Studies of the Binding of α-Lactalbumin to Immobilized Peptide Ligands

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The present work investigates the mechanism of binding of α-lactalbumin to the peptide ligand WHWRKR and its variants HWRKR and acetylated WHWRKR immobilized on a polymethacrylate chromatographic resin. The presence of two temperature-dependent binding mechanisms and one temperature-independent mechanism was demonstrated. Injections of different forms of α-lactalbumin (*apo*-α-lactalbumin, D87A mutant α-lactalbumin) displayed similar behaviors when compared to native α-lactalbumin, while lysozyme showed little or no binding to the WHWRKR and AcWHWRKR resins. An alternative process for isolation of α-lactalbumin from WPI was shown, using consecutive injections of WPI with limited elution.

INTRODUCTION

The major proteins present in whey are β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), lactoferrin, lactoperoxidase, and immunoglobulins. Horton (*1*) proposed that the use of individual proteins could be more valuable than their use as a mixture, allowing the production of better-controlled products and the use of proteins with unique or desirable characteristics in specialized products.

The globular protein α-lactalbumin is a mammary-specific modifier protein of the lactose synthase complex, comprised of galactosyltransferase and α-lactalbumin. Its high tryptophan content, good digestibility, and solubility combined with its low allergenicity (*2*) make this protein an excellent candidate for the main protein source in infant formulas, power drinks, and clear beverages. α-Lactalbumin also has potential value for the pharmaceutical industry because of the three bactericidal domains identified in its structure (*3*). Markus et al. (*4*) linked the consumption of dietary proteins enriched in tryptophan, using α-lactalbumin as a model, with the improvement of coping ability in stress-vulnerable patients, probably through modulations of brain serotonin.

The isolation of α-lactalbumin has been reported using several methods, such as centrifugation (*5*), ion-exchange chromatography (*6*), gel filtration chromatography (*7*), and immobilized metal ion affinity chromatography (*8*), among others. We have previously reported the identification of a hexapeptide ligand (WHWRKR) that displays affinity to α-lactalbumin (*9*) and developed purification strategies for production of α-lactalbumin using bioselective adsorption to the peptide ligand immobilized onto a stable matrix (*10*). The method produced a highly pure α-lactalbumin fraction, with good recovery yields, while avoiding common problems associated with bioselective adsorption such as leakage of ligand into the product stream, stability, and harsh elution conditions.

Although the method allows the production of pure α-lactalbumin, it was noticed that part of the target protein was eluted at different steps in the gradient used (*10*). Moreover, binding of all the major whey proteins to the matrix occurred, which could potentially compete for sites or sterically block specific sites for binding α-lactalbumin.

Permyakov et al. (*11*) reported the interaction of α-lactalbumin with the 26-residue peptide melittin, from bee venom. This peptide also binds to *apo*-α-lactalbumin and the acidic state of α-lactalbumin (also known as the molten globule state). This interaction decreased the α-lactalbumin binding affinity for calcium by 3 orders of magnitude and altered the conformation of the peptide. Calcium-binding proteins are known to interact with peptides containing clusters of basic amino acid residues in close proximity with hydrophobic sequences, such as melittin. Our peptide WHWRKR has two hydrophobic residues (tryptophan) and three basic residues (lysine, arginine), suggesting that one of the binding sites may be through the calcium-binding domain in α-lactalbumin. Other potential candidates for binding sites are hydrophobic patches on the surface of the protein and nonspecific interactions that may occur via dipole–dipole, van der Waals, and London forces.

The present work investigates the nature of the binding of α-lactalbumin to the peptide WHWRKR and its variants HWRKR and acetylated WHWRKR immobilized to a stable matrix. The effect of temperature on the interaction and the interaction with different forms of α-lactalbumin were examined.

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MATERIALS AND METHODS

All chemicals, including α-lactalbumin, were purchased from Sigma Chemical Co. (St. Louis, MO). Mutant α-lactalbumin D87A was kindly provided by Kenya Stokes (Department of

Animal Science, North Carolina State University) and Charles Brooks (Department of Veterinary Biosciences, Ohio State University). Whey protein isolate was obtained from Davisco Foods International (Minneapolis, MN).

apo-α-Lactalbumin was prepared according to the methodology described by Anderson et al. (*12*), using Microcon filtration cartridges (Millipore, Bedford, MA) with an exclusion limit of 3000 Da instead of gel filtration to remove EGTA.

The peptide resins were produced by synthesizing the peptides H₂N−WHWRKRA−COOH (WHWRKR resin), H₂N−HWRKRA−COOH (HWRKR resin), or the acetylated form of H₂N−WHWRKRA−COOH (AcWHWRKR resin) onto a poly-hydroxylated methacrylate polymer (TosoHaas AF Chelate 650, TosoHaas, Montgomeryville, AL) modified to generate free amino groups, following the procedure described by Buettner et al. (*13*).

Unless otherwise noted, all high-performance liquid chromatography runs were done using a step gradient composed of 20 min of 50 mM phosphate buffer, pH 7.0, followed by 20 min each of 0.1 M NaCl in phosphate buffer, 0.25 M NaCl in phosphate buffer, 0.5 M NaCl in phosphate buffer, 2% acetic acid in water, and a final rinse with phosphate buffer at 0.15 mL/min. The sample size was 0.5 mL, and the protein concentration was 1 mg/mL for α-lactalbumin and lysozyme and 5.55 mg/mL for WPI and soy protein isolate. The columns were packed using a slurry of resin in phosphate buffer, with the aid of a vibrator to ensure proper packing. The column and the solutions were kept in a water bath at constant temperature.

The elution was monitored by absorption at 280 nm, and the protein compositions were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) with a 4−12% acrylamide gradient gel, at 200V, 120 mA, and 25 W.

RESULTS AND DISCUSSION

Effect of Temperature on the Adsorption of α-Lactalbumin to the WHWRKR Resin. The chromatograms obtained for the WHWRKR resin at different temperatures are given in Figure 1. At low temperatures, a sharp peak eluting at 0.1 M NaCl can be observed, followed by a peak eluting at 2% acetic acid and another peak eluting during the regeneration of the column with 50 mM phosphate buffer. An initial plateau, due to unbound protein, was also observed at the beginning of the run. This general profile is the same for temperatures of 5 and 10 °C. Starting at 15 °C, small peaks eluting in 0.25 and 0.5 M NaCl start to appear. There is a dramatic increase in the height of the peak eluting in 0.25 M NaCl as the temperature is raised, while the peak eluting in 0.5 M NaCl increases in a much more modest way.

Between 25 and 30 °C, the largest changes in the profiles occur. The 0.1 M NaCl peak degenerates into a less sharp, plateaulike structure, while the 0.25 M NaCl peak increases. The 2% acetic acid peak becomes sharper, and the unbound fraction is reduced.

Because the injection of pure α-lactalbumin gives different peaks at any given temperature, it is reasonable to assume that more than one binding mechanism is involved in this interaction. At lower temperatures, electrostatic forces are dominant, indicating that this is the primary mechanism involved in binding of the fraction that elutes in 0.1 M NaCl. Because of the weak binding of α-lactalbumin to amino groups, the electrostatic forces must arise from dipole−dipole interactions and other less strong bonds that combined are enough to bind the protein to the chromatographic resin.

As temperature increases, there should be a decrease in the dominance of the electrostatic forces, and hydrophobic interactions may play a more important role in
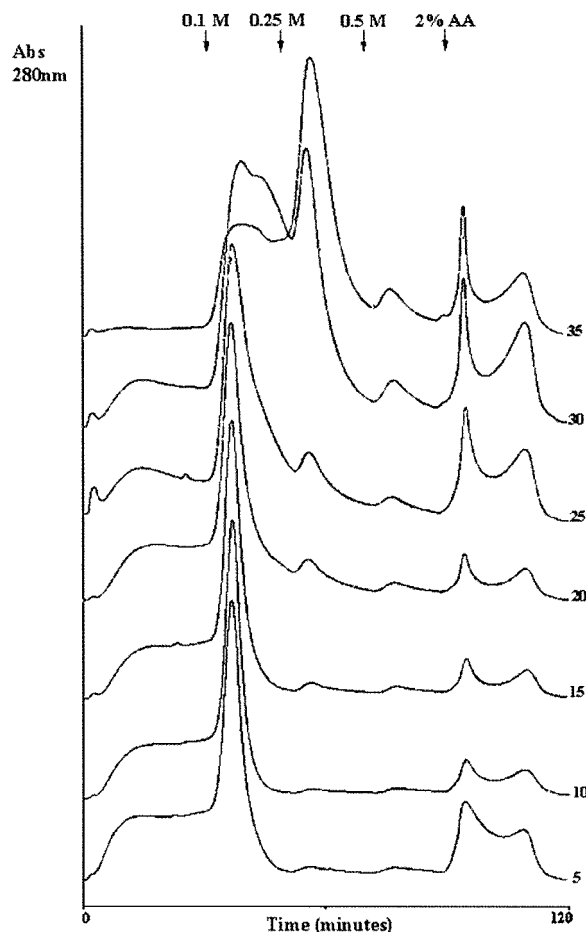

Figure 1. Effect of temperature on the binding of α-lactalbumin to the WHWRKR resin. Labels refer to the temperature of the run (in °C) and to elution steps.

binding. The introduction of 0.25 M NaCl in the system is enough to displace most of the bound protein. Judging from the increase in the area of the 0.25 M NaCl fraction as temperature increases, it is suggested that this fraction might have a predominance of hydrophobic interactions as the binding force. This fraction of α-lactalbumin may bind to the more hydrophobic amino acid residues present in the peptide, viz., the two tryptophan residues. α-Lactalbumin is a globular protein with several hydrophobic patches exposed on its surface, and any of those patches are candidates for binding sites. It is possible that a tryptophan residue in α-lactalbumin forms a tryptophan bridge with the tryptophan residues in the peptide ligand.

Effect of Temperature on the Adsorption of α-Lactalbumin to the AcWHWRKR Resin. Acetylation of the terminal amino group of the peptide ligand has a dramatic impact on the binding behavior of α-lactalbumin, as seen in the chromatograms in Figure 2. The first noticeable difference is the absence of an unbound fraction at any of the temperatures tested. The bound fraction that is eluted in 0.1 M NaCl is fairly large at low temperatures but decreases rapidly as the temperature increases. At 25 °C, this fraction is no longer detectable. The increase in temperature causes an increase in the peak eluted with 2% acetic acid. This fraction starts as a shoulder at 5 °C and grows into a sharp peak at 35 °C. Another remarkable feature of the profiles is the presence of a large, temperature-independent fraction that elutes after the acetic acid elution. This occurs when the mobile phase is changed back to
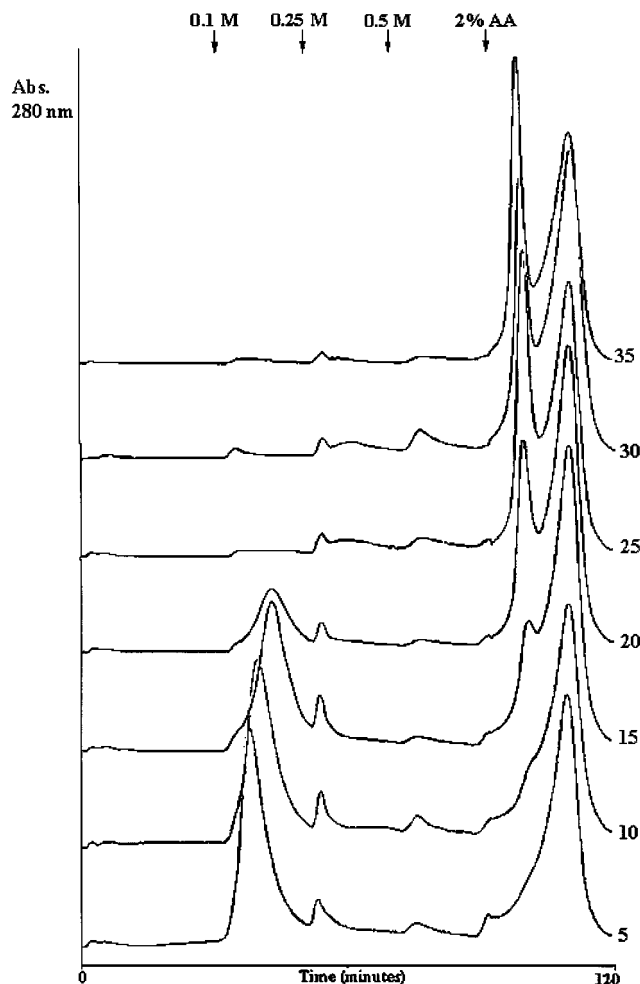
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Figure 2. Effect of temperature on the binding of α-lactalbumin to the AcWHWRKR resin. Labels refer to the temperature of the run (in °C) and to elution steps.

the initial condition using phosphate buffer, suggesting the presence of a nonspecific binding mechanism of the protein to the resin.

As expected, the increase in temperature shifts the dominant binding force from electrostatic to hydrophobic in the acetylated form of the peptide WHWRKR resin. As compared to WHWRKR resin, the acetylated form seems to bind α-lactalbumin more tightly at higher temperatures; the protein is eluted at a later step in the method.

Effect of Temperature on the Adsorption of α-Lactalbumin to the HWRKR Resin. The removal of a terminal tryptophan residue did not greatly change the profiles when compared to the original WHWRKR resin (data not shown). That is not surprising, because of the presence of another tryptophan residue in the peptide ligand, which should be enough to bind to hydrophobic patches in α-lactalbumin. A shift from electrostatic to hydrophobic interactions with an increase in temperature was also observed for this resin, yielding a pattern more similar to that obtained for the WHWRKR resin than for AcWHWRKR, with the shift occurring from 0.1 to 0.25 M NaCl. An unbound fraction was detected at low temperatures, similar to the observations with WHWRKR. The acetic acid peak was small and gained sharpness as the temperature was raised. Another similarity between WHWRKR and HWRKR was the presence of a small, broad peak at 0.5 M NaCl. Despite all of the similarities, use of the HWRKR resin
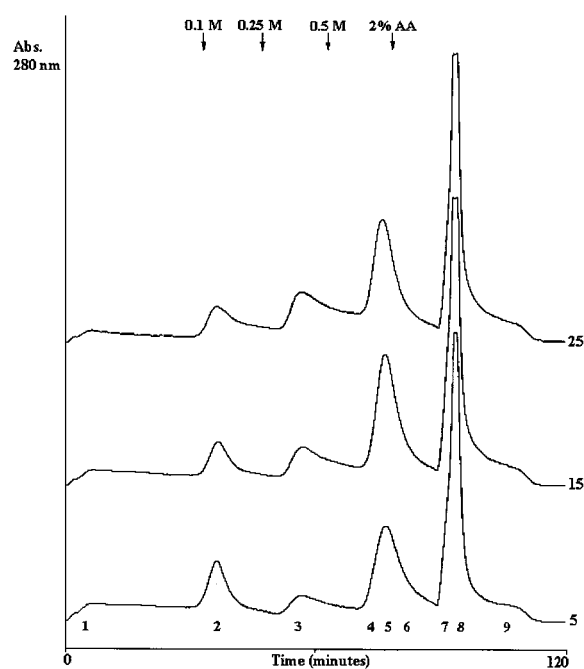

Figure 3. Effect of temperature on the binding of WPI to the WHWRKR resin. Labels on the right side refer to the temperature of the run (in °C), while labels on the bottom refer to the fractions analyzed by SDS−PAGE (Figure 4).

for protein isolation seems less promising than the WHWRKR resin due to the broadness of the peaks and the presence of several shoulders, especially at lower temperatures.

Effect of Temperature on the Adsorption of WPI to the WHWRKR Resin. Profiles of the chromatography of WPI in the WHWRKR resin are presented in Figure 3. The unbound fraction is small for all of the temperatures, and protein eluted during each gradient step, including a shoulder after the elution with 2% acetic acid. The most noticeable difference between the profiles for the three temperatures tested is the shape and size of the peak eluting with 0.1 M NaCl. As expected, the protein concentration eluting decreased as the temperature increased, in conformity with the observations with pure α-lactalbumin. In a previous publication (*10*), we reported a method of purification of α-lactalbumin using a combination of an aminated resin and the peptide WHWRKR resin at room temperature (22−25 °C). The results obtained in this study suggest that the yield for that isolation could be improved by performing the peptide resin step at 5 °C.

The composition of the peaks for the run at 5 °C can be observed in Figure 4. The first peak is composed of α-lactalbumin, with small amounts of lactoferrin and lactoperoxidase also detected. There is no detectable BSA or β-lactoglobulin in this fraction. Only at 15 °C was a β-lactoglobulin band detected.

For all temperatures, the peak eluting with 0.25 M NaCl appears asymmetrical. Its area increases with an increase of temperature. Its composition, however, a mixture of α-lactalbumin and β-lactoglobulin, was similar at all temperatures tested. The composition of the remaining peaks did not appear to change substantially when the temperature was changed (data not shown).

Effect of Temperature on the Adsorption of WPI to the AcWHWRKR Resin. The chromatograms of WPI obtained with the AcWHWRKR resin at 5 and 25 °C are presented in Figure 5. Consistent with the

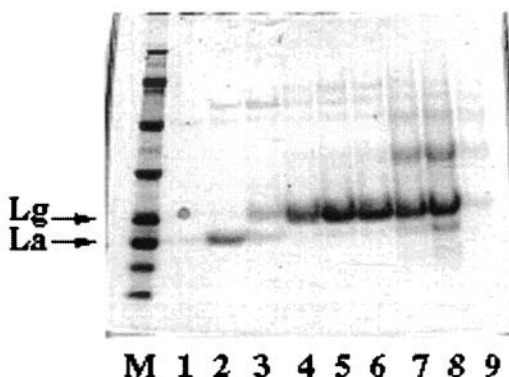**5768** *J. Agric. Food Chem.,* Vol. 49, No. 12, 2001

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Figure 4. SDS−PAGE profile of the fractions obtained at different run temperatures with WPI in WHWRKR resin at 5 °C. M denotes the molecular weight markers (from top to bottom: 188, 97, 52, 33, 19, 12, 6, and 3 KDa), and the numerals refer to the corresponding fraction in Figure 3. La is α-lactalbumin, and Lg is β-lactoglobulin.
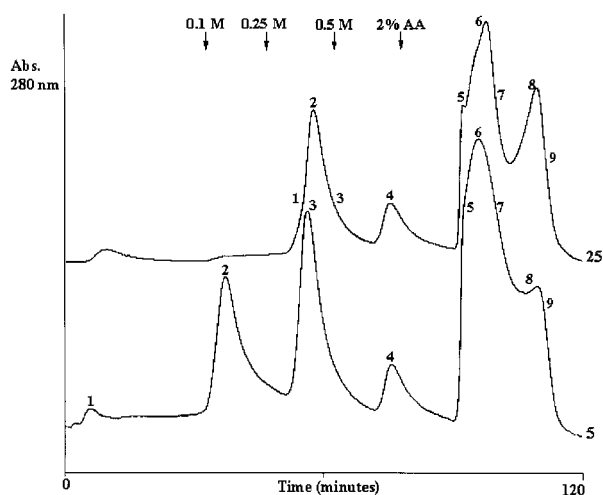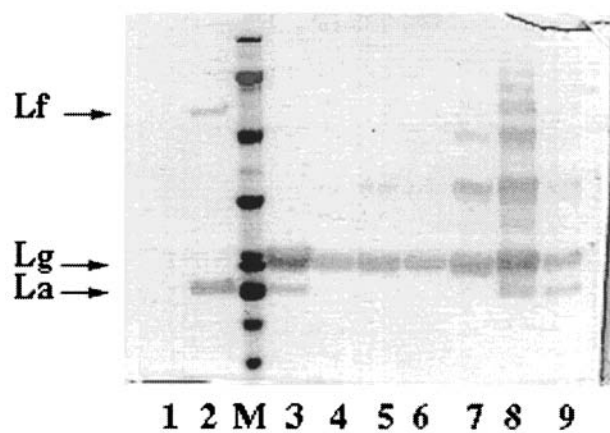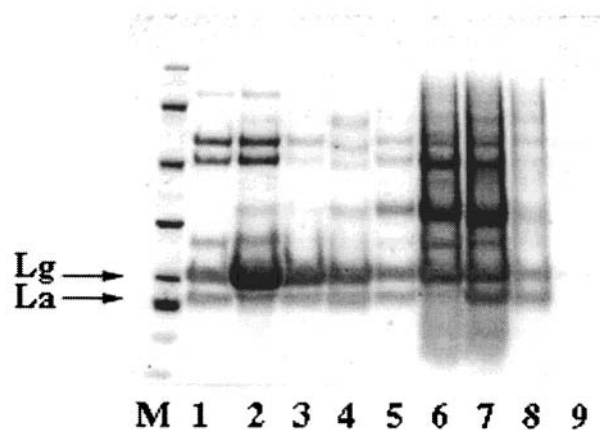

Figure 5. Effect of temperature in the binding of WPI to the AcWHWRKR resin. Labels on the right side refer to the temperature of the run (in °C), while labels along the chromatograms refer to the fractions analyzed by SDS−PAGE (Figure 6).

Figure 6. SDS−PAGE profile of the fractions obtained at different run temperatures with WPI in AcWHWRKR resin. (A) 5 and (B) 25 °C. M denotes the molecular weight markers (from top to bottom: 188, 97, 52, 33, 19, 12, 6, and 3 KDa), and the numerals refer to the corresponding fraction in Figure 5. Lf is lactoferrin, La is α-lactalbumin, and Lg is β-lactoglobulin.

observations for pure α-lactalbumin, at the higher temperature, there was no elution peak during the first step of the elution gradient (0.1 M NaCl), but the proteins eluting in each subsequent step represent virtually every major protein present in WPI (Figure 6B). A decrease of the temperature caused not only the appearance of a fraction eluting at 0.1 M NaCl, as with pure α-lactalbumin, but also a change in the composition of the fractions that allowed the isolation of a fraction containing only α-lactalbumin and lactoferrin (Figure 6A, lane 2). The fraction eluted in 0.25 M NaCl was composed of β-lactoglobulin and α-lactalbumin, while the fraction eluting with 0.5 M NaCl contained predominantly β-lactoglobulin and BSA. The 2% acetic acid fraction and the following peak were composed of a mixture of all proteins present in WPI arguing for a nonspecific binding mechanism.

Densitometric measurements of the electrophoretic bands of the fractions obtained indicated that the α-lactalbumin-rich fraction is composed of 87% α-lactalbumin and 13% lactoferrin. Considering the difference in size between these two proteins, it should not be difficult to produce homogeneous fractions from this sample using a method based on size exclusion, such as membrane fractionation or gel filtration. The pres-

ence of lactoferrin may be considered an advantage if this fraction is to be used as the protein source for infant formulas, because of its potential as an immunological system booster for infants.

Binding of Lysozyme. Lysozyme did not bind to the acetylated resin, while a small fraction bound to the WHWRKR resin, eluting at 0.1 M NaCl. Considering that the net charge of lysozyme at pH 7.0 is positive, because of its higher content of lysine and arginine residues as compared to the number of aspartic acid and glutamic acid residues (*14*), it is reasonable to suggest that this fraction is bound by a negatively charged patch on the surface of the protein. This suggestion is reinforced by the fact that blocking of the amino terminal of the peptide ligand prevents binding, as observed by comparing the binding patterns observed with WH-WRKR and AcWHWRKR. The overall positive charge of lysozyme most likely inhibits binding to the peptide resin. It is also important to notice that the hydrophobic interactions observed when both resins were used with α-lactalbumin were not observed with lysozyme. This may occur because of the higher repulsive forces caused by the presence of positive charges on the surface of the protein, or it may be due to an absence of the hydro-
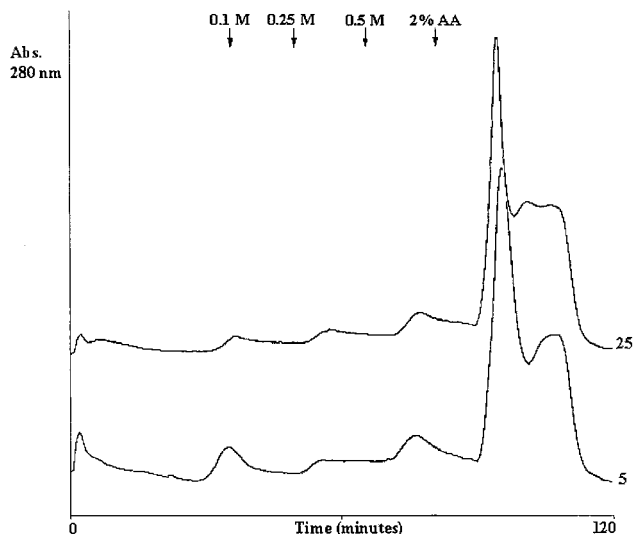
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Figure 7. Chromatograms of 0.5 mL of a 1 mg/mL mutant α-lactalbumin (D87A) solution using the AcWHWRKR resin, at 5 and 25 °C.

phobic region that is the target for binding of α-lactalbumin to the peptide ligands. As reported by McKenzie and White (*14*), there are 26 hydrophobic residues in α-lactalbumin and 24 in lysozyme, with 10 residues not showing direct correspondence or chemical similarity. Another possible explanation for the absence of the hydrophobic interactions is that binding occurs at the calcium-binding site, not present in lysozyme.

Binding of *apo*-α-Lactalbumin. The binding of *apo*-α-lactalbumin has a general profile similar to that for native α-lactalbumin at the same temperatures. A large fraction of the protein was eluted in 0.1 M NaCl at low temperature, with a shift to a later step in the gradient upon an increase of the temperature. There was, however, a considerable amount of protein eluting at each step of the gradient at 25 °C.

Native α-lactalbumin contains two domains: a large α-helical domain and a small β-sheet domain that are connected by a calcium-binding loop (*15*). The two domains are separated by a cleft and are held together by two cysteine bridges (73–91 and 61–77) with the bridge between residue 73 and residue 91 forming the calcium-binding loop (*15*). The presence of calcium stabilizes the molecule, making it more resistant to shifts in temperature (*16*), denaturing agents (*17*), pH (*17*), and pressure (*18*). However, even in the absence of calcium, α-lactalbumin retains its lactose synthase activity (*12*) and its native conformation (*19*) at physiological conditions. Thus, it is not surprising that *apo*-α-lactalbumin behaves in a similar manner when compared to native α-lactalbumin at pH 7.0 and low ionic strengths such as those used in this work. The differences observed may be explained by the presence of disturbing forces caused by the peptide ligand that has not only a hydrophobic patch (with the two tryptophan residues) but also charged residues (lysine, arginine). The forces posed by those residues may be enough to cause a disturbance in the folding of the molecule that has lost calcium, one of its stabilizers.

Binding of Mutant α-Lactalbumin. The mutant α-lactalbumin D87A (aspartate 87 changed to alanine) (*12*) was injected onto the WHWRKR column at the temperatures of 5 and 25 °C, and the profiles for these runs are shown in Figure 7. At both temperatures, there is a predominance of nonspecific binding, although at 5
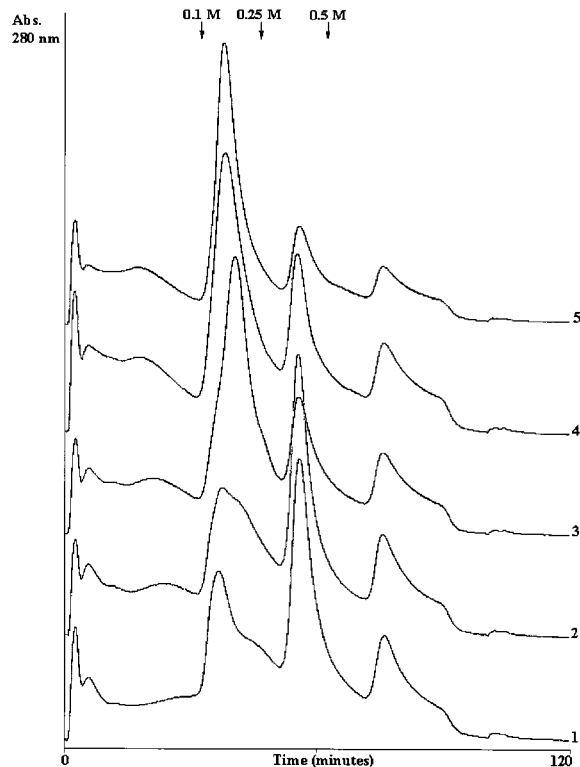

Figure 8. Chromatograms of the injections of 0.5 mL of a 5.55 g/L WPI solution using the AcWHWRKR resin at 5 °C with incomplete elution. Numerals refer to injection sequence.
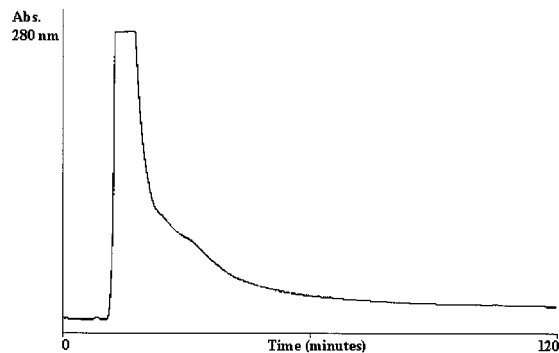

Figure 9. Elution of five injections of WPI in AcWHWRKR at 5 °C with 2% acetic acid.

°C there still is some binding by the electrostatic-driven mechanism, as evidenced by the peak eluting in 0.1 M NaCl. The chromatogram obtained at 25 °C is similar to the one for *apo*-α-lactalbumin at the same temperature, which is consistent with the observation that the D87A mutation interferes with the calcium-binding site (*12*), making both less stable to external factors.

Attempts to Block the Sites of Nonspecific Binding by Repeated Injection of WPI with Incomplete Elution. Because there is a high prevalence of nonspecific binding when using WPI with the peptide resins, an attempt to block these sites with proteins was made. Several injections of WPI were performed using the elution gradient without the last step (2% acetic acid) after each injection. After 5 injections, the column was washed with 2% acetic acid. The chromatograms obtained for the injections are shown in Figure 8, and the peak obtained upon elution with acetic acid is shown in Figure 9.

The first peak after the unbound fraction is the α-lactalbumin-rich fraction. The area of this fraction

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increases, while the other peaks decrease (Figure 8). Assuming that nonspecific binding favors non-native conformations of the proteins, there must be a generation of nonspecific binding sites on the surface of partially unfolded bound proteins. Because α-lactalbumin is in its native calcium-saturated form, it may have sufficient stability to resist unfolding and binding to other proteins. Hence, the relative amount of α-lactalbumin competing for the specific binding sites increases, in agreement with the model proposed by Lauffenburger and Linderman (*20*) for processes where specific and nonspecific binding occur simultaneously. According to that model, nonspecific binding follows a linear relationship with the protein concentration, while specific binding follows a saturation type curve. At low protein concentrations and high concentrations of free specific binding sites, there is a prevalence of specific binding. In this context, this method appears to be effective for the isolation of α-lactalbumin from WPI.

CONCLUSIONS

The experiments have shown that there are multiple mechanisms accounting for the binding of α-lactalbumin to the peptide ligands and its variants and possibly the matrix backbone. The variation of the binding behavior suggests that there are at least three distinct mechanisms of binding. One that is mainly electrostatic and predominates at low temperatures, one that is hydrophobic and thus more prevalent at high temperatures, and one that is nonspecific and acts over a wide range of temperatures. The same behavior was observed for α-lactalbumin when WPI was used rather than the pure protein, indicating that there are specific and nonspecific binding sites and that other proteins present in WPI do not compete for the specific sites.

The WHWRKR and AcWHWRKR peptide resins showed superior performance for α-lactalbumin separation and were studied in further detail. A protein similar to α-lactalbumin, hen egg lysozyme, displayed little or no affinity to the resins tested, while *apo*-α-lactalbumin showed a behavior similar to the native α-lactalbumin, as did the D87A mutant α-lactalbumin.

The injection of WPI without elution of nonspecifically bound protein suggested that there is no competition between α-lactalbumin and other proteins for the specific binding sites. Hence, this approach appears to be a feasible method for the isolation of α-lactalbumin from WPI using bioselective adsorption by peptide ligands.

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