure of these proteins at temperatures above 30°C could take place when they are eluted through the chromatographic system. Wu et al. [1] have related the change in the slope of the plot of retention time vs. temperature to a change in β-LG A structure. Ingraham et al. [5], studying the effect of temperature on some proteins eluted by HIC, have indicated that the broadening of chromatographic bands suggests a conformational change of proteins which enhances their hydrophobicity and increases their retention time. Heat denaturation of β-lactoglobulins has been studied in different conditions and has been shown by different methods, including a recent study by capillary electrophoresis [6]. Values proposed for transition temperature, i.e., the temperature at which concentrations of native and denatured forms are equal, are in the range 66–76°C for pH about 6 [7,8]. Therefore, transformation starts at lower temperatures. In our study, it seems that a structural change could take place at about 30°C. Probably this change could be related to the beginning of the dissociation of the dimer toward the monomer and to a reversible ionization-linked transition mentioned by some authors as taking place at this pH [9,10]. At those temperatures at which several conformations of the protein exist a broadened peak or more than one chromatographic peak could be seen [11].

The effect of the pH of the mobile phases, relative to the isoelectric point (p*I*) of β-lactoglobulins, on the chromatographic behavior was studied by working with mobile phases with the same components as the above mentioned, but with pH lower than the p*I* of β-lactoglobulins (β-LG A p*I*=5.09, β-LG B p*I*= 5.23) [12]. Mobile phases containing acetate buffer (pH 4.5) and 3 *M* ammonium sulfate as modifying salt were employed. At temperatures between 2°C and 40°C, β-LG B was eluted as a single peak, for which the width generally decreased when the temperature increased. The same behavior was observed for β-LG A when temperature was above 20°C, but at lower temperatures a second peak was observed. The proportion of this second peak increased as the temperature decreased. This peak could also be observed at low temperatures when the mixture of both β-lactoglobulins was eluted (Fig. 1). The electrophoretic pattern of β-lactoglobulins obtained by a modified [13] Hillier method [14] and detected by employing an improved silver staining method [15] showed purity higher than 98% (data not shown) and excluded the possibility of the second peak being due to contaminants present in the sample.

The influence of the nature of mobile phases was investigated by employing the same modifying salt and the same pH (4.5) as in the previous experiment, but changing the nature of the buffer to HEPES and MES (10 m*M* each). The chromatograms obtained at different temperatures are shown in Fig. 2. At temperatures above 20°C both genetic variants and their mixture are eluted as a single peak which becomes narrower as the temperature increases. At temperatures below 20°C, the chromatographic band of β-LG B shows a widening that at 2°C can be seen as a second peak smaller than the first one. At low temperature β-LG A elutes as a double peak, but in this case the peak eluting later is taller than the first one, which is opposite to the behavior observed with acetate buffer mobile phase (Fig. 1).

The second peak observed at pH 4.5 is thought to

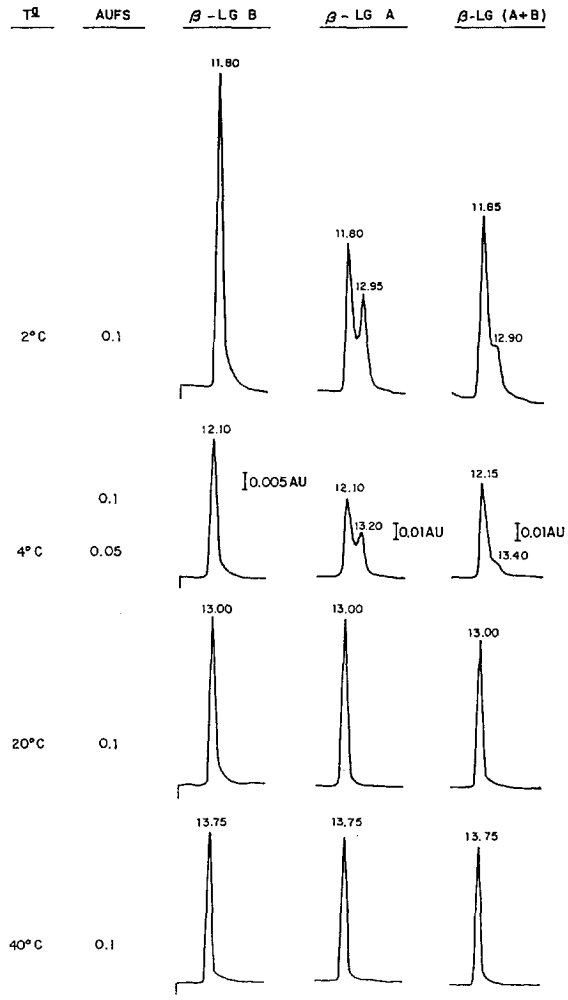

Fig. 1. HIC chromatograms of β-lactoglobulins in gradient 0% B to 100% B in 20 min (flow-rate=1 ml min^{-1}). Mobile phase, component B: 0.5 *M* buffer acetic acid–acetate (pH 4.5); component A: 3 *M* ammonium sulfate dissolved in component B. Injection volume 7.5 μl. Concentration of aqueous solution of β-lactoglobulins 10 mg ml^{-1}. UV detection 280 nm. Numbers on top of the peaks correspond to retention time in minutes.

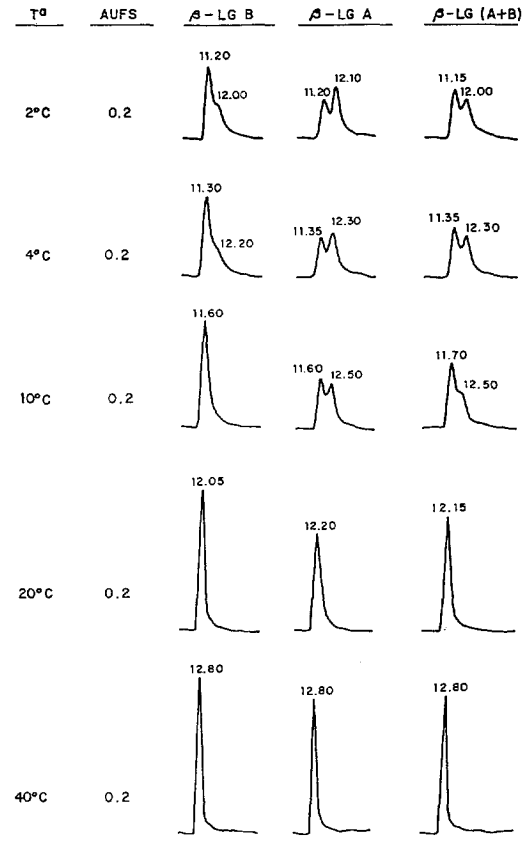

Fig. 2. HIC chromatograms of β-lactoglobulins. Mobile phase, component B: 10 m*M* HEPES, 10 m*M* MES, acetic acid (pH 4.5), component A: 3 *M* ammonium sulfate dissolved in component B. Rest of conditions as in Fig. 1.

be due to aggregation. It is known [16] that below its isoelectric point β-lactoglobulin undergoes aggregation which has a maximum at pH between 4.40 and 4.65 and that is strongly dependent on temperature, reaching the maximum near 0°C. The idea of the aggregation also agrees with the results shown by Grinberg et al. [17] for variant A of β-lactoglobulin. For simplicity, and similarly to these authors from now on we will call our last eluting peak the octamer and the previous one the tetramer of β-lactoglobulin.

At pH 4.5 the second peak observed for β-LG B is always smaller than that observed for β-LG A. These results agree with those from Kumosinski and Timasheff [18], who in the study of octamerization of β-lactoglobulin found that β-LG B aggregation increased when temperature decreased, but it was always less intense than that obtained for β-LG A. According to these authors, the different behavior of both β-lactoglobulins could be explained because one of the four carboxylic groups which are protonated during the octamerization of the β-LG A, could correspond to the aspartic acid (residue 64) which in β-LG B is substituted by a glycine residue.

As shown in Fig. 2 multiple peaks are only detected at low temperatures. This fact can be understood by taking into account that the octamerization reaction has a negative enthalpy and a negative entropy, and thus the polymerization is favored at low temperature [19]. This theory has been confirmed by the electrophoretical and ultracentrifugal studies performed by Townend et al. [16].

Another experiment was performed to study the influence of pH on the chromatographic behavior. It is known that the acidity of the surrounding medium affects the folding and aggregation of β-lactoglobulins. Dufour et al. [20] indicated that at pH 3.0, a destabilization of the close packing due to mutual repulsion of ionized side chains could take place because at this pH the net positive charge can be up to 15 per monomer. Swaisgood [21] and Townend et al. [22] showed that at pH lower than 3.5, the reversible dissociation of the dimer happens. In this way, if stationary phase had no influence, aggregates heavier than dimer should be absent in experiments performed using a mobile phase with acetate buffer (pH 3.5) and ammonium sulfate as modifying salt. The results obtained with this mobile phase at different temperatures showed that both β-lactoglobulins and their mixture were eluted as a single peak when working at low temperatures and that an increase in temperature caused a decrease in peak size, a decrease that is more significative for β-LG A (results not shown). This behavior can be explained through the Townend et al. [22] theory, which indicates that during the dissociation of β-lactoglobulin, hydrophobic surface becomes exposed to the surrounding medium. Moreover, Timasheff and Townend [23] demonstrated that the dissociation of both β-lactoglobulins at acidic pH increases with temperature and that at any temperature β-LG A is more dissociated than β-LG B. Thus, it can be thought that under our experimental conditions β-lactoglobulins suffer a dissociation process, exhibiting the hydrophobic surface to the exterior of the protein, which promotes their denaturation and probably their adsorption to the packing material.

The results shown up to here demonstrate that pH of the mobile phases has a major effect on the chromatographic behavior of β-lactoglobulins. This result is easy to understand taking into account the tendency of β-lactoglobulins to aggregate under the effect of the pH of the surrounding medium and considering that when the structure of a protein changes its interactions with the stationary and mobile phases, which control the chromatographic behavior, also change. Particularly, it seems according to our data that the maximum aggregation of β-LG A at pH 4.5 happens at low temperature and that aggregation is slightly more intense in the presence of HEPES 10 m*M* and MES 10 m*M* than when acetate buffer is used as mobile phase.

3.2. Influence of other factors over aggregates formation

To gain some knowledge about the chromatographic factors that could control β-lactoglobulin aggregation, the influence of other factors such as buffer concentration, solvent sample, amount of injected protein, and incubation in the chromatographic system on aggregation was studied. Since in our previous experiments better chromatographic resolution of aggregated species was obtained at low flow-rates (results not shown), the next study was performed at 0.5 ml min^{-1} instead at 1 ml min^{-1} flow-rate.

Fig. 3 shows under several conditions a new peak eluting before the tetramer, which had not been observed in the previous conditions. This peak was described by Grinberg et al. [17] as the dodecamer of β-LG A when working with mobile phases containing buffer 20 m*M* HEPES, 20 m*M* MES and acetic acid at pH 4.5.

By employing mobile phases containing HEPES and MES at concentration 10 m*M* or 20 m*M* and acetic acid, a dependence of aggregation on injected mass of protein can be observed. In this way, when an increase of mass is obtained by injecting constant volumes of increasing concentration samples, more aggregation corresponding to more concentrated samples is observed (as an example, Fig. 3b with g, Fig. 3c with h and Fig. 3d with i can be compared). However, at any given conditions, aggregation not only depends on β-LG A injected mass, but it is also a function of the concentration and volume of the sample. In this way, if Fig. 3c and d are compared with Fig. 3e and f respectively, higher aggregation is shown for high concentration and low volume for the

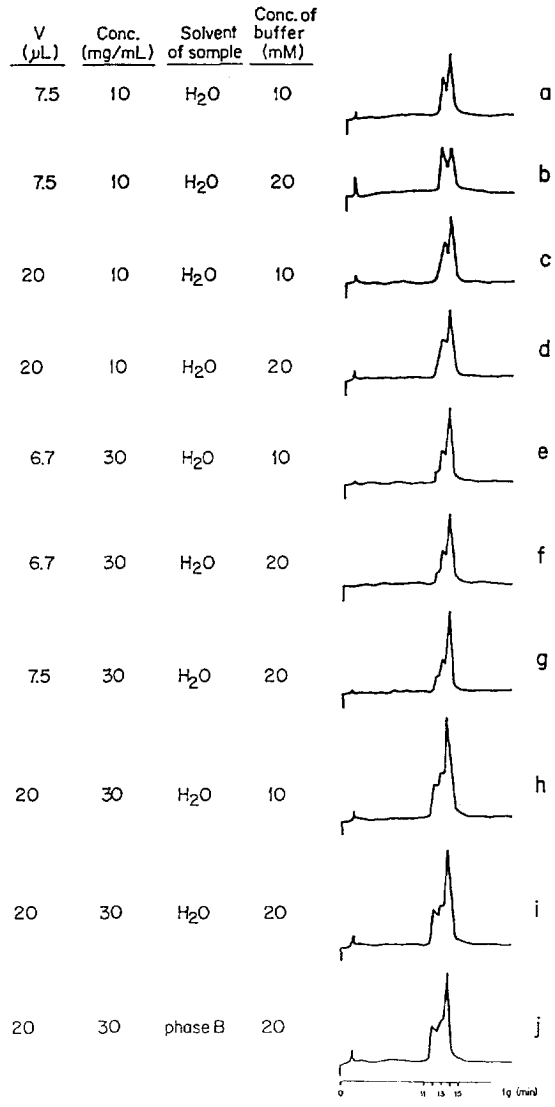

Fig. 3. Evolution of the HIC chromatographic profile of β-LG A eluted in gradient 0% B to 100% B in 20 min with mobile phases containing HEPES, MES and acetic acid when amount of injected protein, buffer concentration and solvent of sample are changed. Mobile phase flow-rate: 0.5 ml min^{-1}. UV detection at 280 nm. (a) and (b) 0.2 AUFS, (c to g) 0.5 AUFS, (h to j) 1 AUFS. Temperature 4°C.

same injected mass. Confirmation of these results and explanation for them will be presented below.

No significant differences in aggregation are found by increasing the concentration of HEPES and MES from 10 m*M* to 20 m*M*. A slightly larger proportion of the peak that, for the same reasons mentioned previously, we call the dodecamer is found in the most aggregating conditions (high amount of injected protein) when the buffer concentration is 20 m*M* (see Fig. 3h and i). By comparing Fig. 3i with j another conclusion can be drawn: no influence over aggregation is observed by employing component B of mobile phase instead of water as the sample solvent.

The results obtained (not shown) when mobile phases containing buffer with HEPES and MES at pH 4.5 and 4°C are employed to elute β-LG B indicate that tendency to aggregation of variant B depends on the same factors as β-LG A. Aggregation increases with the amount of injected sample, and for the same mass, aggregation is more extensive for higher concentrations. Species larger than octamer have not been found; a peak eluting at a retention time of about 11.8 min (the retention time of the peak called β-LG A dodecamer) can not be observed even when 20 μl of β-LG B at 30 mg ml^{-1} are injected. As expected, β-LG B aggregation happens to a lesser extent than does aggregation of β-LG A.

The study shown above and carried out using acetate buffer as mobile phase had not shown aggregation further than octamer for β-LG A. The possibility of observing more than two peaks with this mobile phase was then studied by modifying mobile phase flow-rate and amount of injected protein.

If elution is carried out at 1 ml min^{-1}, different aggregation can be observed as a function of protein mass (Fig. 4). The larger the injected mass, the larger the ratio of octamer/tetramer. At a lower flow-rate (0.5 ml min^{-1}) a qualitatively similar result is obtained (Fig. 4): larger mass implies more aggregation. When 20 μl of β-LG A at a concentration of 30 mg ml^{-1} is injected, the octamer proportion is so big that only one peak is observed (probably the chromatographic resolution is not enough to show the small proportion of tetramer if it exists). Fig. 4 also shows that slower flow-rates lead to more intense aggregation. Therefore, the contact of the protein with either the stationary phase or with the mobile phase are thought to be responsible for aggregation.

To investigate the influence that the time the protein is in contact with the stationary phase has over aggregates formation some experiments were

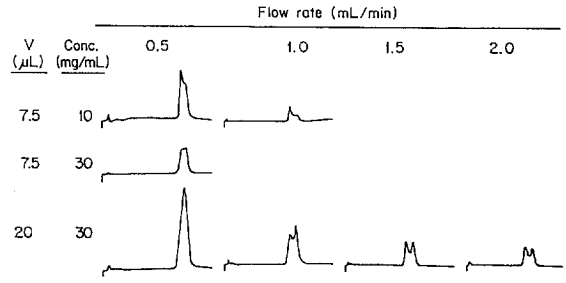

Fig. 4. Effect of the mobile phase flow-rate and the amount of β-LG A injected over aggregates formation when mobile phases containing acetate buffer (pH 4.5) are employed. Gradient 0% B to 100% B in 20 min. UV detection at 280 nm. Chromatograms of samples 7.5 μl×10 mg ml^{-1} acquired at 0.2 AUFS, for 7.5 μl×30 mg ml^{-1} acquisition at 0.5 AUFS and for 20 μl×30 mg ml^{-1} acquisition at 1 AUFS. Temperature 4°C.

conducted. Incubation of protein with stationary phase was performed by injecting the β-LG A solution onto the column when the gradient composition was 0% B and holding it at this composition for 20 minutes before initiating the gradient. Fig. 5 shows the chromatograms corresponding to the injection of 20 μl of a 30 mg ml^{-1} β-LG A solution

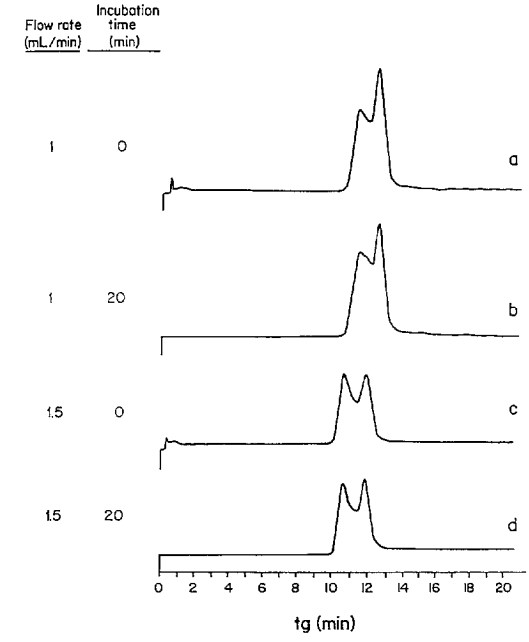

Fig. 5. Influence of contact time protein–stationary phase over aggregation. Injection of 20 μl of β-LG A 30 mg ml^{-1}. UV detection at 280 nm, 1 AUFS. Separation conditions as in Fig. 4.

with no incubation (Fig. 5a and c) and after 20 min of incubation (Fig. 5b and d). Elutions were carried out at 1 ml min^{-1} (Fig. 5a and b) and 1.5 ml min^{-1} (Fig. 5c and d) flow-rates, at which two peaks can be observed. No influence of contact time between protein and stationary phase over aggregation was observed.

Another group of experiments were performed at flow-rate 0.5 ml min^{-1} at which, as mentioned above, better resolution of peaks was expected. It is shown (Fig. 4) that at this flow-rate only one peak can be observed when injecting 20 μl of 30 mg ml^{-1} protein solution. Under the same conditions, the same amount of β-LG A was injected at different times after starting the gradient. It is observed (Fig. 6) that the later the sample is injected, the wider the peak becomes, increasing the proportion of the less retained species ($t_g \approx 15$ min). Since according to the

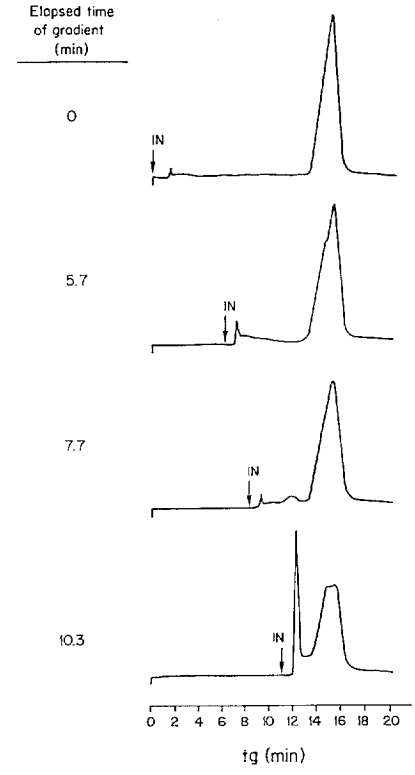

Fig. 6. Injection of 20 μl of β-LG A (30 mg ml^{-1}) at different times after starting the gradient 0% B to 100% B in 20 min. Mobile phase acetic–acetate buffer (pH 4.5). Mobile phase flow-rate 0.5 ml min^{-1}. UV detection at 280 nm, 1 AUFS. Temperature 4°C.

previous experiment the incubation time with the stationary phase do not seem to have a noticeable effect on β-lactoglobulin aggregation (Fig. 5), it is reasonable to suppose that another chromatographic parameter should be at the origin of the peak widening observed in this experiment carried out at flow 0.5 ml min^{-1}. This parameter could be the mobile phase as discussed next. When the protein is injected, it gets in contact with mobile phase which is circulating through the system until the protein is retained by the stationary phase. The higher is the flow-rate, the shorter is the contact time between mobile phase and protein before this one reaches the packing material in the column. Thus, if aggregation is promoted by mobile phase, lower flow-rates would be responsible for higher degree of aggregation. Incubation time inside the column would not lead to further aggregation because during incubation the protein is retained by the stationary phase. When protein is injected some time after starting the gradient, the mobile phase that it meets is not the initial composition of the gradient but that corresponding to the gradient time at which it is injected. In this way, the later it is injected, the lower is the concentration of ammonium sulfate that the protein meets with a consequent reduction in aggregation. It has been shown that addition of different salts can bring about different effects on unfolding or conformational distortion of β-lactoglobulin [24]. Thus it seems that the contact time between protein and mobile phase, and the composition of the mobile phase have a noticeable effect on β-lactoglobulin aggregation.

To confirm this hypothesis other experiments were performed by changing the injection procedure. The injection valve was substituted by a syringe injector. By doing that the protein can be injected straight onto the packing material of the column head, avoiding the contact between mobile phase and protein which happens in the loop and in the tube which connects column and injection valve. In this way, the contact between protein and mobile phase took place only for the short period of time since sample was injected until it was retained by the stationary phase. By using this method, β-LG A aliquots of different volume and concentration were injected. The study was carried out with acetate buffer mobile phases (flow-rates: 0.5 and 1.5 ml min^{-1}) and with mobile phases containing HEPES and MES (20 m*M* each) (flow-rate 0.5 ml min^{-1}). In Fig. 7, the results obtained when the injection was performed by syringe for acetate mobile phase flow-rates of 0.5 and 1.5 ml min^{-1} are compared. The results achieved with 20 m*M* HEPES and MES mobile phase at 0.5 ml min^{-1} for both injection methods (valve and syringe) are shown in Fig. 8. Comparison of the chromatograms obtained with both procedures shows that aggregation is more extensive when the injection valve is employed than when the syringe is used. For both methods, the slowest flow-rate leads to the greatest aggregation. These results show that the longer the contact time between protein and mobile phase, the higher the degree of aggregation. Experiments carried out using the syringe injector show the same result observed with the injection valve: for the same injected mass

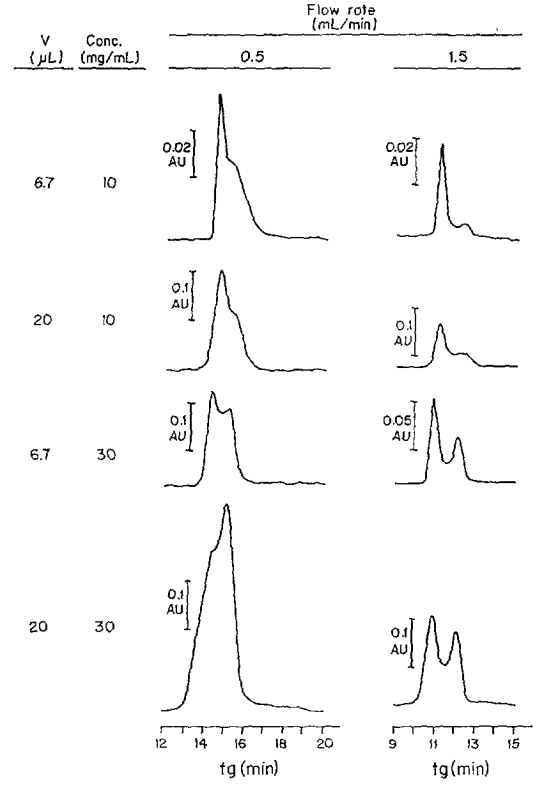

Fig. 7. Influence of mobile phase flow-rate, volume and concentration of injected β-LG A over aggregation when a syringe injector is employed. Mobile phase acetic acid–acetate buffer (pH 4.5). Temperature 4°C.

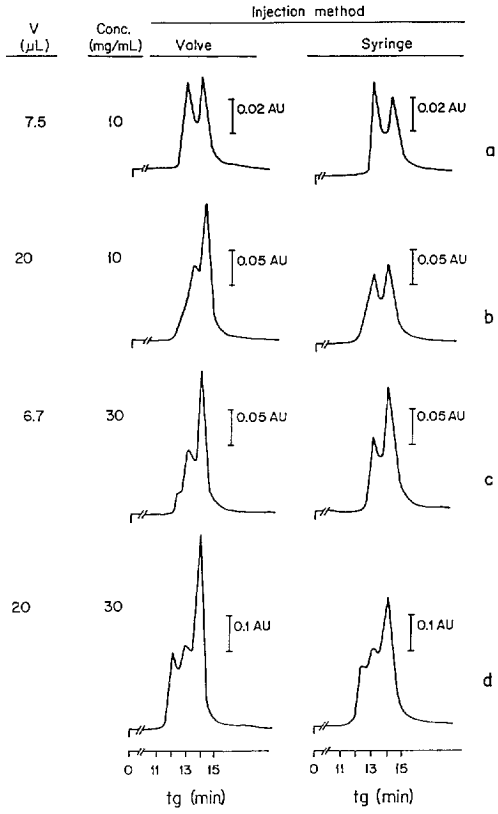

Fig. 8. Influence of the injection method over β-LG A aggregation when mobile phase 20 m*M* HEPES, 20 m*M* MES, and acetic acid (pH 4.5) is employed. Mobile phase flow-rate 0.5 ml min^{-1}. Temperature 4°C.

of protein, aggregation is greater when the volume is small and the concentration high (Fig. 8b and c). We have shown above that the higher the salt concentration in the mobile phase in contact with the sample during injection, the more aggregated the protein becomes. For this reason, for the same mass, the smaller the sample volume, the lower the dilution of the mobile phase and the more extensive is the aggregation.

4. Conclusions

The study of the chromatographic behavior of β-lactoglobulins in hydrophobic interaction chromatography systems as a function of pH shows broadening, disappearance or splitting of peaks. These phenomena are related to denaturation, dissociation or aggregation of β-lactoglobulins at different pH values and temperatures. Maximum aggregation has been observed at pH 4.5 and low temperature, and it has been found to increase with the amount of injected β-LG and with the contact time and salt concentration of mobile phase. More intense aggregation is always found for β-LG A than for the B variant.

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