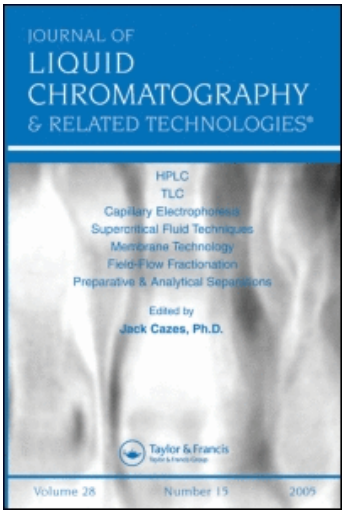

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INTERFERENCE DUE TO NON-SPECIFIC ADSORPTION IN ION EXCHANGE CHROMATOGRAPHY OF PROTEINS: THE ROLE OF INITIAL SALT CONCENTRATION IN THE SEPARATION AND ANALYSIS OF LYSOZYME

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ABSTRACT

In this paper the influence of NaCl concentration in the binding buffer on the analysis of lysozyme in chicken egg white using MonoQ® anion exchange column is examined. When using a mobile phase having pH 9.5, lysozyme (being positively charged at that pH) is not expected to bind to the anion exchange column. However, when the NaCl concentration in the binding buffer is lower than 70 mM, lysozyme is found to bind to the column, probably due to non-specific adsorption. This results in lysozyme splitting into two peaks; a primary peak in the column void volume, and a secondary lysozyme peak in the NaCl salt gradient. The proportion of lysozyme in the secondary peak decreases with an increase in NaCl concentration in the binding buffer. No secondary peaks are observed when the NaCl concentration in binding buffer is greater than 70 mM. This observation is confirmed by experiments carried out with pure lysozyme.

INTRODUCTION

Lysozyme (E.C. 3.2.1.17) is a useful enzyme which occurs naturally in chicken egg white.[1] In addition to its many established uses,[2] it has several potential new applications, such as, for cancer chemotherapy and for monoclonal antibody production.[3,4] The major proteins present in chicken egg white, along with their physical properties, are shown in Table 1. Different chromatographic methods for separation and analysis of lysozyme have been reported.[5,6] The high pI of lysozyme, together with the significantly lower pIs of the other egg white proteins, makes it convenient to separate and analyze lysozyme using anion exchange chromatography. If a mobile phase having a pH in between the pIs of lysozyme (11.0) and conalbumin (6.1) is used, lysozyme (being positively charged) should not bind to an anion exchange column, and should be obtainable as a peak at the end of the column void volume. All other egg white proteins (being negatively charged) would be expected to bind to the column and would need to be eluted out either using high salt concentration or pH change.

In this paper the FPLC® (Fast Protein Liquid Chromatography) separation and analysis of lysozyme in chicken egg white using Mono Q® anion exchange column is examined. Preliminary experiments suggest that non-specific adsorption of lysozyme to the column could give rise to misleading results. The initial salt concentration in the binding buffer was found to influence the chromatograms obtained with chicken egg white. The role of initial salt concentration on lysozyme separation is investigated and the reasons for formation of secondary lysozyme peak under certain operating conditions are hypothesized.

Table 1

Major Proteins in Chicken Egg White

Protein	Molecular Weight	Isoelectric Point (pI)	% of Total Protein
Ovalbumin	45000[a]	4.5[a]	~ 54[a]
Conalbumin	80000	6.1[a]	~ 12[a]
Ovomucoid	28000[a]	4.1[a]	~ 11[a]
Ovomucin	5.5 - 8.3 x 10^{6}[a]	4.5 - 5.0[a]	~ 3.5[a]
Lysozyme	14300[a]	11.0	~ 3.4[a]
G_2 globulin	3.0 - 4.5 x 10^{4}[a]	5.5[a]	~ 4.0[a]
G_3 globulin	12700[a]	4.8[a]	~ 4.0[a]
Ovoinhibitor	49000[a]	5.1a	~ 1.5[a]

[a] Reference 1.

EXPERIMENTAL

Lysozyme (L-6876) and crude dried chicken egg white (A-5253) were purchased for Sigma Chemical Company. A Mono Q® HR 5/5 (Pharmacia Biotech) anion exchange column was used for the FPLC® (Pharmacia Biotech) experiments. FPLCdirector® (Pharmacia Biotech) was used to operate the FPLC®. All mobile phase was prepared using double distilled water, degassed and microfiltered through 0.2-micron membrane (Whatman). A mobile phase flow rate of 0.7 mL/min was used for the chromatographic runs. The two pumps (A and B) of the FPLC® were connected to the two buffer reservoirs containing buffer A (50 mM Tris-HCl, pH 9.5) and buffer B (50 mM Tris-HCl, pH 9.5 and containing 1 M NaCl) respectively. The concentration of NaCl in the binding buffer was varied by adjusting the proportion of liquid pumped by the two pumps as shown in Figure 1. The initial NaCl concentration was varied between 0 and 80 mM in the chicken egg white experiments and between 0 to 90 mM in the pure lysozyme experiments. Elution of the bound proteins was achieved using a linear NaCl gradient as shown in Figure 1. When the required initial NaCl concentration in the binding buffer was 0, pump A delivered 100% of the liquid for the first 5 minutes. Between 5 and 18 minutes, the percentage

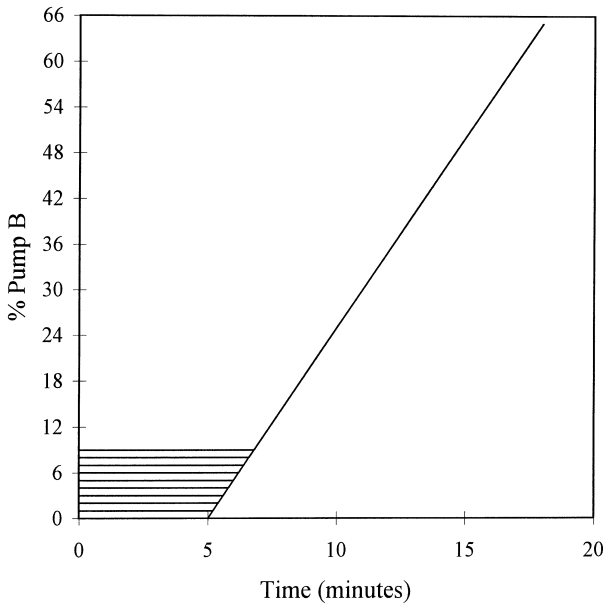

Figure 1. Gradients for FPLC runs using different initial NaCl concentration.

of liquid delivered by pump B increased linearly from 0% to 65%. When a higher initial NaCl concentration was required in the binding buffer, the initial proportion of liquid delivered by pump B was maintained at the desired level (e.g. 10% for 10 mM and so on) till the gradient line was encountered. After that the proportion of liquid delivered by pump B was increased along the gradient line. This scheme was used in order to keep the retention time of proteins eluted in the salt gradient region constant. Preliminary experiments have shown that bound proteins start getting eluted after about 8 minutes. An AUFS of 0.05 was used in order to get sufficient sensitivity of analysis while keeping the noise signals to a minimum. All samples were prepared by dissolving the proteins in appropriate binding buffer. Prior to injection, samples were centrifuged at 13000 rpm for 10 minutes to get rid of particulate matter. A 100 μL sample loop was used for sample injection. Protein peaks were monitored using a UV-detector set for 280 nm wavelength.

RESULTS AND DISCUSSION

Figure 2 shows the chromatograms for chicken egg white samples (concentration = 3 g/L) obtained using different initial NaCl concentrations in the binding buffer (i.e. 0, 20, 40 and 80 mM). The egg white proteins other than lysozyme were eluted as a broad peak in the salt gradient between 10 and 16 minutes. When no NaCl was present in the binding buffer, a very tiny (almost undetectable) primary lysozyme peak was obtained at a retention time of about 1.23 minutes. This retention time corresponds to the column void volume. Theoretically, all the lysozyme in the chicken egg white samples should have come out in this peak. However, in this case most of the lysozyme remained bound to the column and was eluted as a secondary peak in the salt gradient (at a retention time of around 8.9 minutes). As the NaCl concentration in the binding buffer was increased, greater amounts of lysozyme were obtained in the primary peak (retention time = 1.23 minutes). When the binding buffer NaCl concentration was 80 mM, all the lysozyme was obtained in the primary peak and no secondary lysozyme peak could be detected in the salt gradient. The homogeneity of the primary and secondary lysozyme peaks were checked by SDS-PAGE.

The presence of a secondary lysozyme peak (which can lead to misleading results) is unexpected since at pH 9.5 lysozyme is positively charged and hence should not bind to an anion exchange column. Ionic interaction of lysozyme with the column is probably not responsible for the secondary lysozyme peak. Hydrophobic interaction can also be ruled out since with this phenomenon, increase in NaCl concentration should encourage more binding of lysozyme to the column. The binding of lysozyme to the column is probably due to non-specific adsorption, which is discouraged at higher salt concentrations. Undesirable interference due to separation mechanisms, other than the one being utilized, is not totally uncommon. Interference due to hydrophobic and

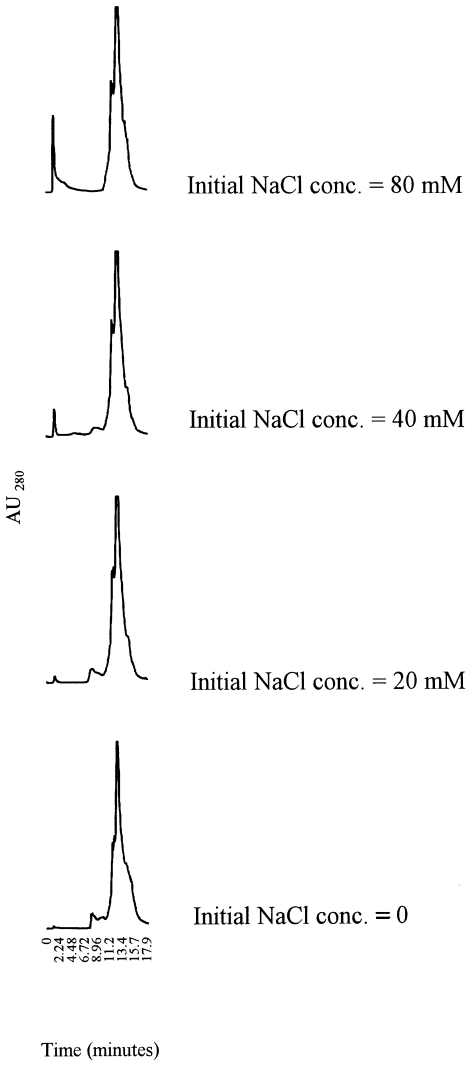

Figure 2. Effect of NaCl concentration in binding buffer on chromatograms obtained with chicken egg white.

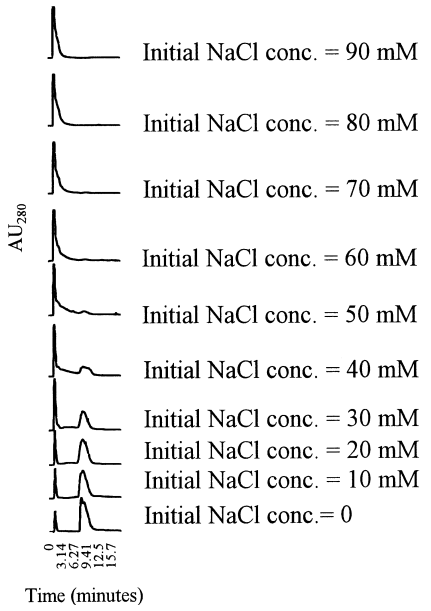

Figure 3. Effect of NaCl concentration in binding buffer on chromatograms obtained with pure lysozyme.

ionic interactions is often observed in gel filtration. Effect of pH dependent ionic interaction in the gel filtration of lysozyme using Superose® 12 HR 10/30 column has been discussed by Golovchenko et al.[7]

In order to conclusively prove that lysozyme splits into a primary and a secondary peak under certain operating conditions, FPLC® experiments were done using pure lysozyme (concentration = 0.5 g/L). The lysozyme was found to give a single band in SDS-PAGE. Figure 3 shows the chromatograms for pure lysozyme obtained with different concentrations of NaCl in the binding buffer. These results quite clearly demonstrate that lysozyme splits into two distinct and well-separated peaks under certain conditions and that the proportion of lysozyme in the two peaks depended on the NaCl concentration in the binding buffer. The percentages of lysozyme obtained in the secondary peak at different binding buffer NaCl concentrations are shown in Figure 4. These values were calculated from chromatogram peak integration data. At initial NaCl concentrations of 70 mM and higher, lysozyme was obtained as a single primary peak.

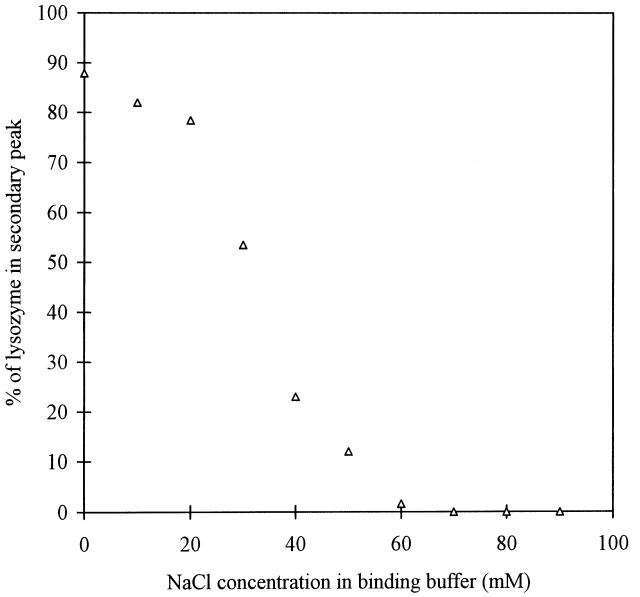

Figure 4. Effect of initial salt concentration on percentage of lysozyme in secondary peak.

Thus, for separating or analyzing lysozyme with a Mono Q® anion exchange column, the binding buffer NaCl concentration should be 70 mM or slightly higher in order to prevent lysozyme from binding to the column. If lower initial NaCl concentrations are used, secondary lysozyme peaks will be obtained and this will cause confusion in interpreting the chromatograms. On the other hand, if a very high NaCl concentration is used in the binding buffer, adsorption of anionic proteins will be affected. While developing chromatographic analytical and separation protocols, such possibilities must always be investigated.

CONCLUSIONS

From the experimental results, the following can be concluded:

When the NaCl concentration in the binding buffer is lower than 70 mM, a certain proportion of lysozyme binds to the Mono Q® anion exchange column even when lysozyme is positively charged. Thus, for the analysis of lysozyme in chicken egg white using this column, the NaCl concentration in the binding buffer should be at least 70 mM.

Such binding can be ascribed to non-specific adsorption and this results in secondary lysozyme peak in the salt gradient.

The percentage of lysozyme in the secondary peak is a function of the NaCl concentration in the binding buffer. The percentage decreases with increase in the salt concentration.

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