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Original research article

The efficacy of albumen from different egg sources to generate thermally denatured protein and their IC_{50} in an anti-inflammatory assay

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ABSTRACT

Hen egg albumen is commonly used for *in vitro* anti-inflammatory assay by thermal protein denaturation. There are many sources of albumen such as from eggs of quail, pigeon, hen, duck, goose, and crocodile. Thus, this study aims to investigate alternative sources of albumen that can be used for this assay. Albumen specimens collected from quail, pigeon, hen, duck, goose, and crocodile eggs were employed for investigating protein denaturation by turbid formation after 5 min incubation at 70ºC and 85ºC, following standard protocol. The amount of protein of each albumen was determined spectrophotometrically at 280 nm using bovine serum albumin as standard protein. Then, albumen exhibiting a turbidity response similar to that of hen egg albumen was selected to determine anti-inflammatory activity using diclofenac sodium as a positive control. Our results show that only quail egg albumen became turbid after being heated at 70ºC for 5 min in a way similar to hen albumen, with absorbance values of 1.341 ± 0.006 and 1.134 ± 0.016 Absorbance units (AU), respectively. The other egg albumen specimens showed low turbidity values of between 0.013-0.175 AU. After increasing the incubation temperature to 85ºC, the turbidity of quail, duck, and goose egg albumen specimens was increased. The least amount of protein was evident for crocodile egg albumen (5.64 ± 0.39 mg/0.2 ml of albumen), while the other sources contained 26.4-31.7 mg/0.2 ml of albumen. There was no relation found between the turbidity and amount of protein except for crocodile egg albumen. The IC_{50} values for hen and quail egg albumens were 1092 and 625 µg/ml, respectively. This indicated that quail egg albumen exhibited higher sensitivity for thermal protein denaturation than hen egg albumen. We propose that quail egg albumen, which is

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inexpensive and available in some countries, may be used instead of hen egg albumen for *in vitro* anti- inflammatory activity assay using the thermal protein denaturation inhibition technique.

Keywords*: in vitro,* anti-inflammatory, thermal protein denaturation, albumen

1. Introduction

There are various protocols used for searching anti- inflammatory compounds depending on their mechanism of action.[1,2] Inhibition of thermal protein denaturation is a well-known technique used to study anti-inflammatory activity *in vitro*.[2-7] The proteins used in this protocol are bovine serum albumin[3,4] or those contained in egg albumen.[5-7] Albumen contains about 54% ovalbumin, 12% ovotransferrin (conalbumin), 11% ovomucoid, and 18.8% other proteins.[8] There are many kinds of eggs available in Thailand. Different kinds of the egg have different protein concentrations. For example, hen, duck and crocodile eggs contain 11.49, 10.83, and 2.5 g/100 g protein per edible portion and this might cause different results using the standard anti-inflammatory activity protocol.[9] Some research articles describe using albumen of unknown origin,[2,5] others the use of fresh hen's albumen.[6,7] However, no research article describes the use of other animal sources of fresh albumen. Thus, it was the aim of our research to study the albumen from eggs of duck, goose, quail, pigeon, and crocodile that could be used instead of fresh hen egg albumen for *in vitro* anti-inflammatory activity assay using thermal protein denaturation inhibition. It is of interest that the albumen from different animal eggs may contain different amounts of proteins which could affect denaturation conditions.

2. Materials and Methods

2.1 Chemicals and reagents

Diclofenac sodium was kindly provided by T.O. Chemicals (1979) Ltd., Bangkok, Thailand. Hen, duck, and quail eggs were bought from a market in Nakhon Pathom province, Thailand. Pigeon eggs were from their nest in Silpakorn University, Nakhon Pathom, Thailand. Goose eggs were bought from a farm in Nakhon Pathom Province, Thailand. Crocodile eggs were provided by Sam-phran elephant ground and zoo, district of Sam-Phran, Nakon Pathom, Thailand. The photos of all kinds of eggs used in this research are shown in (Fig. 1). Potassium dihydrogen orthophosphate (lot no.: E23W60, Ajax Finechem, Australia) and sodium hydroxide (lot no. AF 310204, Ajax Finechem, Australia) were used as constituents in phosphate buffer. Bovine serum albumin 20 mg/ml (BSA, BioLab®, New England) was used to make a calibration curve for protein quantification.

2.2 Preparation of albumen

For each kind of egg, albumen was separated from the yolk. Three eggs of each hen, duck, goose, and crocodile albumen and 6 to 8 eggs of pigeon and quail albumen were pooled together before being used in the anti-inflammatory assay.

2.3 Testing of thermal protein denaturation with different kinds of albumen in the *in vitro* anti-inflammatory assay

A solution of 5 ml was prepared by mixing 2.8 ml phosphate-buffered saline (PBS) pH 6.4, 0.2 ml albumen protein from each fresh egg, and 2 ml double-distilled water. This mixture (solution A) was incubated at 37ºC for 15 min and then heated in a water bath at 70ºC or 85ºC for 5 min. The absorbance of the mixtures was measured at 660 nm using a UV-Vis spectrophotometer (Hitachi U-2000, COAX Group Corporation Ltd.) after cooling down to room temperature. Each sample was measured in triplicate. The mean ± standard deviation of absorbance was recorded and values were compared. Samples exhibiting absorbance values comparable to hen's albumen were tested with the positive control

(diclofenac sodium) to find the IC_{50} value of the anti-inflammatory activity. For this purpose, various concentrations of diclofenac sodium solutions were used instead of 2 ml of double- distilled water when preparing solution A. The percent inhibition was calculated with this formula: percentage inhibition = (Absorbance of negative control - Absorbance of sample) X 100/Absorbance of the negative control. The 50% inhibition concentration (IC_{50}) was calculated by using linear regression with Microsoft Excel.

2.4 Protein quantification of albumen

Ten microliters of solution A from the previously described experiment were diluted with 90 µl PBS (except for crocodile egg albumen, see below) and the absorbance was measured using a microvolume UV-Vis spectrophotometer (NanoDrop™ One/OneC, Thermo Fisher Scientific) at the wavelength of 280 nm. The sample volume per measurement was 3 µl. Measurements were made in triplicate. For crocodile egg albumen, undiluted solution A was used to determine the protein content in order to stay within optimal absorbance as calculated by the standard curve. The PBS was used as blank. The BSA standard curve was used for calculating the protein concentrations.

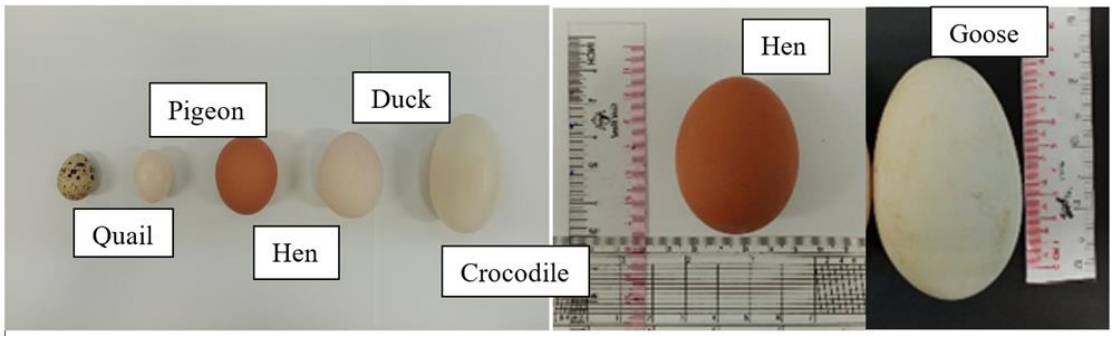

Fig. 1. Photos of the various kinds of eggs used in this experiment

3. Results

3.1 Testing of thermal protein denaturation with different kinds of albumen protein

Protein denaturation at 70°C and 85°C for 5 min, represented as turbidity (change of absorbance at 660 nm) of different kinds of albumen, is shown in (Table 1). The standard protocol using hen albumen resulted in a turbidity value of 1.134 ± 0.016 AU, which was close to the turbidity value of quail albumen of 1.341 ± 0.006 AU, after heating at 70°C. In comparison, albumen from eggs of pigeon, duck, goose, and crocodile turned less cloudy, with turbidity values of 0.123 ± 0.002, 0.175 ± 0.005, 0.172 ± 0.006, and 0.013 ± 0.001 AU, respectively. When heated at 85°C, the turbidity values of quail, duck, and goose egg albumen increased to 2.172 ± 0.012, 0.666 ± 0.021, and 0.623 ± 0.019 AU, respectively. However, the turbidity of hen, pigeon, and crocodile egg albumen changed little after heating at 85°C compared to 70°C.

3.2 Protein determination of albumen

The amount of protein in each albumen solution was calculated using BSA as standard protein. The standard curve of BSA is shown in (Fig. 2). The linear regression coefficient (R^2) was equal to 0.9997. The amount of protein was calculated as mg of BSA in 0.2 ml albumen which was equivalent to the amount of protein in the volume of albumen used per reaction in this assay. The concentrations of protein in each kind of albumen are exhibited in (Table 1). The result showed that the amounts of protein in duck,

goose, quail, hen, pigeon, and crocodile egg albumen were 31.7 ± 0.39, 28.1 ± 0.39, 27.3 ± 0.39, 26.7 ± 0.47, 26.4 ± 0.68, and 5.64 ± 0.39 mg/0.2 ml, respectively. The highest amount of protein content was evident in duck egg albumen whereas the lowest was found in crocodile egg albumen.

Table 1. Color of albumen, turbidity (absorbance) after heating at 70°C and 85°C, and protein concentration of albumen from different eggs.

Albumen from different eggs	Color of albumen	Absorbance 660 nm		Amount of protein (mg/0.2 ml of albumen) (n=3)
		70°C 5 min (n=3)	85°C 5 min (n=3)	
Hen	yellow	1.134 ± 0.016	1.242 ± 0.016	26.7 ± 0.47
Quail	yellow	1.341 ± 0.006	2.172 ± 0.012	27.3 ± 0.39
Pigeon	clear	0.123 ± 0.002	0.132 ± 0.003	26.4 ± 0.68
Duck	clear	0.175 ± 0.005	0.666 ± 0.021	31.7 ± 0.39
Goose	clear	0.172 ± 0.006	0.623 ± 0.019	28.7 ± 0.39
Crocodile	clear	0.013 ± 0.001	0.042 ± 0.002	5.64 ± 0.39

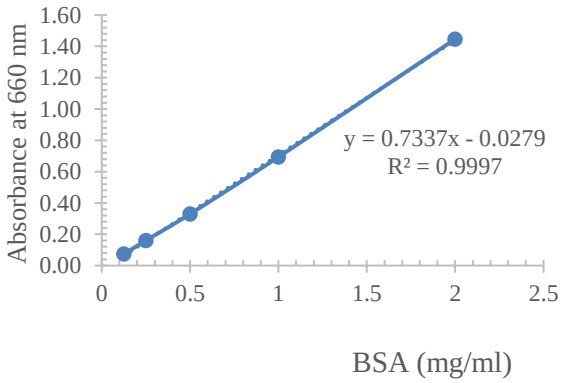

Fig. 2. Standard curve of bovine serum albumin for protein content analysis.

Table 2. Dose dependence of percentage inhibition, linear regression equation, and anti-inflammatory activity expressed as IC_{50} values using diclofenac sodium as a positive control in standard protocol thermal protein denaturation inhibition with hen and quail albumen.

Kind of eggs	Diclofenac sodium concentration (µg/ml)	%Inhibition (n=3)	Linear regression equation	IC_{50} values (µg/ml)
Hen	800	23.4 ± 0.97	y = 0.0962x - 55.1 R² = 0.996	1092
	1000	39.6 ± 0.67		
	1200	59.2 ± 0.55		
	1400	81.0 ± 0.27		
Quail	400	4.7 ± 0.55	y = 0.204x - 77.4 R² = 0.999	625
	500	23.6 ± 0.57		
	600	44.6 ± 0.54		
	700	67.2 ± 0.34		
	800	84.9 ± 0.81		

4. Discussion

Our results have shown that different kinds of albumen generate different heat-induced turbidity indicating different responses of protein denaturation under the same experimental conditions.

Previous reports have shown the percentages of crude protein of duck, goose, quail, hen, and pigeon albumen to be 10.7 ± 0.54, 8.5 ± 0.55, 10.6 ± 0.62, 10.8 ± 0.59, and 9.1 ± 0.66 %, respectively.[10] Thus, the trend of the protein amounts of egg albumen from duck, goose, quail, hen, and pigeon measured in this study was not apparently different from the previous report. The percentage of crude protein in crocodile egg albumen in this experiment was lower than the value reported previously to be about 2.5%.[9]

When analyzing the experimental results of protein content and turbidity after thermal decomposition of proteins, we found that the protein concentration did not correlate with turbidity, probably owing to the variety of amounts and compositions of proteins in the different kinds of albumen. There are many kinds of protein in albumen, such as ovalbumin, ovomucoid, ovotransferrin, ovomucin, lysozyme, flavoprotein, avidin, and others.[8] In addition, the denaturation temperature of each protein varies. The denaturation temperature of ovalbumin is 75-84ºC, that of ovomucoid 77ºC, for ovotransferrin it is 61-65ºC, and lysozyme denatures at 69-77ºC.[11] Hen egg albumen comprises 14% ovalbumin (heat- resistant) and 10% ovotransferrin (heat-sensitive protein), more than in duck egg albumen.[12] The low thermally induced turbidity of crocodile egg albumen may be due to its low protein content. Moreover, there was a relationship between the color of albumen and the turbidity after heating. In the case of the presence of a yellowish shade of albumen, the turbidity could be present when heating at 70ºC. Only the color of albumen samples from hen and quail eggs was yellowish and their turbidity could be observed after heating at 70ºC. However, there has been no other information to support this observation.

Quail egg albumen exhibited the same turbidity as hen egg albumen (standard protocol) after heating. Thus, both hen and quail egg albumen specimens were selected to determine the anti-inflammatory activity compared with the positive control, diclofenac. Diclofenac sodium is an anti-inflammatory drug commonly used as a positive control in the thermal protein denaturation anti-inflammation model.[2,13,14] The IC_{50} values of thermal protein denaturation inhibition for quail and hen egg albumen samples were 624 and 1092 µg/ml, respectively, as shown in (Table 2). The lower the IC_{50} value, the higher the sensitivity. Therefore, the albumen from quail egg appears to have a higher anti-inflammatory test sensitivity than that of hen egg.

Some factors affecting the denaturation of protein in albumen for this anti-inflammatory assay model are pH, salt, sugar additive, and freshness of the eggs. The lower the pH, the more turbidity was found after heating at a fixed temperature in hen egg albumen.[15] A decrease in heat used for ovalbumin denaturation was caused by an increase in NaCl concentration. However, the denaturation temperature of ovalbumin tended to shift higher when the sucrose concentration was increased.[16] Long-term storage induced the conversion of ovalbumin to S-ovalbumin up to 50%. S-ovalbumin is a more heat-stable form than ovalbumin causing a shift of protein denaturation temperature from 84.5ºC to 92.5ºC.[17] These factors must be controlled for good laboratory practice.

5. Conclusion

According to the standard protocol of thermal protein denaturation for the anti-inflammatory model using diclofenac sodium as positive control, quail egg albumen showed higher sensitivity than hen egg albumen. Moreover, quail eggs are widely available and not expensive in some countries. These findings indicate that quail

albumen exhibits the potential to be used as an alternative to hen albumen for *in vitro* anti-inflammatory activity assays.

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Conflicts of Interest

The authors declare no conflicts of interest.

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