OVALBUMIN ISOFORMS - PURIFICATION AND DENATURATION / RENATURATION STUDIES

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Abstract: Under certain temperature and/or elevated pH conditions, native ovalbumin is transformed into thermostabilized forms: S and I .

The purpose of this article is to investigate both urea denaturation and renaturation of ovalbumin isoforms by fluorescence studies.

INTRODUCTION

Ovalbumin is a major protein of avian egg white with a molecular mass of 45 kDa that represents about 45% from the egg white proteins. This protein is a non-inhibitory member of serine proteinase inhibitors (serpin). It is classified as a serpin based on its three-dimensional structural similarity to the inhibitory serpins.

Under certain temperature and elevated pH conditions, the native ovalbumin is transformed into a heat–stabilized form, S-ovalbumin. Under normal conditions, the native ovalbumin is transformed via an intermediate state into S-ovalbumin. This form assures the storage of non-fertilized eggs for a month at a temperature of 30ºC. The S form has a higher thermal stability and is more negative than the native protein. S-ovalbumin is more sensitive to proteolytic cleavage than the native protein, a property that led to the suggestion that it has more distorted α-helix at its reactive site loop (Huntington J. A. et al., 1995).

Another form of ovalbumin is I-ovalbumin (inhibitory ovalbumin) that is obtained by heating treatment of the native form at 97ºC. I-ovalbumin is a potent reversible competitive inhibitor of human neutrophil elastase and cathepsin G, bovine trypsin and chemotrypsin and porcine elastase and α-lytic proteinase. It has 8% less α-helices and 9% more β-sheet structures than the native ovalbumin. I-ovalbumin differs from the active serpins by its inability to form irreversible complexes with proteinases (Mellet P. et al., 1996).

The role of these ovalbumin isoformes is not yet understood. The goal of this study is to purify the native ovalbumin and to compare the profiles of urea denaturation/ renaturation of ovalbumin isoforms by fluorescence studies for a better understanding of their possible role.

MATERIALS AND METHODS

Materials: fresh chicken eggs.

Isolation and purification of native ovalbumin

Egg white was shaken for breaking up the membranes for 30 minutes. After filtering through 3 single layers of cheesecloth, an equal volume of saturated solution of ammonium sulfate was added. After 5 minutes of stirring, the precipitate was centrifugated for 20 minutes at 4000 x g. Then the supernatant was collected.

While stirring, 2 M acetic acid solution was slowly added to the supernatant until the pH reached 4.6 (the isoelectric point of ovalbumin). After the centrifugation (20 minutes at 4000x g), the pellet was solubilized in 50 mM sodium phosphate buffer (Sigma), pH 7. The purification of native ovalbumin was performed by affinity column chromatography using Cibacron Blue Sepharose (BioRad). This matrix can not retain the ovalbumin but retains the majority of egg white proteins (Lascu I. et al., 1984; Li S. et al., 2000). Therefore the ovalbumin eluted with the washing buffer (50 mM sodium phosphate, pH 7). The elution fractions were monitored by absorbance at 280 nm (Jasco V-530 UV-VIS spectrophotometer). Quantification of native protein was performed by Gornall and Bradford methods. The examination of protein fractions was made by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gel.

Preparation of ovalbumin isoforms

S-ovalbumin was prepared by incubating the native ovalbumin in 50 mM sodium phosphate buffer, at 55ºC, for 16 hours at pH 10 (Onda M. et al., 1997; Yamasaki M. et al., 2003). I-ovalbumin was prepared by incubating the native ovalbumin in the same buffer, at 97ºC, for 30 minutes at pH 7 (Mellet P. et al., 1996).

Denaturation studies

All isoforms of ovalbumin (20-25 μM) were incubated in 0-9 M urea at 25ºC, for 3 hours. For each isoform the state of protein denaturation was studied by measuring the changes in both intrinsic and extrinsic fluorescence intensities (Jasco FP-750 spectrofluorometer). In the case of intrinsic tryptophan fluorescence recording, the urea denaturation mixture was dilluted ten times with 50 mM sodium phosphate buffer, pH 7.

The excitation wavelength was set at 295 nm and the fluorescence emission spectra were recorded in the range 300-420 nm.

The extrinsic fluorescence was obtained by using 25 μM ANS (1-anilino-8-naphthalene sulfonate) that binds to cationic groups of proteins (Desai U. R., 2002; Matulis D., Lovrien R., 1998). After denaturation, each mixture was dilluted four times with 50 mM sodium phosphate buffer, pH 7. The excitation wavelength was set at 405 nm and the fluorescence emission spectra were recorded in the range 450-600 nm.

Renaturation studies

Each mixture of urea denaturation was dilluted ten times with 50 mM sodium phosphate buffer (renaturation buffer), pH 7 in the case of measuring of intrinsic fluorescence intensity and it was dilluted four times with the same buffer in the case of measuring of extrinsic fluorescence intensity. The time of incubation was 3 hours at 25ºC. The fluorescence parameters are the same with those used for denaturation studies.

RESULTS AND DISCUSSION

The purity of native ovalbumin was checked by SDS-PAGE. As control, the ovalbumin (45 kDa) (Amersham) was used among molecular weight markers. The native ovalbumin purified by affinity chromatography showed the same molecular weight with the control ovalbumin (Fig.1).

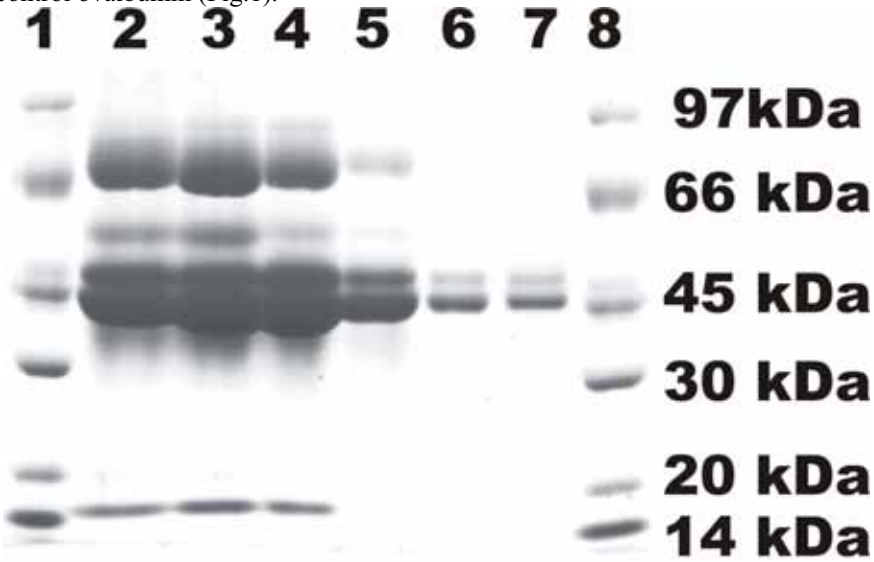

Fig.1 The elecrophoregram of native ovalbumin purification steps: lanes 1 and 8: molecular weight markers; lane 2: protein fractions from egg white; lane 3: protein fractions from egg white after filtration; lane 4: protein fractions after precipitation with saturated solution of ammonium sulfate; lane 5: protein fractions after precipitation at isoelectric point of ovalbumin; lanes 6 and 7: purified native ovalbumin

After preparing of ovalbumin isoforms (S and I), the differences of three-dimensional structures were monitored by both intrinsic and extrinsic fluorescence spectra. The intrinsic tryptophan fluorescence emission spectra of native and S-ovalbumin are similar, with the peak at 338 nm and almost the same values of fluorescence intensity (189.00 % for native ovalbumin, 195.25% for S-ovalbumin). The maximum fluorescence intensity of I-ovalbumin at 338 nm was less high (149.60%) than the above mentioned values (Fig.2). The concentration for each type of ovalbumin is 2-2.5μM.

The emission fluorescence spectra of ANS bound to each type of ovalbumin were different. The maximum fluorescence intensities at the peak wavelength (474 nm) were 1.52% for the native form (6.5 μM), 8.12% for the S-form (6.5 μM) and 45.42% for the I-form (6.5 μM) (Fig.3).

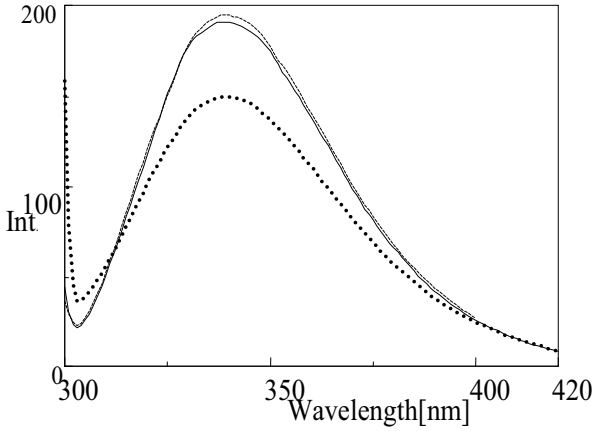

Fig. 2 The intrinsic tryptophan fluorescence emission spectra of ovalbumin isoforms: solid line for native ovalbumin; dashed line for S-ovalbumin, dotted line for I-ovalbumin; Int.-fluorescence intensity (%)

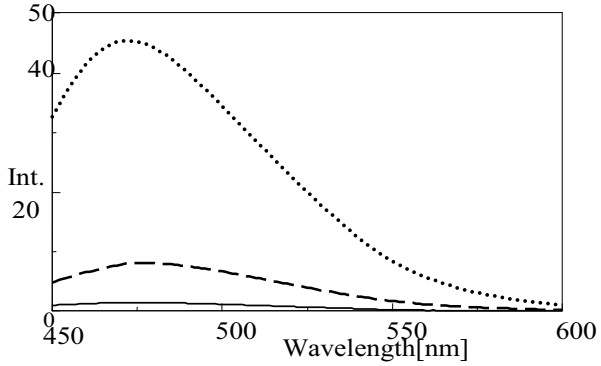

Fig. 3 The fluorescence emission spectra of ANS bound to the ovalbumin isoforms: solid line for native ovalbumin; dashed line for S-ovalbumin, dotted line for I-ovalbumin; Int.-fluorescence intensity (%)

The patterns of denaturation-renaturation of the three types of ovalbumins were different and they are shown in Fig.4.

For each of the isoform the state of denaturation-renaturation was studied by measuring the changes of intrinsic fluorescence intensity at 338 nm and of extrinsic fluorescence intensity at 474 nm.

For the intrinsic and extrinsic fluorescence studies, the concentrations of each type of ovalbumin were 2-2.5 μM and 6.5 μM respectively.

For the native ovalbumin, the intrinsic fluorescence intensities were constant (about 245%) up to 4.5 M urea. In the range of 4.5-9 M urea, the fluorescence intensity decreased to 127.5%. After renaturation, the values of fluorescence intensity at all urea concentrations were similar with those from denaturation (Fig. 4-a).

The ANS-binding affinity was weak for the native ovalbumin. In the case of the ANS binding to the native ovalbumin, the values of extrinsic fluorescence intensity were constant (about 1.5-2%) up to 4 M urea. In the range of 4-9 M urea, the values of fluorescence intensity increased to about 23%. After incubation in the renaturation buffer, the fluorescence intensities at all urea concentrations were similar with those from denaturation step (Fig. 4-b). The results of both types of fluorescence could suggest that the native form was denatured irreversibly at a urea concentration higher than 4 M.

The intrinsic fluorescence intensities of S-ovalbumin were constant (about 210%) up to 8.5 M urea. The fluorescence intensity decreased slightly at 9 M urea (135.5%).

After incubation in the renaturation buffer, the fluorescence intensities at all urea concentrations were also similar (215%) (Fig. 4-c). These results could suggest that S-ovalbumin is resistant to denaturation in the range of 0-8.5 M urea and it was a little denatured at 9 M urea.

The ANS-binding affinity was also weak for the S-ovalbumin. The values of extrinsic fluorescence intensity of the ANS bound to S-ovalbumin were constant (7-9%) in the range of 0-9 M urea. After renaturation, the values of fluorescence intensity at all urea concentrations were similar with those from denaturation (Fig. 4-d). These results could suggest that S-ovalbumin is resistant to denaturation in the range of 0-9 M urea.

Below 3 M urea, the intrinsic fluorescence intensities of I-ovalbumin were constant (about 145%). In the range of 3-9 M urea, the fluorescence intensities slightly decreased (to 105%). Following the incubation in the renaturation buffer, the values of fluorescence intensity increased to 141% in the range of 3-6.5 M urea (Fig. 4-e) suggesting a reversible denaturation. The fluorescence intensities were unchanged in the range of 6.5-9 M urea.

The ANS-binding affinity of the I-ovalbumin was high. In this case, the extrinsic fluorescence intensities were similar (about 40-45%) up to 3.5 M urea. In the range of 3.5-9 M urea, the values of fluorescence intensity decreased to about 20%. After the incubation in the renaturation buffer, the values of fluorescence intensity increased to 35-40% in the range of 3.5-5.5 M urea suggesting a reversible denaturation. A possible partial renaturation is suggested by the increasing in fluorescence intensities at 20-25% in the range of 5.5-9 M urea (Fig. 4-f).

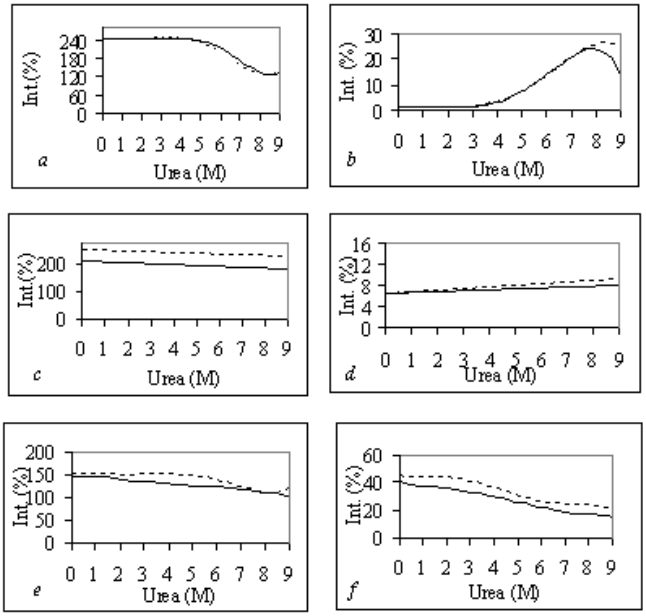

Fig.4 Comparison of urea denaturation–renaturation profiles of ovalbumin isoforms

a - denaturation-renaturation of native ovalbumin (intrinsic fluorescence); b - denaturation-renaturation of native form (extrinsic fluorescence); c - denaturation-renaturation of S-form (intrinsic fluorescence); d - denaturation-renaturation of S-form (extrinsic fluorescence); e - denaturation-renaturation of I-form (intrinsic fluorescence); f - denaturation-renaturation of I-form (extrinsic fluorescence); solid line–denaturation; dashed line–renaturation; Int.(%)-fluorescence intensity

CONCLUSIONS

The affinity chromatography using Cibacron Blue Sepharose can not retain ovalbumin and retains the majority of egg white proteins (Lascu I. et al., 1984, Li S. et al., 2000). Therefore this method assured a high purity of ovalbumin (about 95-98%).

The patterns of urea denaturation-renaturation of three types of ovalbumins were different. The native form was denatured irreversibly at higher urea concentration than 4-4.5 M. The S-form was not denatured in the range of 0-8.5 M urea and it was sligthly denatured at 9 M urea. The I-form was denatured reversibly in the range of 3.5-5.5 M urea. Between 5.5 and 9 M urea, the I-form was partialy renatured.

BIBLIOGRAPHY

Desai U. R., Johns Jennifer L., Lahaye Laura, Wright H.T., 2002. Anal. Biochem., 302, 81-87

Huntington J. A., Patston P. A., Gettins P. G. W., 1995. Protein Science, 4, 613- 621

Lascu I., Porumb H., Porumb T., Abrudan Ileana, Tarmure Cornelia, Petrescu I., Presecan Elena, Proinov I., Telia M., 1984. J. Chromatogr., 283, 199-210
Li S., Bohui X., Runzi C., Junde W., 2000. Chromatogr. 3, 21, 221-224
Matulis D., Lovrien R., 1998. Biophys. J., 74, 422-429
Mellet P., Michels B., Bieth J. G., 1996. J. Biol. Chem., 48, 271,. 30311-30114
Onda M., Tatsumi E., Takahashi N., Hirose M., 1997. J. Biol. Chem., 7, 272, 3973-3979
Yamasaki M., Takahashi N., Hirose M., 2003. J. Biol. Chem., 37, 278, 35524-35530

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