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Heterogeneity of Egg Albumin in Calcium Phosphate Chromatography. Part VIII

Proline Contents of the Chromatographically Separated Fractions

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Crystalline egg albumin shows three compnents; A_1, A_2 and A_3; electrophoretically[1]. It was shown by Perlmann[2] that the mobility differences among these three components can be attributed to their phosphate contents, that is, 2, 1 and 0 phosphate residues per mole protein for A_1, A_2 and A_3 respectively.

Another heterogeneity of egg albumin was pointed out by Tiselius et al.[3] through the calcium phosphate column chromatography. They showed the existence of at least four components. But the differences among them were not described.

We are investigating this heterogeneity of egg albumin[4,5]. It was shown first that this heterogeneity was not accounted for by the heterogeneity of A_1, A_2 and A_3. When egg albumin was divided into the chromatographically different fractions, EaI and EaII, A_1 came into both of the fractions as a main component, indicating that A_1 itself was heterogeneous chromatographically. There must be differences among the components of egg albumin other than the phosphate content. One possibility is some variation in the amino acid residues of the polypeptide chain of egg albumin.

Pauling[6] pointed out that the unique characteristics of proline residue may play a dominant part in the folding of a polypeptide chain. If there occurs variation in the proline residue, it may cause a change of the comformation of the polypeptide chain and hence affect remarkably the chromatographic behavior of the protein. Therefore, the proline residue was first investigated.

Materials and Methods

1. *Egg albumin.* Crystalline egg albumin was prepared by ammonium sulfate method[7] and recrystallized three times. It was freeze-dried after removal of salts by dialysis and stocked in a refrigerator for use. EaI and EaII, the fractions of egg albumin different in chromatographic behavior on calcium phosphate column, were separated and purified as described in the previous paper[5].

2. *Hydrolysis of protein.* Each 100 mg. of protein preparations was hydrolyzed in sealed tubes with 6 N hydrochrolic acid at 110°C for 24 hours. After hydrolysis excess hydrochrolic acid was removed in vaccum desiccator over sodium hydroxide.

3. *Determination of protein concentration.* The concentration of protein was determined by a photometric method. The molar extinction coefficient of 3.2×10^4 was used at 280 mμ (M.W.=45,000).

4. *Method of photometric estimation of proline in protein hydrolyzate.* Chinard's[7] photometric method was modified for the determination of proline in protein hydrolyzate.

Reagent solution.—Twenty-five mg. of ninhydrin was added per ml. of the acid mixture

containing 0.4 ml. of 6 M phosphoric acid and 0.6 ml. of glacial acetic acid for each ml. After the addition of ninhydrin, the aicd mixture was heated to about 70°C to insure solution of the ninhydrin.

Color development.—To 1.0 ml. of the solution to be analyzed were added 1.0 ml. of glacial acetic acid and 1.0 ml. of the reagent solution. A sample blank was prepared by adding 1.0 ml. of glacial acetic acid and 1.0 ml. of acid mixture without ninhydrin to 1.0 ml. of the solution to be analyzed. A reagent blank was prepared by adding 1.0 ml. of the reagent solution and 1.0 ml. of glacial acetic acid to 1.0 ml. of water. After mixing, tubes were capped and heated in a boiling water bath for 60 minutes. Reaction mixtures were extracted with 5ml. of benzene after cooling to a room temperature.

Estimation.—The optical density of the red benzene extract was determined at 515 mμ. The proline content was estimated by using a standard curve given in Fig. 1.

Results and Discussion

1. *Determination of proline in protein hydrolyzate.*

Standard curve. Fig. 1 shows the relationship between the proline concentration and the optical density at 515 mμ. As shown in Fig. 1, a linearity was observed up to 0.25 μ mole per ml.

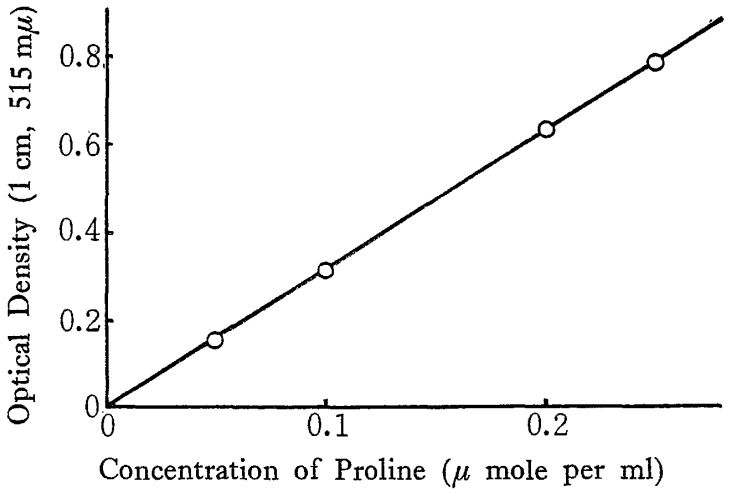

Fig. 1. Relationship between the concentration of proline and the optical density at 515 mμ.

Table 1. Recovery of added proline to egg albumin hydrolyzate

Added proline	Total proline in hydrolyzate	Recovered proline
0 μmole	0.105 μmole	— μmole
0.030	0.134	0.029
0.060	0.164	0.059
0.090	0.195	0.090
0.120	0.220	0.115
0.150	0.256	0.151

Each ml. of sample solution contained 0.5ml. of hydrolyzate and 0.5ml. of standard solution of proline.

Recovery test.—After adding a known amount of proline to a protein hydrolyzate, the proline content was determined. As shown in Table 1, added proline was well recovered.

Influence of hydroxyproline.—According to Cannan et al.[8], hydroxyproline forms a red product with ninhydrin as well as proline. The product is soluble in benzene. Therefore, the influence of hydroxyproline on the optical density at 515 mμ was investigated. The results are shown in Table 2.

Table 2. Influence of hydroxyproline on the optical density at 515 mμ.

Concentration in sample solution, mole/ml.		Optical density 515 mμ, 1 cm.
Proline	Hydroxyproline	
0.12	0	0.374
0.12	0.02	0.372
0.12	0.04	0.367
0.12	0.06	0.364
0.12	0.08	0.380
0.12	0.10	0.382
0.15*	0	0.463
0.15*	0.04	0.468
0.15*	0.10	0.475

* Hydrolyzate of egg albumin

As seen in Table 2, even at the presence of equimolar concentration of hydroxyproline, the increase of optical density was within the limit of experimental error. In the determination of proline with a hydrolyzate of egg albumin, the interference by hydroxyproline can be ignored, since hydroxyporline has not been detected in this protein.[9]

2. *Proline contents of EaI and EaII.*

The results of the determination of proline with EaI, EaII and original egg albumin are shown in Table 3.

Table 3. Proline contents of EaI, EaII and original egg albumin (M.W.=45,000)

Samples	Proline mole/mole protein
Original egg albumin	12.8
EaI preparation	12.9
EaII preparation	12.3

No significant difference was observed among the proline contents of these three preparations. From the results, it is unlikely that the heterogeneity of egg albumin comes from the variation of proline residues in polypeptide chain.

Summary

The modified method of photometric estimation of proline was described.

The proline contents of the chromatographically separated fractions of egg albumin were estimated by the modified method. There was no significant difference in the proline content between the fractions.

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