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preliminary communication

Activity of Various Hydrolytic Enzymes in Chicken Egg-white during Egg Storage

*Tamara Tripić, † Ivan Škrtić and Ljubinka Vitale**

Dept. of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička c. 54, 10001 Zagreb, Croatia

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Summary

In order to obtain an insight into possible changes of proteins in chicken egg-white upon aging, its pH, protein concentration and activities of hydrolytic enzymes were followed during four months of eggs storage. In eggs kept after laying a rise of egg-white pH and a slow decrease of soluble proteins content, were observed. Activity of broad specificity aminopeptidase, glutamyl aminopeptidase and cholinesterase persisted during the whole storage period. A small but significant fall of the broad specificity aminopeptidase activity and a rise of activity of the other two enzymes, were late events of egg's aging. On the contrary, activity of egg-white N-acetyl-β-glucosaminidase had a steep decline and was completely lost after 30 days of storage. Thus, aminopeptidases and cholinesterase by their hydrolytic activity can influence a quality of eggs during their storage, whereas N-acetyl-β-glucosaminidase activity could be used as a marker of their freshness.

Keywords: chicken egg storage, egg-white, aminopeptidase, cholinesterase, *N*-acetyl-β-glucosaminidase

Introduction

Chicken eggs, though best when fresh, often have to be stored before consumption. Their quality upon this process can be influenced, not only by a possible microbial contamination, but also by changes due to chemical reactions and interactions of their constituents. As some of deteriorating changes, thinning of egg-white, weakening of yolk membrane and reactions of egg-white proteins with reducing sugars can be mentioned *(1,2)*. Among egg-white proteins there are various hydrolytic enzymes that could catalyze reactions in this egg-compartment. At the same time enzymes could be targets for other agents action. This makes their activity measurement a suitable approach to assess changes occurring during egg storage. Thus, early loss of egg-white *N*-acetyl-β-glucosaminidase was reported *(3,4)*. Lysozyme activity decrease was also reported *(5)*, whereas α-amylase, whose test has been recommended by FAO/ WHO for distinguishing pasteurized and unpasteurized egg products, did not change significantly in the stored eggs *(6,7)*. The data on other enzymes are not available. In this work a fate of two egg-white aminopeptidases *(8,9)*, cholinesterase *(10)* and *N*-acetyl-β-glucosaminidase during aging of unfertilized chicken eggs has been followed.

Materials and Methods

Eggs and egg-white samples

Fresh infertile eggs from Hissex-Brown breed were obtained from the local farm. They were stored at 12 °C and constant moisture (approx. 90%) and turned once a week. After different time intervals from day of laying (zero day) eggs were taken for analysis. Whites were quantitatively separated from yolks, groups of three whites were pooled and homogenized with 1.5 volumes of 0.9% aqueous NaCl (15 min, immersible mixer at 500 r.p.m. + ultrasound treatment for 5 min, 20 KHz s^{-1} on ice, MSE 100 W London, UK). The solution was clarified by centrifugation at 5000 *g* for 20 min, and supernatant used for pH, protein concentration and enzyme activity determination.

* Corresponding author: Tel: (01) 468 0115, E-mail: vitale@rudjer.irb.hr

pH was measured with Iskra MA 5705 pH meter equipped with a combination electrode (Iskra, Kranj, Slovenia).

Enzyme and protein assay

Aminopeptidase activity was determined by a colourimetric method as previously described, using 2-naphthylamides of amino acids as substrates *(9)*. The reaction mixture contained 50 mM Tris-HCl solution (pH = 7.5), 0.1 mM of leucine-2-naphthylamide (Leu-2NA) or methionine-2--naphthylamide (Met-2NA) and an appropriate amount of the sample. When glutamic acid-2-naphthylamide (Glu-2NA) was the substrate, 5 mM $CaCl_2$ was also present in the reaction mixture. After 30 min of incubation the reaction was stopped by addition of Fast Blue B salt reagent, and the absorbance was measured at 530 nm (PyeUnicam SP8-100 spectrophotometer). A calibration curve was prepared with freshly crystallized 2-naphthylamine. Substrates and other chemicals were products of Serva, Heidelberg, Germany.

Cholinesterase activity was determined according to Ellman *et al. (11)* in the reaction mixture containing 0.1 M sodium-phosphate buffer (pH = 7.4), 5 mM S-butyrylthiocholine iodide (Merck, Darmstadt, Germany) and egg-white sample. After 30 min of incubation, the reaction was stopped by the addition of 5 mM 5,5'-dithio-2--nitrobenzoic acid (Sigma, St. Louis, Missouri, USA) solution in the same buffer (0.1 mL/mL), the mixture was clarified by centrifugation (10 min at 18000 *g*) and absorbance was measured at 405 nm. The enzyme units were calculated with absorptivity of ε = 13.6 mM cm^{-1} for the formed 5-thio-2-nitrobenzoate.

Activity of *N-acetyl-β-glucosaminidase* was determined according to Winn and Ball *(4)*. The reaction mixture, containing 25 mM sodium citrate buffer (pH = 4.4), 5 mM p-nitrophenyl-*N*-acetyl-β-glucosaminide (Serva, Heidelberg, Germany) and egg-white sample, was incubated for 30 min, then the same volume of 0.2 M sodium-carbonate was added, the solution clarified by centrifugation and absorbance was measured at 430 nm. An absorptivity for the liberated *p*-nitrophenol of ε = 10.75 mM cm^{-1} was used for calculations.

All reactions were carried out at 37 °C in a water bath shaker, and were linear under the conditions used for an assay. One enzyme unit was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per minute.

The *protein* content was assayed by the method of Bradford *(12)* using bovine serum albumin (BSA) as a standard.

Several consecutive experiments were carried out and each analysis was performed in duplicate. A natural variability was determined by analyzing separately 10 one-day-old eggs or 5 three-eggs-pools after 1, 30 and 60 days of storage. For evaluation of activity changes upon storage the Student *t*-test was applied, and when the probability value (P) was below 0.05, the obtained mean was considered to be significantly different from that determined for the one-day old egg.

Results

Measurement of pH in egg-whites revealed its increase for one pH unit (approx. pH = 8.5 to pH = 9.5) during the first 30 days of egg storage, and a slow decline thereafter (Fig. 1). Protein concentration, regardless of the obtained dissipation of values, has shown a tendency to decrease with time. Correlation coefficient calculated by linear regression analysis of results obtained in four consecutive experiments was r = –0.8748 (Fig. 2).

The presence of a broad specificity aminopeptidase and glutamyl aminopeptidase in the egg-white was established *(8,9)*, therefore aminopeptidase activity was followed using three substrates: Met-2NA, Leu-2NA, and Glu-2NA. Activity curves towards Met- and Leu-2NA, (Fig. 3) had almost the same shape. (Met-AP versus Leu-AP activity correlation was r = 0.8772). After a rise at day 7 and 28, they had a tendency to decrease, and became significantly lower after 40 days of storage. Egg-whites from the group of fresh eggs were less active towards Glu-2NA. This aminopeptidase activity did not follow a particular pattern of changes, except a tendency to increase after three months (Fig. 4). When the activiti-

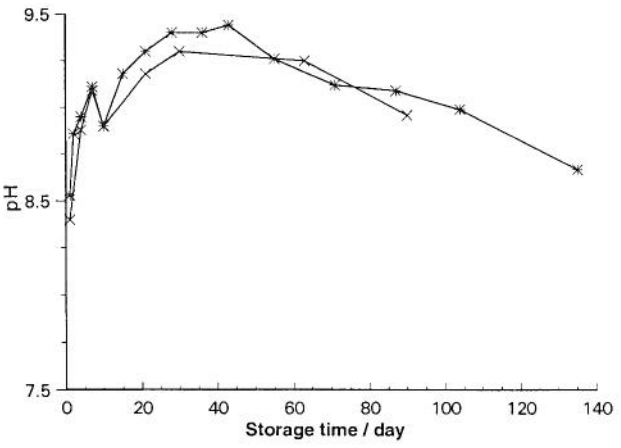

Fig. 1. pH of egg-white from eggs stored for different time intervals. Results of two consecutive experiments are presented.

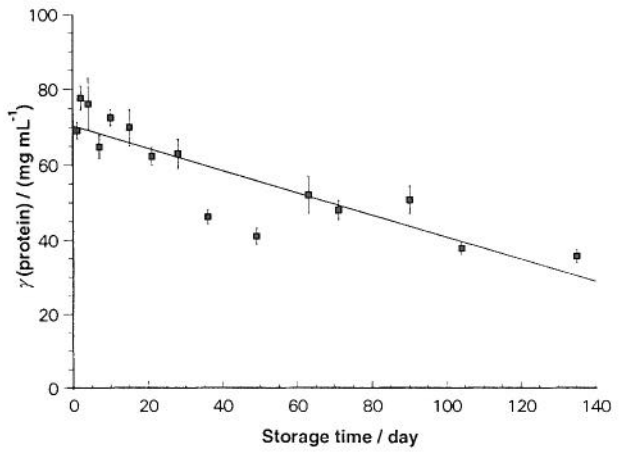

Fig. 2. Protein content of egg-white from eggs stored for different time intervals. Results obtained in four consecutive experiments are presented as mean ± standard error.

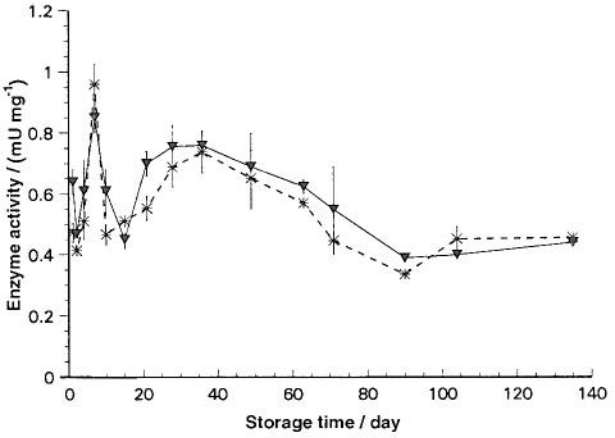

Fig. 3. Hydrolytic activity of egg-white from eggs stored for different time intervals: aminopeptidase determined with methionine-2-naphthylamide (four consecutive experiments) and with leucine-2-naphthylamide (three consecutive experiments) as substrates. Results are presented as mean ± standard error. (▼Leu-2NA, * Met-2NA)

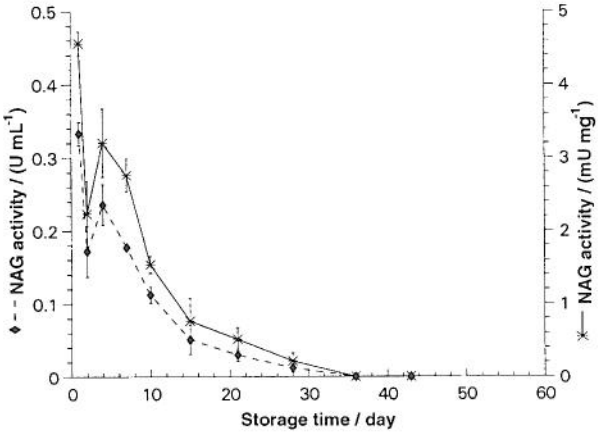

Fig. 5. *N*-acetyl-β-glucosaminidase (NAG) activity of egg-white from eggs stored for different time intervals. Results obtained in three consecutive experiments are presented as mean ± standard error.

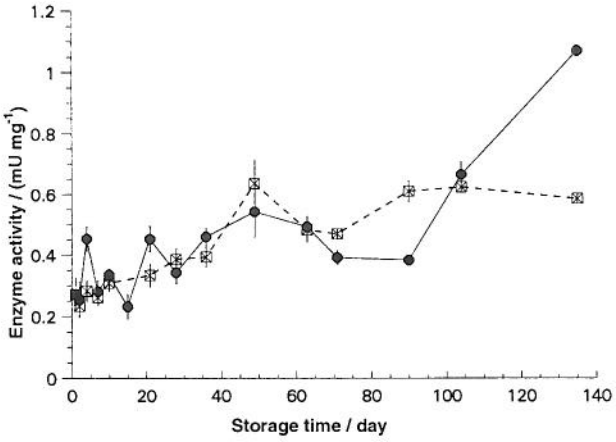

Fig. 4. Hydrolytic activity of egg-white from eggs stored for different time intervals: aminopeptidase determined with glutamic acid-2-naphthylamide as substrate and cholinesterase (ChE). Results obtained in four consecutive experiments are presented as mean ± standard error. (● Glu-2NA, ⊠ ChE)

es towards all three substrates were expressed per mL of egg-white the same results were obtained.

Cholinesterase activity per mL of egg white determined with butyrylthiocholine did not vary significantly during the storage period. When expressed per mg of protein an increase was observed after 40 days of storage (Fig. 4).

On the contrary, *N*-acetyl-β-glucosaminidase activity was reduced by half after 10 days, and was completely lost after 20-30 days of storage (Fig. 5) irrespective of the way of its expression. Decrease of enzyme activity followed an exponential curve.

Discussion

Being simultaneously possible targets and causes of reactions that affect egg-white proteins, hydrolytic enzymes could be considered as likely indicators of changes occurring in stored eggs. Follow up of pH, proteins and activity of four hydrolases during 4 months period, has revealed a change of the first two parameters and different behaviour and fate of the examined enzymes.

The rise of pH in chicken egg-white found during the first month after laying is in the agreement with observations made by Winn and Ball *(4)*. As previously suggested, it most probably contributes to the chemical modifications of egg-white proteins *(13)*. The fall of soluble proteins content during egg storage could be explained by their denaturation and partial hydrolysis. However, from egg-white only two aminopeptidases have been described *(8,9)*. Proteinases, that have been found in egg-yolk, could have escaped detection in egg-white due to the presence of high amounts of proteinase inhibitors *(14)*. The present study has revealed that aminopeptidase activities persist during egg storage. Moreover, comparison of curves presenting aminopeptidase activity expressed per mg of protein and per mL of egg-white, has shown that these enzymes are more stable in relation to other proteins. Pattern of aminopeptidase activity changes suggests that Met-2NA and Leu-2NA hydrolysis is catalyzed by the same enzyme, which is in accordance with the presence of a broad specificity aminopeptidase (AP M) in the chicken egg-white *(8)*. For its activity increase, observed reproducibility on day 7 and 21–27, there is no clear explanation. It could be ascribed to release of the enzyme from membrane fragments originating from oviduct or from an endogenous inhibitor, but this remains to be proved. Activity towards Glu-2NA originates from another enzyme, glutamyl aminopeptidase *(9)*.

Cholinesterase is the most abundant enzyme of egg-yolk, but its low activity has been detected in egg-white as well *(10,15)*. This activity was present during the whole storage period. Its slow increase after 40 days might reflect a diffusion of the enzyme from yolk due to the weakening of yolk-sac membrane.

The enzyme that has undergone a drastic change upon aging was *N*-acetyl-β-glucosaminidase. As it is a classical lysozomal enzyme, a disappearance of its activity from egg-white, is most probably a result of the enzyme inactivation at higher pH.

The described data suggest that aminopeptidases and cholinesterase activities could influence the quality of stored eggs, but obviously are not suitable as markers of their freshness or age. On the contrary, degradation of polysaccharide components by *N*-acetyl-β-glucosaminidase would not proceed in egg-white. However, determination of this enzyme activity can be applied to estimate how long eggs have been stored. The assay, if performed in a semiquantitative mode, does not require instrumentation and can be used »in field«.

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Aktivnost nekih hidrolitičkih enzima u bjelancu kokošjeg jajeta tijekom pohrane jaja

Sažetak

Da bi se dobio uvid u moguće promjene proteina bjelanca za vrijeme starenja kokošijih jaja, praćeni su pH, koncentracija proteina i aktivnost hidrolitičkih enzima bjelanca tijekom četiri mjeseca nakon nesenja jaja. U bjelancu pohranjenih jaja opažen je porast pH i spori pad količine topljivih proteina. Aktivnosti aminopeptidaze široke specifičnosti, glutamil aminopeptidaze i kolinesteraze bile su prisutne u bjelancu tijekom cijelog razdoblja pohrane. Mali, ali signifikantan pad aktivnosti aminopeptidaze široke specifičnosti i povećanje aktivnosti drugih dvaju enzima opaženi su u kasnim fazama ispitivanog vremena starenja. Nasuprot tome, aktivnost N-acetil-β-glukozaminidaze naglo je padala i potpuno nestala nakon 30 dana pohrane. Stoga se može zaključiti da aminopeptidaze i kolinesteraza svojim djelovanjem mogu utjecati na kakvoću pohranjenih jaja, a da bi se aktivnost N-acetil-β-glukozaminidaze mogla koristiti kao biljeg za procjenu svježine jaja.