Characterization of the Exudate from Cooked Shell Eggs

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Boiled and peeled eggs stored in Cryovac bags at 5°C produced a greenish-yellow fluorescent exudate. The amount of exudate increased from 1.26% at 1 day to 2.7% at 7 days, with no further increase on storage. The freeze-dried exudate contained 61% protein, 21.25% carbohydrate, and 8.9% minerals. Chromatography of the exudate on Sephadex G-50 produced five fractions. The first two fractions, based on biological activity and chemical analysis, appeared to be ovomucoid-like. Fractions 3 and 4 contained large amounts of carbohydrate but their exact nature could not be established. Fraction 5 was fluorescent and appeared to be a component of the flavoprotein moiety in albumen.

A greenish-yellow liquid, the exudate, accumulates on refrigerated storage of hard-cooked eggs packaged in Cryovac bags. This is an important industrial problem, as the manufacture and distribution of boiled, peeled eggs is becoming popular at the institutional level due to labor costs and at retail levels due to convenience. As to the nature of this exudate and the methods of its prevention or control, no information is available in literature. This study was undertaken to elucidate the chemical nature of the exudate.

MATERIALS AND METHODS

Cooking and Packaging Eggs. One-day-old eggs from a single strain of White Leghorn chickens were cooked in a thermostatically controlled water bath at 93 ± 1°C for 20 min. Following heating, the eggs were chilled for 5 min in ice water (1–2°C) containing 200 ppm of available chlorine and were hand-peeled. Six to eight eggs were placed in a Cryovac bag and packaged under vacuum. The bags were placed in boiling water for 5 min and were stored at 5°C for 1 week. The cooking time and temperature and other procedures used in this study were similar to those used in the industry.

Chemical Analysis. Protein, bound carbohydrate, and hexosamine in the exudate were determined according to the procedures of Gornall *et al.* (1949), Tillmans and Philippi (1929), and Boas (1953), respectively. Reducing sugar was determined by the procedure of Nelson (1944). The total sialic acid content in the exudate was determined in the hydrolyzed (0.05 *N* H_2SO_4 at 80°C for 1 hr) samples by the thiobarbituric acid procedure of Warren (1959). Tryptophan in the exudate fractions was determined according to the procedure of Gaitonde and Dovey (1970).

Gel Filtration. Sephadex G-50 (fine) was used for column chromatography of egg exudate. Sephadex was suspended in 15–16 vol of 0.5 *M* acetic acid and allowed to swell for 24 hr. Fines were removed by decanting the supernatant several times. The gel suspension was poured into a vertically mounted column (2.5 × 37.5 cm) filled one-third with 0.5 *M* acetic acid. The column was packed at a pressure head of 50 cm and equilibrated with 0.5 *M* acetic acid overnight. Three milliliters of egg exudate was passed through a Millipore filter (0.45 μ) and was applied on the dried bed surface of the column. The eluate was monitored through an Isco-automatic recorder and 4-ml fractions were collected. The material under each peak was freeze-dried.

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Electrophoretic Analysis. The electrophoresis was performed using a 7.5% polyacrylamide gel in tris-borate buffer at pH 8.9. The gels were stained with 0.2% amido black and destained with 7% acetic acid.

Trypsin Inhibitor Assay. Trypsin assay with casein substrate was performed essentially by the procedure of Sale *et al.* (1957). One milliliter (100 μg/ml) of a bovine trypsin solution (crystalline, Sigma) was mixed with an equal volume of 0.1% total exudate or exudate fractions dissolved in 0.1 *M* phosphate buffer, pH 7.4. The mixture was incubated at 25–26°C for 30 min, followed by the addition of 2 ml of 1% casein solution in phosphate buffer at pH 7.4. The samples were incubated for an additional 30 min at 35°C. The reaction was stopped at the end of the incubation period by adding 5 ml of 5% trichloroacetic acid (TCA). Reference blanks were prepared similarly except for the incubation at 35°C. The TCA precipitate was removed by filtration (Whatman No. 42) and the absorption of the supernatant was measured at 280 nm. The control represents the activity of crystalline trypsin on 1% casein solution. Antitrypsin units were calculated by the following equation.

$$\text{antitrypsin units} = 100 - \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{absorbance of casein} - \text{absorbance of blank}} \times 100$$

Spectral Analysis. The freeze-dried material was dissolved in distilled water (0.5% solution) and the spectral analysis was performed using a Beckman DB recording spectrophotometer.

RESULTS AND DISCUSSION

Rate of Accumulation of Exudate. The data on accumulation of egg exudate are shown in Table I. The exudate appeared within 1 day of storage at 5°C and reached a level of 2.56% within 4 days. The amount increased slightly and leveled off after 7 days of storage.

Chemical Composition. The egg exudate was analyzed for gross chemical composition and the results are summarized in Table II. The dried exudate is a proteinaceous material containing large amounts of carbohydrate. The exudate on boiling showed no coagulation and no turbidity was observed over the entire pH range except for pH 5.2–5.5. There was no distinct precipitate with 5 or 10% TCA; however, the exudate resulted in a heavy precipitate with 3 vol of chilled acetone at pH 5.2–5.5. Electrophoretic analysis of the exudate showed a smear with pronounced staining on the edges between the area corresponding to post-albumin and G-3 region of an electrophoretogram of fresh egg albumen. This electrophoretic behavior and the chemical analysis

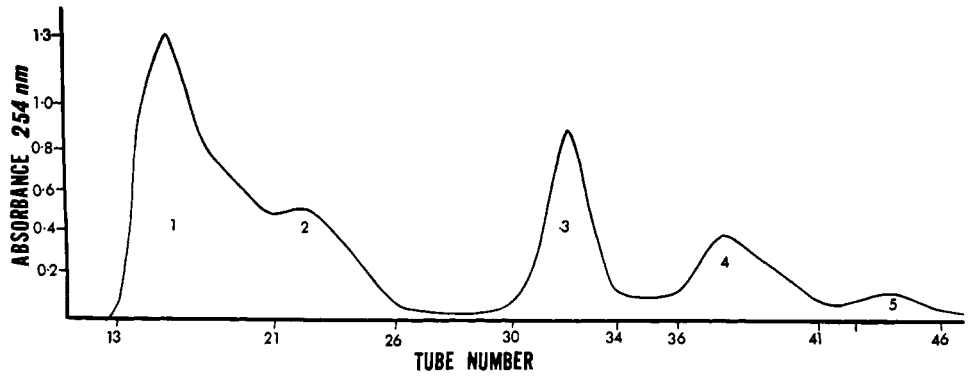

Figure 1. Chromatographic pattern of egg exudate on Sephadex G-50, eluent 0.5 *M* acetic acid

Table I. Rate of Accumulation of Egg Exudate during Storage in Cryovac Bags at 5 °C

Storage time, days	Amount of exudate, % of egg weight
1	1.26
4	2.56
7	2.70
14	2.70

Table II. The Gross Chemical Composition of Freeze-Dried Egg Exudate from Eggs Stored for 1 Week at 5 °C

Description	Percent
Moisture	8.61
Protein	61.00
Carbohydrate (total)	21.25
Sialic acid	0.35
Ash	8.91

would indicate that the exudate contains ovomucoid or an ovomucoid-like moiety (Baker and Manwell, 1962).

Gel Chromatography. Chromatography of the exudate on Sephadex G-50 showed five peaks (Figure 1). The carbohydrate, hexosamine, protein, and sialic acid contents of the freeze-dried fractions are presented in Table III. It appears that the fractions 1 and 2 conform more to the composition of ovomucoid than do fractions 3 and 4. This apparent heterogeneity of the exudate would indicate the presence of other components of egg proteins. Fractions 1 and 2 did not contain tryptophan and their carbohydrate analysis was similar to that of pure ovomucoid (Bragg and Hough, 1960).

Antitryptic Activity. Lineweaver and Murray (1947) identified ovomucoid as the heat-stable trypsin inhibitor in egg white. Its relatively high heat stability was further confirmed by the work of Deutsch and Morton (1956) and Stevens and Feeney (1963). The antitryptic activity of five fractions of the exudate was determined and the data are shown in Table IV. Fractions 1 and 2 showed the greatest amount of antitryptic activity. These results appear similar to those of Rhodes *et al.* (1960), who obtained three ovomucoid fractions with antitryptic activity for egg white fractionated on carboxymethylcellulose. Fractions 3 and 4 were devoid of antitryptic activity and the carbohydrate in these fractions was in the reducing form, indicating that it may be originating from free glucose or some degradation of the carbohydrate-rich moieties in the albumen. However, the precise origin of fractions 3 and 4 is not yet known.

Table III. Chemical Analysis of Various Egg Exudate Fractions

Fraction	Protein, %	Carbohydrate: Unhydrolyzed (reducing sugar[a]), %	Carbohydrate: Hydrolyzed (total), %	Hexosamines, %	Sialic acid, %
1	84.3	4.9	15.0	11.05	0.405
2	91.5	4.0	13.0	10.92	0.394
3	0.004[b]	14.0	14.0	1.38	0.240
4	14.3	34.0	34.0	0.0	0.220
5	n.d.	n.d.	1.0	0.0	n.d.

[a] Reducing power expressed as % of hexose. n.d. = not determined. [b] Samples insoluble at neutral/alkaline pH.

Table IV. The Antitryptic Activity of Egg Exudate and Its Fractions on Casein

Fraction	Antitryptic activity, units: per ml sample	Antitryptic activity, units: per mg protein
1	100	118
2	100	109
3	2.0	17
4	2.0	17
5	n.d.	...
Total exudate	99.6	163

$$\text{Antitrypsin units} = 100 - \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{absorbance of casein} - \text{absorbance of blank}} \times 100.$$

Spectral Analysis. The exudate was yellow-green and fluoresced in ultraviolet light. Spectral analysis showed absorption maxima at 275 and 442 nm. These results indicate the presence of riboflavin as well as the flavoprotein system of the egg albumen (Rhodes *et al.*, 1959).

Pure riboflavin absorbs maximally at 445 nm, and the absorption is quenched by the addition of sodium hydrosulfite (Rhodes *et al.*, 1959). The peak at 442 nm in the exudate may be due to the presence of riboflavin, and this was further indicated by the addition of sodium hydrosulfite (Figure 2).

Rhodes *et al.* (1959) reported that the absorption maxima of egg flavoprotein was 276 nm. Fraction 5 in this study had maximum absorption at 275 nm, which would indicate the presence of a flavoprotein component; however, this fraction had a negligible amount of protein, thus indicating that it may be a degradation product of flavoprotein rich in fluorescent material.

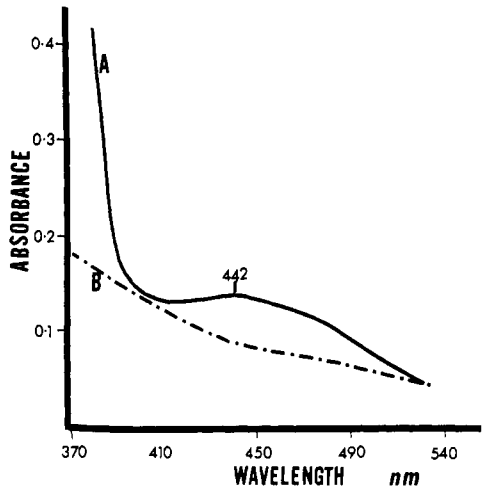

Figure 2. Spectral analysis of egg exudate with and without sodium hydrosulfite (A = egg exudate; B = reduced egg exudate)

Thin-layer chromatographs of fractions 4 and 5 on cellulose layers were developed with 1-butanol–acetic acid–water (4:1:5) and these showed one fluorescent spot each, with R_f values of 0.24 and 0.25, respectively. Pure riboflavin had an R_f value of 0.46 in the same solvent system. These differences in R_f values may be due to the conjugation of riboflavin with other components in fractions 4 and 5.

The exact identity of fraction 4 could not be established by spectral analysis, as the patterns did not agree with the absorption maxima either for riboflavin or flavoprotein.

In conclusion, it appears that the egg exudate is a heterogenous mixture of at least five moieties, as determined by gel filtration. Fractions 1 and 2, on the basis of their biological activity and chemical analysis, would appear to be similar to ovomucoid in the native egg albumen. The exact nature of fractions 3 and 4 could not be fully established, although their analysis would indicate that these are derived from carbohydrate-rich albumen moieties, while fraction 5 appears to be a degradation product of egg flavoproteins.

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Improved Method for Determination of Sulfur-35 in Plant Material Using Oxygen Flask Combustion and Liquid Scintillation Counting

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A safe oxygen flask combustion procedure is described for the preparation of ^{35}S-labeled plant material for liquid scintillation counting. Hazards associated with flammable absorbing solutions are eliminated by using deionized water to trap the sample combustion products from the oxygen flask. Sulfur-35 activity in the resulting aqueous solution is then determined in a 1:1:2 mixture of toluene, Triton X-100, and water with an efficiency of 73%. With a 3-l. oxygen flask and a 20-min absorption period, the relationship between sample size and activity recovered was linear up to a sample weight of 1.2 g. Recoveries of ^{35}S added to plant material as sodium sulfate-^{35}S or cysteine hydrochloride-^{35}S were 100.0 ± 2.3% and 99.1 ± 1.2%, respectively. Using five combustion flasks and an automatic liquid scintillation counter, a single operator can process about 50 samples per day.

Oxygen flask combustion offers a convenient means of oxidizing biological materials prior to the estimation of stable or ^{35}S-labeled sulfur (MacDonald, 1961). Since the operation is conducted in a closed system, the volatile losses of sulfur which can occur with wet or dry ashing of samples in open vessels are largely prevented (Beaton *et al.*, 1968).

For the estimation of ^{35}S, it has been usual to trap the combustion products in absorbing solutions similar to those used when estimating ^{14}C (Kalburer and Rutschmann, 1961; Dobbs, 1963). However, the presence of flammable solvents such as methanol, ethanol, and toluene in these solutions greatly increases the fire and explosion hazard of the method. Various procedures have been used to minimize these hazards. Kalburer and Rutschmann (1961) added the trapping solution to the flask before sample ignition but chilled the solution in a Dry Ice–acetone bath before and during sample combustion. Others have avoided the hazard by admitting the trapping solution to the flask only after the combustion had finished (Martin and Harrison, 1962; Dobbs, 1963). However, to introduce a solution at this stage, the internal pressure of the flask must be lowered, either by a lengthy chilling period (Martin and Harrison, 1962) or through the use of elaborate

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