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CHEMICAL CHARACTERIZATION OF THE HEN EGGSHELL MATRIX: ISOLATION OF AN ALKALI-RESISTANT PEPTIDE

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SUMMARY

The eggshell matrix was obtained from hen eggshells using EDTA solutions. The water-soluble organic material was separated on a DEAE-cellulose column equilibrated with 8 *M* urea using Tris–hydrochloric acid buffer as eluent with a linear gradient of sodium chloride.

Five main fractions were obtained which differ in amino acid composition and sugar contents. As is shown from the uronic acid content, the first two fractions eluted from the column are glycoproteins, while the other three contain proteins and glycosaminoglycans.

From the alkaline hydrolysate of the eggshell matrix, a peptide was isolated which is composed of aspartic acid, threonine, serine, glutamic acid, proline, glycine and alanine in a molar ratio of 2:1:3:7:1:3:1 with a minimum molecular weight of 2158 daltons.

The calcium ion binding to this peptide was studied, at different pH values, with both free and blocked carboxyl groups, using murexide as an indicator of free Ca^{2+}. The importance of this acidic peptide in the calcification process of the eggshell matrix is discussed.

INTRODUCTION

The chicken eggshell is composed of a protein–polysaccaride and glycoprotein mixture, as already described by numerous workers[1–5]. The organic eggshell matrix represents approximately 2% (w/w) of the total calcified material in which calcium carbonate is the major inorganic component. The shell matrix, as other calcified substrates[6], seems to influence the mineral deposition, even though the binding sites in the calcifying processes are still a matter of discussion.

As far as the organic eggshell matrix of the chicken is concerned, the importance of the ionizable carboxyl groups in the calcium ion-binding processes has been demonstrated[5]. In fact, the calcium ion binding is pH dependent and rapidily decreases if the carboxyl groups of the shell matrix are previously blocked with a water-soluble carbodiimide.

Many calcifying systems, such as bone, dentine and ectopic calcifications, have

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been shown to contain γ-carboxyglutamic acid[7]. This calcium ion-binding amino acid seems to play an important role in the regulation of calcium salt deposition, as has been demonstrated for the process of vitamin K activation[9,10]. The presence of this particular amino acid is not obligatory, however, for the calcification process in invertebrates and in the eggshell of the chicken, as has been shown by King[11].

In our work we have separated the eggshell matrix on a DEAE-cellulose column and the fractions obtained have been chemically characterized. In addition we have investigated the presence of particular compounds such as the γ-carboxylglutamic acid, phosphoserine or similar substances, which could be involved in the calcium ion-binding process. For this purpose we have studied the amino acid composition of the eggshell matrix after alkaline and acid hydrolysis in order to observe the eventual presence of particular amino acids or peptides.

The calcium ion binding to a peptide isolated from the alkaline hydrolysate of the eggshell matrix has been also studied.

MATERIALS AND METHODS

The eggshell matrix was obtained by extraction of the broken shells with EDTA solutions as previously described[1,5]. Briefly, the broken eggs were washed with running tap water and immersed in 5% (w/v) EDTA solution adjusted at pH 7–7.5 in order to remove the cuticles. The membranes were peeled off and then the shells were crushed and decalcified with 20% EDTA solutions by stirring overnight (pH 7–7.5). The mixture was dialysed against distilled water until no salts were detectable in the tubes and then lyophilized. The organic material which represents *ca.* 2% of the starting shells was suspended in water and any precipitate was centrifuged off. Only the water-soluble material was used for the experiments.

Hexosamines were determined according to the method described by Cessi and Piliego[12]. The hydrolysis was performed 4 *M* hydrochloric and for 8 h at 110°C. Uranic acid was determined by the modified Dische procedure as described by Davidson[13] and Dische[14]. The neutral sugars were determined according to the method described by Scott and Melvin[15]. The hydrolysis was performed at 100°C in 0.5 *M* sulphuric acid for 3 h. Sialic acid was determined according to the procedure described by Warren[16].

The chromatographic separation of the eggshell matrix was performed on a DEAE-cellulose (DE 32 microgranular) column equilibrated in 8 *M* urea. Aliquots (100 mg) of the material were applied on a 30 × 2 cm column and the elution was carried out with Tris–hydrochloric acid buffer (0.05 *M*, pH 8.5) in 8 *M* urea with a sodium chloride gradient from 0 to 0.5 *M*. The column was washed with 1 *M* sodium chloride followed by 0.1 *M* sodium hydroxide.

The amino acids were determined, after hydrolysis with 6 *M* hydrochloric acid for 22 h at 110°C, on a JLC-5AH amino acid analyser.

The alkaline hydrolysis of the eggshell matrix was performed with 2 *M* sodium hydroxide at 100°C for 24 h. The hydrolysate was then neutralized with concentrated hydrochloric acid and applied on to the amino acid analyser which was provided with a split stream device in order to collect part of the effluent from the column.

For desalting, a Bio-Gel P-2 column (100 cm × 2 cm) was used with 1% acetic acid as eluent.

Calcium ion binding was determined as described elsewhere, using murexide as an indicator of free Ca^{2+}[17]. A 50-μM solution of murexide in 10 mM Tris–hydrochloric acid buffer from pH 5 to 9 was titrated with additions of $1.1 \cdot 10^{-5}$ M calcium chloride. The reaction was measured spectrophotometrically at 540–510 nm, with a dual-wavelength spectrophotometer (Johnson Research Foundation). If at the same pH value the murexide solution was titrated in the presence of 0.5–1 mg of peptide, smaller absorbance changes were initially obtained under the same experimental conditions. From the difference of the two titration curves, in the presence and in the absence of the peptide, it was possible to calculate the amount of the Ca^{2+} bound to the peptide at pH 5, 6, 7, 8 and 9 (see Results). The carboxylic groups of the protein were blocked with the glycine ethyl ester in the presence of the water-soluble carbodiimide 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, according to the method of Hoare and Koshland[18]. The reaction was performed at pH 4.75. The pH was maintained constant by automatic titration with 0.5 M hydrochloric acid.

RESULTS

Fig. 1 shows the chromatographic separation of the eggshell matrix on DEAE-cellulose. Five main fractions are obtained. The first two peaks are eluted with the void volume, the third large fraction is eluted with the sodium chloride gradient and the last two fractions with the 1 M sodium chloride and 0.1 M sodium hydroxide washings, respectively. The five fractions represent respectively 10, 4.0, 12, 4.0 and 70.0% of the weight of the starting material.

Table I reports the amino acid composition of the eggshell matrix and its

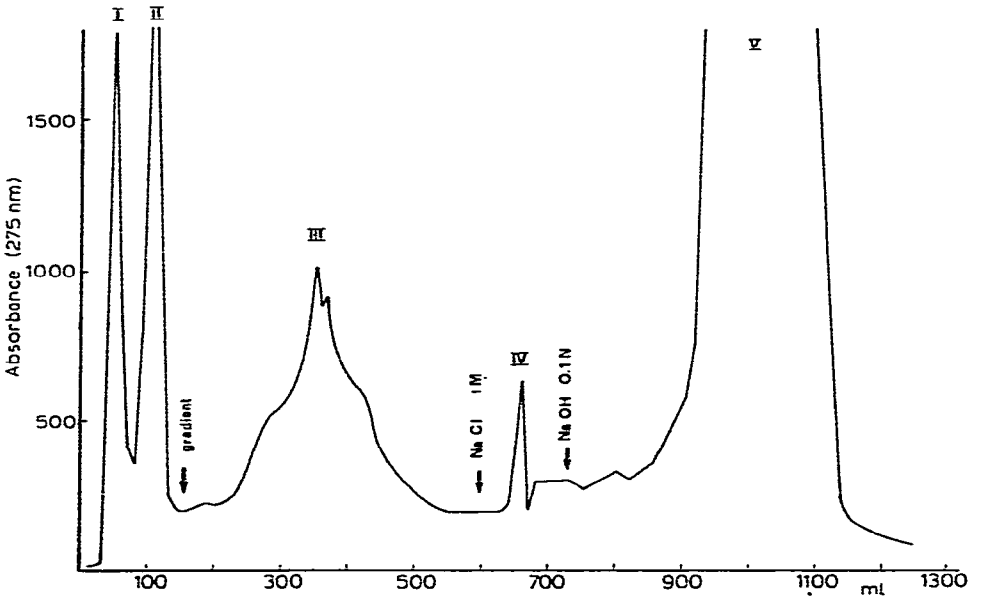

Fig. 1. Chromatographic separation of the eggshell matrix on a column (30 × 2 cm) of DE 32 DEAE-cellulose equilibrated in 8 M urea. The elution was carried out with Tris–hydrochloric acid buffer (0.05 M, pH 8.5) in 8 M urea with a sodium chloride gradient from 0 to 0.5 M. Five fractions (I–V) were collected.

TABLE I

AMINO ACID COMPOSITION OF THE EGGSHELL MATRIX AND ITS FIVE FRACTIONS

Amino acid residues per 1000 residues.

Amino acid	*Water-soluble eggshell matrix*	*Fractions isolated from the DE-cellulose column (see Fig. 1)*				
		I	*II*	*III*	*IV*	*V*
Hyl	–	–	–	–	–	–
Lys	38.7	33.5	44.6	38.3	26.9	41.0
His	22.5	9.3	–	20.9	9.6	20.6
Arg	36.1	29.2	34.1	37.5	50.0	51.3
Hyp	–	–	–	–	–	–
Asp	102.3	104.4	67.3	92.3	67.3	105.7
Thr	73.1	107.4	35.8	67.6	46.1	58.4
Ser	95.4	85.7	54.2	87.9	119.2	85.9
Glu	128.1	52.1	85.7	126.4	155.7	127.8
Pro	62.7	63.1	81.3	65.4	–	68.5
Gly	147.9	115.2	137.3	150.8	251.9	119.1
Ala	73.9	121.3	111.1	77.7	75.0	72.1
1/2 Cys	7.7	–	–	2.3	–	13.0
Val	68.8	82.2	109.3	91.9	78.8	68.7
Met	13.7	–	–	9.2	–	10.5
Ile	36.1	35.8	41.1	29.1	26.9	42.9
Leu	72.2	76.8	70.8	71.4	46.1	90.2
Tyr	7.7	25.0	70.8	10.0	46.1	9.0
Phe	12.8	58.4	55.9	20.6	–	15.6
NP/P*	1.01	1.37	2.10	1.12	1.10	1.04

* NP/P = ratio of non-polar to polar amino acids.

fractions separated on the DEAE-cellulose column. The ratio of non-polar to polar (NP/P) amino acids has been calculated for all the fractions and is expressed by the sum of the moles of proline, glycine, alanine, cystine, valine, isoleucine, leucine, methionine, tyrosine and phenylalanine, divided by that of lysine, histidine, arginine, aspartic acid, serine and glutamic acid.

Table II reports proportions of uronic acid, hexosamines and neutral sugars

TABLE II

URONIC ACID, HEXOSAMINES AND NEUTRAL SUGAR CONTENTS OF THE EGGSHELL MATRIX AND ITS FIVE FRACTIONS

Material	*Content (per 100 g of material)*		
	Uronic	*Hexosamines*	*Neutral sugar*
Water-soluble eggshell matrix	12.8	10.8	3.2
Fraction I	0.6	2.77	9.0
Fraction II	0.4	2.2	8.0
Fraction III	10.3	9.16	5.2
Fraction IV	2.2	4.4	5.1
Fraction V	7.6	8.16	3.84

for alle the fractions. No sialic acid was found in any sample. Fig. 2 shows the elution profile on the amino acid analyser of the alkaline hydrolysate of the eggshell matrix. It can be seen that just before the glutamic acid, an unknown peak is eluted. This material, referred to as GPX, was collected from many runs of the amino acid analyser with a split stream device. When this material was purified on Bio-Gel P-2 (see Fig. 3) it was resolved into two peaks, the first of which was shown on the amino acid analyser (see Fig. 4) to be the unknown substance (GPX) eluted before the glutamic acid, and the second to consist of salts and two free amino acids, serine and glutamic acid. The isolated GPX was further co-chromatographed in the presence of glutamic acid, thus confirming that its position was just before the glutamic acid.

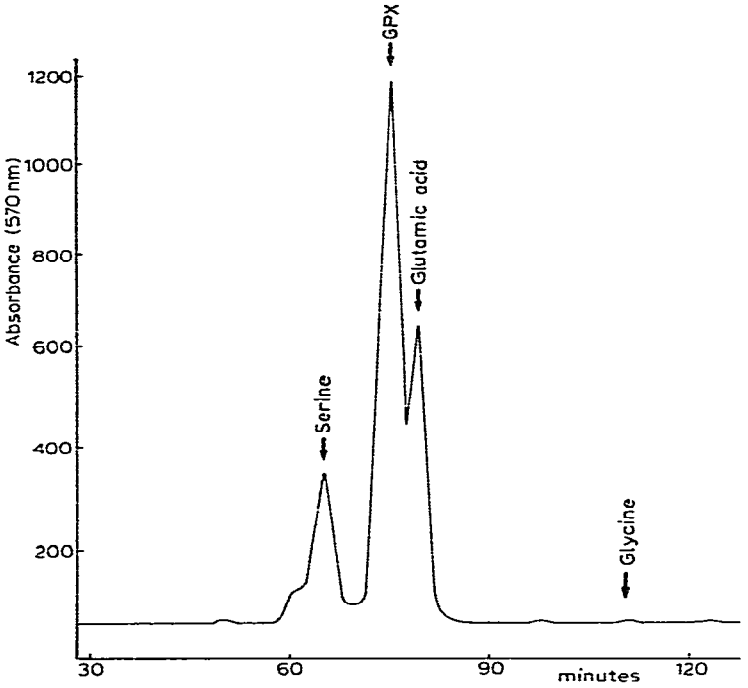

Fig. 2. Chromatographic separation of the alkaline hydrolysate of the eggshell matrix on a JLC-5AH amino acid analyser GPX is the unknown substance which is eluted just before the glutamic acid.

The unknown compound GPX, which represents 2% of the eggshell matrix by weight, was submitted to acid hydrolysis with 6 *M* hydrochloric acid and then applied on to the amino acid analyser. The chromatographic profile (Fig. 5) shows the GPX to be composed of several amino acids; these are summarized in Table III.

The calcium ion binding to the GPX peptide at different pH values under saturation conditions is prepresented in Fig. 6. The upper curve represents the binding to the alkali-resistant peptide (GPX) in which the carboxyl functions are free, whereas the lower curve represents the calcium binding to the peptide in which the carboxyl groups are modified.

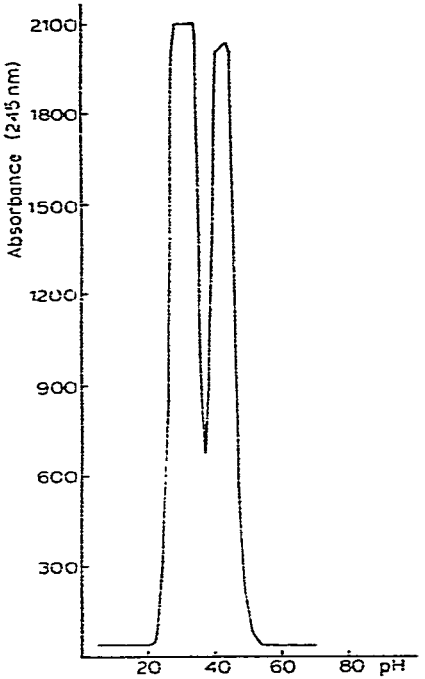

Fig. 3. Chromatographic separation of the GPX material (see Fig. 2) from the salts and amino acids on a Bio-Gel P-2 column (200 × 2 cm). The first fraction is represented by GPX.

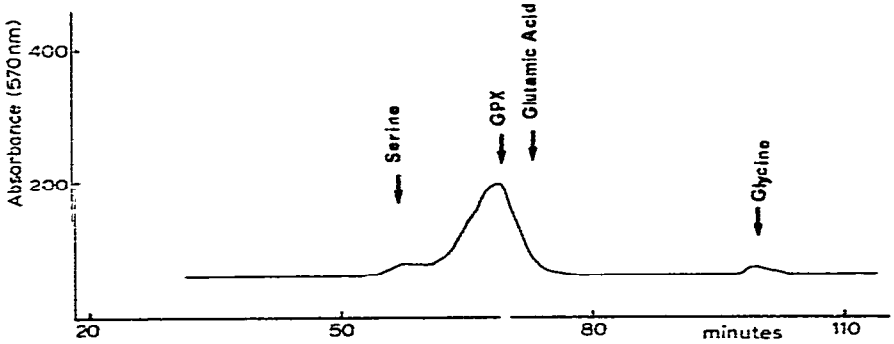

Fig. 4. Chromatographic analysis of the desalted GPX material (see Fig. 2) on a JLC-5AH amino acid analyser. The unknown substance GPX is eluted near the glutamic acid.

DISCUSSION AND CONCLUSION

The eggshell matrix, obtained by extraction of the shells with EDTA solutions, can be partially solubilized in water (*ca.* 50% of the total). When this water-soluble material is applied on to the DEAE-cellulose column, five main fractions are obtained (Fig. 1) which differ in their amino acid composition and sugar content.

From the ratio of the non-polar to polar amino acids, it can be observed that

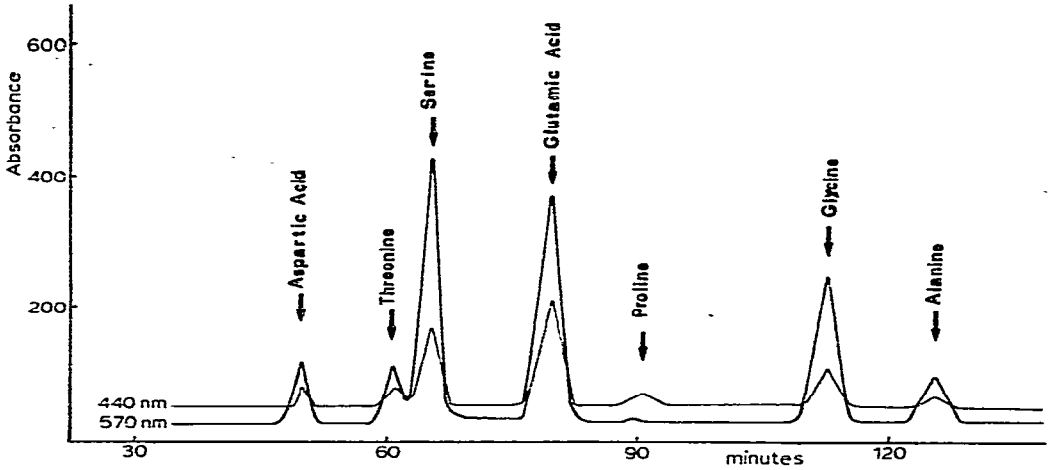

Fig. 5. Chromatographic separation of the GPX material on the amino acid analyser after acid hydrolysis with 6 *M* hydrochloric acid at 110°C for 22 h (see text).

TABLE III

AMINO ACID COMPOSITION OF THE ALKALI-RESISTANT PEPTIDE (GPX)

Amino acid	*Amino acid residues per 1000 residues*	*Molar ratio*
Asp	111.1	2
Thr	55.5	1
Ser	166.6	3
Glu	388.8	7
Pro	55.5	1
Gly	166.6	3
Ala	55.5	1

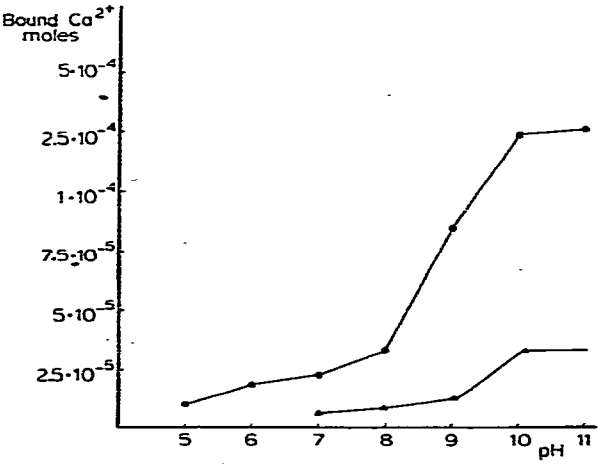

Fig. 6. Binding of Ca^{2+} to the alkali resistant peptide (GPX) under saturation conditions using murexide as indicator of free Ca^{2+}. The upper curve represents the calcium binding to the peptide with the free carboxyl groups; the lower curve represents the calcium binding to the peptide in which the carboxyl functions are modified with the glycine ethyl ester in the presence of a water-soluble carbodiimide.

the first two fractions, eluted in the void volume of the column, are richer in non-polar residues than the other fractions. The distribution of phenylalanine is different among the various fractions and it is not detectable in fraction IV. Tyrosine is more abundant in peaks I, II and IV than in peaks III and V. Only in these last two fractions are methionine and cystine present. A particular difference is also represented by the high content of glycine and glutamic acid in fraction IV. No tryptophan was detected in any of the fractions.

These observations all demonstrate that the eggshell matrix is a mixture of different proteins which possess an abundance of acidic residues (aspartic and glutamic acid). In addition, from the chemical analyses of the sugars, it must be said that the proteins are accompanied by different quantities of glycosaminoglycans (GAGs). Fractions III and V show a significant amount of glycosaminoglycans, as can be derived from the uronic acid and hexosamine content. Fractions I and II seem to be glycoprotein in nature, as shown by the high neutral sugar and hexosamine content and by the very low quantity of uronic acids. Fraction III is eluted in the region where normaliy hyaluronic acid appears.

The chemical analyses and the chromatographic separation clearly confirm that the eggshell matrix is composed of proteins, glyco- and proteoglycans, which show different behaviour on the DEAE-cellulose column.

From the amino acid analyses of the alkaline hydrolysate of the eggshell matrix, no compounds have been isolated which could be related to the prescence of γ-carboxyglutamic acid, which has been shown in other calcified tissues[7–11]. The unknown GPX peak observed in the region of the glutamic acid (Fig. 2) is due to a peptide resistant to the alkaline hydrolysis. A particular aspect of this peptide is represented by the amino acid composition. About 50% of the residues consists of aspartic and glutamic acid. The molar ratio of the detected amino acids aspartic acid, threonine, serine, glutamic acid, proline, glycine and alanine is 2:1:3:7:1:3:1, respectively, with a minimum molecular weight of 2158 daltons.

The peptide we have isolated from the alkaline hydrolysis represents from its chemical composition an interesting stage in the process of the calcium ion binding. The study of the calcium ion binding to the peptide GPX demonstrates the importance of the ionized side chain carboxyl groups. In fact more calcium ion binding occurs with increasing pH values (Fig. 6). The ionic nature of the calcium binding is confirmed also by the absence of calcium bound to the peptide in which the carboxyl groups have been previously blocked with the glycine ethyl ester in the presence of water-soluble carbodiimide. This study confirms the previous observations made on eggshell matrix[5] and on elastin[17,19] which have demonstrated the importance of the carboxyl groups in the calcium ion binding. In the particular case of this alkali-resistant peptide the abundance of the aspartic and glutamic acid residues makes this molecule extremely rich in ionizable acidic groups which in turn are responsible for the binding with calcium.

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