

Effect of storage temperature and time on egg white protein

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Eggs stored up to six weeks at temperatures of 5, 20 and 30 °C at 60% rel. humidity were investigated regarding their egg white protein composition. The proteins were separated on a C₄-column by HPLC. Specific lysozyme activity was determined using the *Micrococcus lysodeikticus* method, and the lysozyme content was quantified via HPLC. The results showed no significant changes in the activity and content of lysozyme, during storage. Conalbumin content was quantified by HPLC and iron binding capacity. No significant changes in the content could

be observed during storage. Changes however, in the HPLC elution pattern of ovalbumin were observed at a reduced retention time. Furthermore, the isoelectric point of ovalbumin became slightly more acidic. These changes of ovalbumin are in accordance with the formation of S-ovalbumin during storage. As the changes are influenced more by the storage temperature than by storage time, this leads to the conclusion that more care should be taken on a consequent storage of eggs under refrigerated conditions.

1 Introduction

In Europe storage of shell eggs is regulated by the *EU regulation 3117/94* [1], in which the height of the air cell serves as criterion for freshness of eggs. In Germany it is decreed by law [2] that from the 18th day after laying, eggs have to be stored at temperatures between 5 and 8 °C. This is based on the observation, that the growth of microorganisms in albumen is reduced in fresh eggs, whereas in older eggs microbes grow relatively fast [3]. In theory this antimicrobial potential is due to egg white proteins such as lysozyme [4] and conalbumin [5] or its viscous consistency [6]. Lysozyme has N-acetylmuramidase activity and thus destroys the cell wall of microorganisms, while conalbumin forms a stable complex with metal ions e. g. iron which are then no longer available for microorganisms [7, 5]. Therefore the question what kind of changes in egg white proteins occur during storage is an important topic which has up to now not been studied sufficiently. The most obvious and important change during storage is the process of egg white thinning. The gel structure which is formed by ovomucin is weakened throughout storage. The amount of aggregated ovomucin decreases due to non-covalent disaggregation of β -ovomucin from its complex with α -ovomucin [8]. Furthermore it is known that ovalbumin, the major egg white constituent, changes to S-ovalbumin, a more heat stable form [9], which has a reduced surface hydrophobicity [10] caused by deamidation of asparagine and glutamine residues [11]. According to Kato [12] and Trziszka and Clostermann [13] lysozyme activity is reduced during storage. There have been different approaches to separate and quantify egg white proteins. The most recent and promising is the separation by HPLC. Different columns from gel permeation to ion exchange and reversed-phase have been used [14]. The best way to separate egg white proteins was achieved by HPLC on a C₄-reversed-phase column [14, 15]. Quantification of egg white proteins by HPLC was performed by Thapon und Brulé [16] for lysozyme on a gel permeation column and by Guerin and Brulé [17] for conalbumin using ion exchange. As the above mentioned investigations are not concerned with the influence of storage on the egg white proteins, this deals with the influence of storage time and temperature on the constituents of the egg white proteins applying HPLC equipped with a reversed phase C₄-column for the separation and quantification of egg white proteins.

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2 Materials and methods

2.1 Materials

Fresh eggs (grade A, weight 60–65 g) were obtained from Shaver hens fed on a commercial diet. The eggs were stored at three different temperatures (5, 20, 30 °C) and 60% rel. humidity for up to six weeks. Investigations were carried out in weekly intervals.

The C₄-reversed phase HPLC-column (214TP5415, 4.6 × 150 mm – ID × length –, 5 µm particle size, 300 Å pore size) with a precolumn (214SFK54) was purchased from Vydac (The Separation Group, Hesperia, CA). HPLC was carried out on a Beckman system (Beckman Instruments GmbH, München) consisting of a pump module 126, a diode array detector module 168, an autosampler module 507 and a Rheodyne 100 µl sample loop. Lysozyme (EC 3.2.1.17) from chicken egg white (~95% protein) was purchased from Sigma (Sigma Aldrich Vertriebs GmbH, Deisenhofen). Electrophoresis standards were products from Sigma and BioRad (BIO-RAD Laboratories GmbH, München). All other chemicals were of analytical grade.

2.2 Sample preparation

The egg white was separated from the yolk with a spoon. Weight and volume of the albumen were determined and pH was measured using a combination pH electrode (406-M6-KN2) purchased from Knick (Elektronische Meßgeräte GmbH & Co, Berlin). To prepare an albumen homogenate about 35 ml of egg white were homogenised with the same volume of H₂O_{bid.} in a Waring Blender 5 × 1 sec. The homogenate was diluted to 100 ml with H₂O_{bid.}, cooled under stirring for one hour and the sediment was removed by centrifugation at 10.000 × g for 20 min.

2.3 HPLC

The albumen homogenate was diluted 1:2 with 50 mM acetate buffer pH 4.0 and separated from sediment by centrifugation 10.000 × g for 20 min. The supernatant was diluted 1:2 with 20% acetonitrile (ACN) containing 0.2% trifluoroacetic acid (TFA). The sediment was separated by centrifugation as described before, the supernatant was filtered through a 0.45 µm membrane filtration unit of regenerated cellulose.

Elution gradient: eluent A (10% ACN/90% H₂O_{bid.}/0.1% TFA), eluent B (85% ACN/15% H₂O_{bid.}/0.1% TFA): 10% B in A for 1 min, 10% B to 33% B in 9 min, 33% B to 45% B in 10 min, 45% B to 51% B in 5 min, 51% B to 53% B in 2 min, 53% to 90% B in 10 min. Sample volume was 20 µl. Flow was adjusted to 1 ml/min. Detection wavelength was 215 nm. Peak area was integrated applying Beckman Gold software vers. 6.01. Lysozyme and conalbumin content were determined using lysozyme (E.C. 3.2.1.17 from chicken egg white, Sigma) and conalbumin (type 1 from chicken egg white,

Sigma) as reference substances. Peak areas of lysozyme and conalbumin were assessed for six different concentrations from 0.053 to 0.60 mg/ml for lysozyme and from 0.76 to 2.00 mg/ml for conalbumin, resulting in standard curves with the following equations: conalbumin content [mg/100 ml] = $0.0071 \times \text{peak area} + 0.0232$ $\times 400$; $r = 0.9967$

lysozyme content [mg/100 ml] = $(0.055 \times \text{peak area} + 0.0052) \times 400$; $r = 0.9988$

Standard reference substances of ovalbumin and ovomucoid were purchased from Sigma (ovalbumin grade VII free of S-ovalbumin, ovomucoid type III). S-ovalbumin was synthesised according to Smith and Back [18].

2.4 Protein content

The protein content was assessed according to the method of Whittaker and Granum [19].

2.5 Specific lysozyme activity

Approximately 25 mg lyophilised *M. lysodeikticus* was suspended in 150 ml 0.1 M potassium phosphate buffer pH 7.0, incubated for 10 min at 37 °C and cooled to 25 °C. The absorption of this suspension at a wavelength of 450 nm was adjusted to 0.75 ± 0.05 absorption units. 900 µl of this suspension were mixed with 100 µl 1 : 250 diluted albumen solution. The absorption was monitored at $\lambda = 450$ nm for 3 min in 30 sec intervals. The decrease in absorption per time interval ($\Delta A/\text{min}$) was subsequently calculated. The specific lysozyme activity was calculated under consideration of the lysozyme content in the assay (assessed by HPLC) according to the following equation:

$$\text{specific activity [U/mg]} = \frac{|\Delta A/\text{min}| \times 1\,000}{\text{lysozyme content in the assay}}$$

2.6 Iron ion binding capacity of conalbumin

500 µl albumen solution was mixed with 480 µl carbonate/phosphate/citrate buffer pH 8.5 (0.1 M Na_2CO_3 /0.1 M sodium citrate/0.2 M Na_2HPO_4 /0.1 M NaH_2PO_4) and 20 µl 20 mM FeCl_3 . The colour developed after 12 min and was monitored at $\lambda = 470$ nm. A standard curve with conalbumin in seven different concentrations from 0.25 to 10 mg/ml in the assay was measured.

2.7 Isoelectric focusing (IEF)

Isoelectric focusing was performed on Servalyt Precotes pH 4–6, 300 µm, 125 mm $\times$ 125 mm with anode fluid pH 3 and cathode fluid pH 7 (2 g glycine ad 100 ml $\text{H}_2\text{O}_{\text{bid}}$) using Serve pK_I-marker (amylglucosidase pI 3.5; glucose oxidase pI 4.2; trypsin inhibitor pI 4.5; β -lactoglobulin pI 5.15/5.3; myoglobin pI 6.9/7.35; lentil lectin pI 7.75/8.0/8.3; ribonuclease A pI 9.45, cytochrom C pI 10.65; carboanhydrase pI 6.0). Electrophoretic conditions: voltage gradient from 100 to 2000 Volt in 200 min and final focusing until amperage is reduced to 2 mA. Silverstaining was performed according to Heukeshoven and Dernick [20].

3 Results and discussion

3.1 Lysozyme content and specific activity

In fresh eggs the lysozyme content was $4.3 \pm 0.4\%$ of the albumen protein. It has been reported that the lysozyme content in hen eggs varied between 2.9 and 8.4% [21, 22]. These variations are likely due to application of different methods of determination and genetic differences between lines and individual hens. During storage the lysozyme content decreased (Table 1). At storage temperatures of 5 and 20 °C the reduction is not significant between the two temperatures while at 30 °C a significant decrease was observed (2.10 ± 0.10) after six weeks of storage.

Table 1. Lysozyme content of the total protein during storage of eggs at different temperatures.

Storage interval [days]	Lysozyme [%]		
	5 °C	20 °C	30 °C
0	4.30 ± 0.41	4.30 ± 0.41	4.08 ± 0.35
7	4.04 ± 0.72	3.78 ± 0.46	3.69 ± 0.14
14	3.73 ± 0.35	3.82 ± 0.41	3.14 ± 0.23
21	3.68 ± 0.40	3.50 ± 0.37	3.05 ± 0.22
28	3.65 ± 0.50	3.62 ± 0.39	2.72 ± 0.11
35	3.41 ± 0.23	3.40 ± 0.39	2.59 ± 0.09
42	3.13 ± 0.36	3.02 ± 0.50	2.10 ± 0.10

Values are means ($\pm$ SD, $n = 8$ at 5 and 20 °C, $n = 3$ at 30 °C).

The reason for this decline may be due to the degradation of SS-bonds in alkaline pH or the formation of a dimer which has no lytic activity [23]. In HPLC (Figure 1) an increase of the peak area just before the lysozyme peak was observed. The isolation of the substance under this peak was not successful. Therefore it is not clear, if it is a lysozyme degradation product or not.

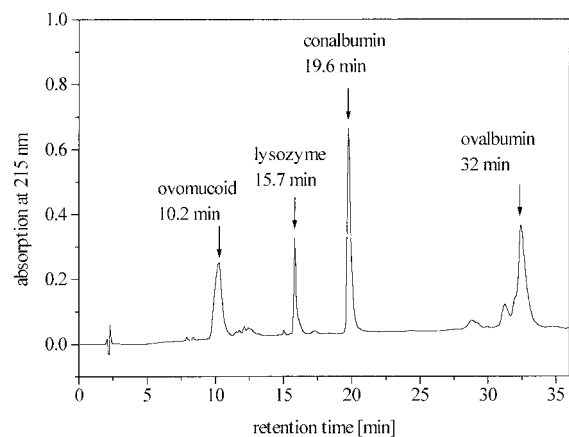


Figure 1. Separation of albumen of fresh eggs on a C₄-column. (Eluent from 17.5–77.5% acetonitrile in 55 min, flow rate 1 ml/min).

The specific lysozyme activity in the albumen varies from 29.500 to 46.700 U/mg (Table 2). No significant changes were observed during storage at pH 7.0 at which the test was performed. The pH in the albumen increases to values higher than 9.6 during storage of eggs. The specific activity at this pH was not investigated but according to the literature it is drastically reduced [24]. The results imply that the lysozyme activity is not irreversibly damaged during the storage procedure.

Table 2. Specific lysozyme activity of albumen at pH 7 during storage of eggs at different temperatures.

Storage interval [days]	Specific lysozyme activity [U/mg]		
	5 °C	20 °C	30 °C
0	$30\,800 \pm 4\,300$	$30\,800 \pm 4\,300$	$34\,700 \pm 1\,900$
7	$31\,000 \pm 2\,300$	$32\,100 \pm 4\,600$	$33\,800 \pm 800$
14	$35\,200 \pm 3\,700$	$35\,100 \pm 2\,300$	$40\,300 \pm 1\,200$
21	$37\,100 \pm 4\,500$	$36\,700 \pm 6\,900$	$46\,700 \pm 10\,500$
28	$35\,700 \pm 3\,900$	$31\,700 \pm 2\,600$	$29\,500 \pm 500$
35	$32\,300 \pm 4\,100$	$32\,900 \pm 3\,800$	$36\,300 \pm 1\,500$
42	$39\,000 \pm 2\,100$	$41\,700 \pm 6\,900$	$31\,100 \pm 1\,100$

Values are means ($\pm$ SD, $n = 8$ at 5 and 20 °C, $n = 3$ at 30 °C).

3.2 Conalbumin content

The conalbumin content in fresh eggs assessed via HPLC was $14.8 \pm 1.74\%$ of the total protein content (Table 3). It has been reported that conalbumin content in hen egg albumen varied between 12 and 21% [25, 16]. The conalbumin content altered during storage but did not show significant changes between the temperatures. The binding capacity of conalbumin for iron ions did not change during storage. Therefore, the calculated quotient of the conalbumin amount determined by HPLC and binding capacity for iron ions (Table 4) varied slightly during storage, but did not exhibit a tendency to increase, which would imply a reduced binding capacity. A value below 1.0 indicates that the application of the method of iron binding capacity leads to higher calculated amounts than the HPLC method.

Table 3. Conalbumin content of the total protein during storage of eggs at different temperatures.

Storage interval [days]	Conalbumin content [%]		
	5 °C	20 °C	30 °C
0	14.8 ± 1.7	14.8 ± 1.7	13.9 ± 1.5
7	14.0 ± 1.6	13.3 ± 1.7	12.6 ± 1.0
14	13.8 ± 1.3	13.9 ± 1.6	13.4 ± 1.8
21	13.8 ± 1.3	14.7 ± 1.1	13.2 ± 0.0
28	14.1 ± 1.3	14.4 ± 1.4	11.3 ± 0.5
35	13.1 ± 1.2	14.2 ± 0.9	11.3 ± 1.0
42	10.8 ± 0.8	12.5 ± 0.6	10.2 ± 0.4

Values are means ($\pm$ SD, $n = 8$ at 5 and 20 °C, $n = 3$ at 30 °C).

Table 4. Quotient of conalbumin content determined via HPLC and iron ion binding capacity during storage of eggs at different temperatures.

Storage interval [days]	Conalbumin quotient		
	5 °C	20 °C	30 °C
0	0.94 ± 0.10	0.94 ± 0.10	0.86 ± 0.06
7	0.85 ± 0.09	0.81 ± 0.08	0.78 ± 0.03
14	0.93 ± 0.05	0.96 ± 0.09	0.95 ± 0.06
21	0.87 ± 0.04	0.93 ± 0.05	0.96 ± 0.05
28	0.89 ± 0.05	0.87 ± 0.12	0.82 ± 0.05
35	0.83 ± 0.09	0.90 ± 0.07	0.82 ± 0.07
42	0.68 ± 0.07	0.86 ± 0.04	0.74 ± 0.01

Values are means ($\pm$ SD, $n = 8$ at 5 and 20 °C, $n = 3$ at 30 °C).

3.3 HPLC chromatography of albumen proteins

The albumen proteins ovomucoid, lysozyme, conalbumin and ovalbumin were separated and identified by HPLC on a C₄-column. The following retention time were determined: ovomucoid 10.2 min, lysozyme 15.7 min, conalbumin 19.6 min, ovalbumin 32 min (Figure 1). Ovalbumin was not eluted as one peak, but showed in fresh egg white two peaks at 31.2 min and 32.5 min with a shoulder at 32.0 min (Figure 2). The percentage of the second peak was about 70% of the total peak area. During storage this peak decreased while the peak area of the shoulder at 32 min increased. At a storage temperature of 5 °C this process slowed down. After two weeks of storage (Figure 2) the peak area decreased to about 35% and became only a shoulder after four weeks of storage. At temperatures of 20 and 30 °C the process was much faster, with the result that at 20 °C the peak was reduced to a shoulder after 2 weeks of storage. At 30 °C it had totally vanished after the first week. The shoulder at 32 min increased at storage temperatures of 5 and 20 °C. At 5 °C it

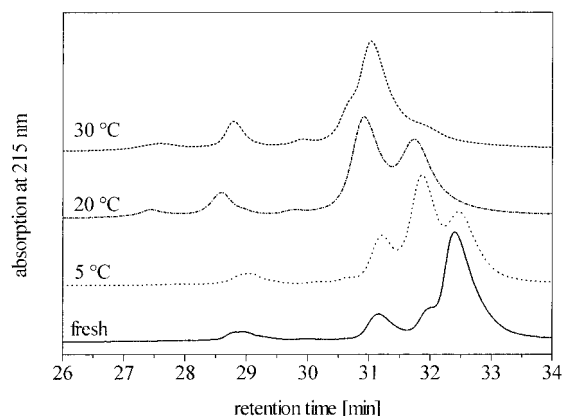


Figure 2. Albumen separation of fresh eggs and eggs stored for two weeks at 5, 20 and 30 °C on a C₄-column. (Eluent from 17.5–77.5% acetonitrile in 55 min, flow rate 1 ml/min).

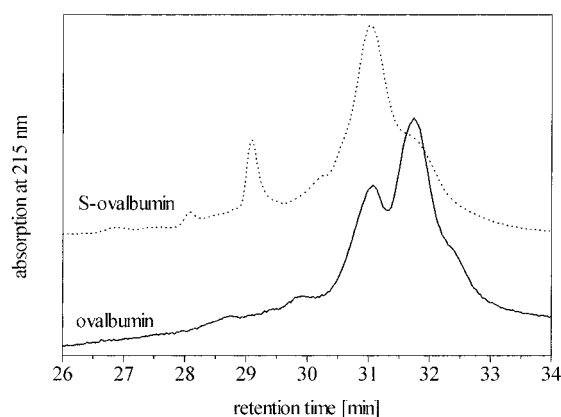


Figure 3. Ovalbumin and S-ovalbumin separation on a C₄-column. (Eluent from 17.5–77.5% acetonitrile in 55 min, flow rate 1 ml/min).

reached a maximum within two weeks, at 20 °C within a period of one week and then decreased in both cases. During storage at an elevated temperature of 30 °C this peak had disappeared during a period of three weeks. The peak at 31.2 min constituted the maximum peak after one week of storage at 30 °C and a new peak at 28.5 min developed. This implies a reduction of retention time during storage, because of a weaker interaction between ovalbumin and the reversed phase of the HPLC column. A comparison with ovalbumin (free of S-ovalbumin) reference substance revealed, that the elution pattern of ovalbumin consisted of a maximum peak at a retention time of 32 min and a shoulder at 32.5 min (Figure 3). This indicates that it had not been isolated from fresh eggs or it has possibly been transformed during the isolation procedure. S-ovalbumin in comparison showed a maximum peak at 31.2 min and an additional peak at 28.5 min. The postulated structural changes in ovalbumin during storage indicates that the S-ovalbumin is less hydrophobic than ovalbumin. This is in accordance with the results of Nakamura [10], who observes a reduced surface hydrophobicity in S-ovalbumin.

3.4 IEF of ovalbumin

Ovalbumin was separated by isoelectric focusing into different bands with isoelectric points from pH 4.50 to 4.65 (Figure 4). The reason for the formation of several bands is the different degree of phosphorylation and based on different carbohydrate residues bound to the molecule. During storage the isoelectric points shifted to slightly more acidic values. The intensity of the

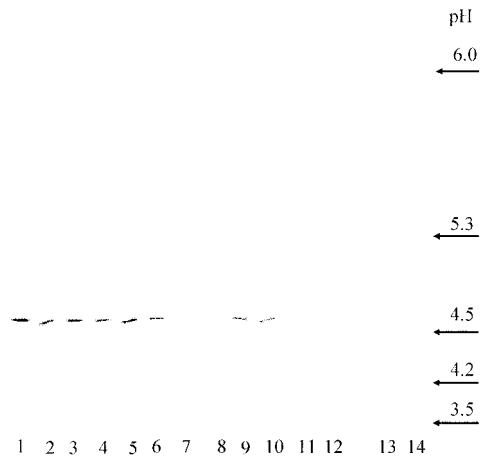


Figure 4. Separation of albumen on a IEF-gel (Servalyt Precotes pH 4–6).

(Silverstaining, sample lanes: (1) fresh eggs; stored eggs; (2) 1 week 5 °C; (3) 1 week 20 °C; (4) 1 week 30 °C; (5) 2 weeks 5 °C; (6) 2 weeks 20 °C; (7) 2 weeks 30 °C; (9) 3 weeks 5 °C; (10) 3 weeks 20 °C; (11) 3 weeks 30 °C; (12) 4 weeks 20 °C; (8) IEF marker proteins; (13) ovalbumin; (14) S-ovalbumin.

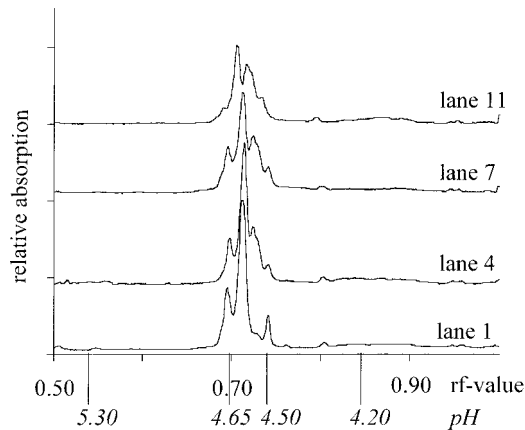


Figure 5. Densitogram of albumen separation on an IEF gel (shown in Fig. 4).

Sample lanes: (1) fresh eggs; (4) eggs 1 week 30 °C; (7) eggs 2 weeks 30 °C; (11) eggs 3 weeks 30 °C.

bands was measured after scanning and densitometric analysis of the gel (Figure 5). The band at pH 4.65 started to disappear gradually with storage time, whereas a new band at pH 4.55 appeared. This process was dependent upon the storage temperature. While at a temperature of 5 °C up to six weeks no band at pH 4.55 could be observed this band was formed after 2 weeks at a temperature of 20 °C and after one week at 30 °C. An increase of the band intensity at pH 4.65 occurred after three weeks at 30 °C, while at 20 °C only after 4 weeks. The comparison between isolated ovalbumin and S-ovalbumin showed a somewhat different pattern with a maximum band intensity not only at pH 4.60 but also at pH 4.65, while S-ovalbumin exhibited the same band intensity at pH 4.65 and 4.60. As the degree of phosphorylation and glycosylation in the isolated ovalbumin is unknown, the difference between the isolated protein and the raw albumen protein might be explained in this way. The same tendency of a shift of the isoelectric points to more acidic values could also be observed.

In conclusion the changes in albumen proteins during storage are more influenced by temperature than by the duration of storage. At a storage under refrigerated conditions practically no

deterioration processes in egg white can be observed, while at elevated temperatures of 30 °C even after a period of one week ovalbumin changes occurred. If refrigeration is recommended only after the 18th day of storage, changes could have taken place in the ovalbumin, which would have been prevented under refrigerated conditions from the start. Therefore, a regulation of the storage temperature at 5 to 8 °C from the very beginning should be recommended. This is preferable for hygienic reasons as well as for egg quality characteristics. Thus it would be possible to prove if appropriate storage conditions have been applied by using an ovalbumin index. Such an ovalbumin index could be a method in the future for the determination of the age of eggs.

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