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Comparative antibacterial activity of avian egg white protein extracts
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Abstract
1. Egg white proteins from the eggs of domestic chicken (Gallus gallus), turkey (Meleagris gallopavo), duck (Anas platyrhynchos) and goose (Anser anser) were analysed in order to compare the antimicrobial activity of these products.
2. Albumen from each species was sampled and analysed by SDS-PAGE and Western blotting. Antimicrobial activity and lysozyme activity were measured.
3. Ovotransferrin and ovalbumin were identified in all species while c-type lysozyme was present in chicken, turkey and duck egg white samples, but not in goose.
4. Galliformes appear to possess albumens with greater antimicrobial activity than those of the Anseriformes. This can be attributed to higher concentrations of ovotransferrin and the broad acting c-type lysozyme.

INTRODUCTION
Avian albumen is a complex multifunctional medium promoting the growth and development of the embryo. In the domestic chicken (Gallus gallus), the albumen is composed of 88% water and 10% protein (Burley and Vadehra, 1989). In addition to providing water and nutrients to the developing embryo, albumen prevents the growth of micro-organisms. The alkaline pH of egg white and the presence of antimicrobial proteins significantly reduce the growth of micro-organisms (Deeming, 2002).

Many albumen proteins, including cystatin (Saxena and Tayyab, 1997; Wesiarska et al., 2005), ovomacroglobulin (Miyagawa et al., 1991) and avidin (Board and Fuller, 1974), have been implicated in the antimicrobial defences of Gallus gallus egg white. Ovotransferrin, a major egg white protein, is considered to be the principal impediment to the growth of bacteria in the albumen of the hen’s egg (Seviour and Board, 1972). Ovotransferrin is composed of two domains that each reversibly bind iron, limiting the amount of this essential element and thereby inhibiting the growth of micro-organisms (Phelps and Antonini, 1975; Valenti et al., 1981). Large microbial populations form in albumen when supplemented with iron (Schade and Caroline, 1944; Seviour and Board, 1972). Lysozyme, or N-acetylmuramidaseglycanohydrolase, is another major egg white component (3-5% of chicken egg white protein). It is an enzyme that splits the bond between the glycosidic beta-1,4-linked residues of N-acetylneuramic acid (NAM) and N-acetylglucosamine (NAG) in the peptidoglycan structure of Gram-positive bacteria and to a lesser extent in some Gram-negative bacteria (Burley and Vadehra, 1989; Bera et al., 2005).

Many studies have investigated the antimicrobial properties of albumen from the domestic chicken. Raw hen egg white inhibits the growth of Staphylococcus aureus, Shigella dysenteriae, Escherichia coli and Saccharomyces cerevisiae (Schade and Caroline, 1944). Wang and Shelef (1991) reported that Listeria monocytogenes, strains Scott A and Brie-1, are highly sensitive to raw chicken albumen. Sahin et al. (2003) reported that the viability of inoculated Campylobacter jejuni was dramatically reduced in albumen
while bacteria were able to survive up to 14 d in chicken egg yolk. Nonetheless, literature describing the antimicrobial properties of albumen or albumen components in species other than the chicken, Gallus gallus, is scarce. In this study, we investigate and compare the antimicrobial properties of albumen from 4 domestic avian species: chicken (Gallus gallus), turkey (Meleagris gallopavo), duck (Anas platyrhynchos) and goose (Anser anser).

**MATERIALS AND METHODS**

**Egg white protein sampling**

Fresh unfertilised eggs of domestic chicken (Gallus gallus), turkey (Meleagris gallopavo), duck (Anas platyrhynchos) and goose (Anser anser) were obtained from a local farm in Perth (Ontario, Canada). After cleaning of the eggshell surface with running deionised water, eggs were cracked open and egg whites sampled. Pooled egg whites were dialysed 6 times, using cellulose dialysis membranes (MWCO 3500 Da, Fisher Scientific, Ottawa, ON, Canada), against a 100-fold greater volume of deionised water at 4°C for 24-h periods, lyophilised and stored at −20°C.

**Analysis of proteins**

Protein concentration of samples used for denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as well as those used for lysozyme and antimicrobial assays was determined by bichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), using bovine serum albumin (Bioshop, Burlington, ON, Canada) as a standard. For SDS-PAGE analysis, samples were dissolved in 4% sodium dodecyl sulphate, 25% glycerol, 1.5% Tris–HCl pH 6.8. Bromophenol blue (0.125 mg/ml) and 1,4-dithiothreitol (7-7 mg/ml) were added to the samples before heating (5 min at 90°C) and loading. SDS-PAGE was carried out on 12% polyacrylamide gels and visualised by Coomassie Blue staining. Molecular weight markers (MBI Fermentas, Burlington, ON, Canada) were included in gels during SDS-PAGE while prestained protein standards (Bio-Rad, Mississauga, ON, Canada) were used for blotting.

Western blotting was performed for selected chicken proteins with rabbit antisem (1:5000) in 0.01 m phosphate buffer, 0.0027 m potassium chloride, 0.137 m sodium chloride, pH 7.4, Tween-20 (0.1%) as described by Hincke et al. (1999). Polyclonal antibody to chicken egg white c-type lysozyme was a kind gift from Dr J. Gautron (SRA, INRA, Nouzilly, France). Polyclonal antibody to chicken egg white ovotransferrin was a kind gift from Dr A.B. Mason (University of Vermont, USA). Polyclonal antibody against chicken egg white ovalbumin was obtained from Chemicon (Temecula, CA, USA). Bovine serum albumin (3% solution) was used during blocking of nitrocellulose membranes and 1:5000 anti-rabbit IgG horse radish peroxidase linked whole antibody (GE Healthcare Bio-Sciences, Montreal, QC, Canada) was used to reveal immunoreactive bands by enhanced chemiluminescence method (PerkinElmer BioSignal, Montreal, QC, Canada).

**Determination of lysozyme concentration by enzymatic activity**

Lysozyme concentration of samples was determined using a bioassay developed by Liao et al. (2001). Protein samples as well as serially diluted chicken egg white lysozyme (Sigma-Aldrich, Oakville, ON, Canada), used as standards, were suspended in 0.01% acetic acid. Samples and standards were added to a 1 mg/ml dry cell wall suspension of Micrococcus lysodeikticus ATCC 4698 (Sigma-Aldrich) in 10 mM sodium phosphate buffer (pH 7.3 and 5.3) and absorbance (600 nm) at room temperature was recorded over time. A standard curve was constructed by plotting the log reciprocal of the time required for a 0.05 unit change in absorbance vs log concentration of chicken egg white lysozyme (mg/ml) standards. Lysozyme activity was expressed as a percentage of total protein concentration within samples.

**Bacteriostatic activity**

Bacteriostatic activity of protein samples was evaluated using an antimicrobial assay adapted from Valenti et al. (1981). A sample of an overnight bacterial culture was grown to log phase in Luria-Bertani (LB) broth (Bioshop) or LB broth supplemented with salt. Gram-negative bacteria were grown in LB broth supplemented with 50 mM sodium bicarbonate while Gram-positive bacteria were grown in LB broth supplemented with 10 mM sodium citrate and 50 mM sodium bicarbonate. Valenti et al. (1981) demonstrated that these salts promote the binding of iron by ovotransferrin, thereby inhibiting the growth of micro-organisms. In Gram-negative micro-organisms, bicarbonate enhanced the antimicrobial action of ovotransferrin while citrate had an antagonising effect since such bacteria possess an iron transport system mediated by citrate (Valenti et al., 1981, 1985). Bacterial inoculum was concentrated by centrifugation at 3000 g, 4°C for 10 min and adjusted to ~1 × 10⁵ CFU/ml. After determination of protein concentration by BCA assay, protein samples in 0.01% acetic acid were added to bacterial inoculum.
Growth at 37°C, 250 rpm (Multitron HT Infors incubator, Rose Scientific, Mississauga, ON, Canada) was monitored by spectrophotometric reading at 600 nm after 24-h incubation. Acetic acid (0.01%) was used as a control and 1 mg/ml of bovine lactoferrin (Sigma-Aldrich) was used as a positive control. Lactoferrin is an iron-binding glycoprotein with similar bacteriostatic activity as ovotransferrin and other transferrins (Ward et al., 2002). Through the use of a low initial bacterial population, the bacteriostatic assay was designed for sensitivity to detect the inhibition of bacterial growth. Antimicrobial activity was evaluated against two Gram-positive (Bacillus subtilis ATCC 19659 and S. aureus ATCC 6538) and two Gram-negative (Pseudomonas aeruginosa ATCC 15442 and E. coli D31) bacteria.

Bactericidal activity
Bactericidal activity of protein samples was evaluated by an antimicrobial assay adapted from the micro-broth dilution assay developed by Steinberg and Lehrer (1997). A sample of overnight bacterial culture was grown to log phase in LB broth, centrifuged (3000 g, 4°C, 10 min), washed and re-suspended in 10 mM sodium phosphate buffer (pH 7.3). Protein samples were dissolved in 0.01% acetic acid and concentration determined by BCA protein assay. Protein samples were further diluted with 0.01% acetic acid, 0.1% bovine serum albumen (BSA) and incubated for one hour at 37°C, 250 rpm with a 10-fold greater volume of bacterial suspension. After incubation, a serial dilution of the sample was plated on LB agar, incubated overnight at 37°C and CFU/ml determined. Acetic acid (0.01%) and 0.1% BSA was used as a control and 100 μg/ml bovine lactoferrin B (Sigma-Aldrich) was used as a positive control. Lactoferrin B is an antimicrobial peptide sequence found within the N-terminus of lactoferrin (Bellamy et al., 1992). The bactericidal assay was designed for sensitivity to detect reductions in bacterial populations due to cell death. Antimicrobial activity was evaluated against two Gram-positive (B. subtilis ATCC 19659 and S. aureus ATCC 6538) and two Gram-negative (P. aeruginosa ATCC 15442 and E. coli D31) bacteria.

Statistical analysis
Data were analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL, USA). An analysis of variance (ANOVA) followed by pair-wise analysis was conducted to identify any significant differences between the growth of bacteria in the presence or absence of protein samples. If the data did not meet the assumptions of the statistical model, the Kruskal-Wallis test followed by a Kolmogorov–Smirnov test was conducted to identify reductions in bacterial populations.

RESULTS

Egg white composition
SDS-PAGE (Figure 1(a)) revealed two major bands, at 45 and 78 kDa, that appeared to be shared across species and were identified as being ovotransferrin and ovalbumin by Western blotting (Figure 1(b)). These bands showed mild differences in intensity across species during SDS-PAGE analysis (Figure 1(a)) and slight differences in reaction intensity across species during Western blotting (Figure 1(b)). A 14 kDa band present in chicken, turkey and duck egg white was immunoreactive with antibody to chicken c-type lysozyme during Western blotting (Figure 1(b)). This band was absent from goose egg white which also failed to react during Western blotting for c-type lysozyme (Figure 1(b)). A 20 kDa Coomassie Blue stained protein band, possibly g-type lysozyme, was detected during SDS-PAGE analysis in goose egg white (Figure 1(a)).

Lysozyme activity of egg white samples was determined by enzymatic activity. Chicken egg white had the highest lysozyme activity, followed by turkey, duck and goose egg white, when tested at pH 7.3 (Table). This activity correlates with the intensity of the 14 kDa band visualised by SDS-PAGE (Figure 1(a)) and Western blotting (Figure 1(b)). When tested at pH 5.3, the lysozyme activity of chicken, turkey and duck egg white did not show significant change, in contrast to goose egg white which showed an almost 9-fold increase (Table). Canfield and McMurry (1967) reported that the enzymatic activity of goose egg white lysozyme is 3 times greater than an equimolar amount of hen egg white lysozyme at pH 6-2. The pH optimum of g-type lysozyme of goose and ostrich was reported to be at pH 5-5 to 6-0 (Pooart et al., 2005) while chicken c-type lysozyme possesses a broad pH range of 5.5 to 7.5 (Thammasirirak et al., 2001). This is visualised in Figure 2 where chicken egg white demonstrates uniformly high lysozyme activity between pH 5 and 8 while goose egg white demonstrates maximal lysozyme activity around pH 5-3. Increased lysozyme activity at pH 5-3 can therefore be used as an indication of the presence of g-type lysozyme.

Antimicrobial activity of egg white samples
The antimicrobial activity of albumen is a combination of bacteriostatic activity, whereby bacterial growth is inhibited, and bactericidal
Figure 1. SDS-PAGE analysis (panel A) and Western blot analysis (panel B) of avian egg white samples. For SDS-PAGE analysis, protein samples (20 µg) were loaded into each well of a 12% polyacrylamide gel and visualised by Coomassie Blue staining. Molecular weight of standard (ST) is indicated on the left. For Western blot, protein samples (1 µg) were loaded into each well. Purified chicken egg white lysozyme, ovotransferrin or ovalbumin (0-1 µg) was used as a positive control (+). Egg white samples are labelled on the X-axis (C = chicken, T = turkey, D = duck, G = goose).

Table. Determination of lysozyme content of pooled avian egg white samples by measurement of enzymatic activity against M. lysodeikticus cell walls at pH 5-3 and 7-3 in 10 mM sodium phosphate buffer. Lysozyme activity is expressed as a per cent by weight (± standard deviation) of total protein. Experiment was performed on two separate occasions with triplicate readings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Per cent lysozyme ± standard deviation</th>
<th>Ratio pH 5-3/ pH 7-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7-31</td>
<td>pH 5-32</td>
</tr>
<tr>
<td>Chicken</td>
<td>2.44 ± 0.22</td>
<td>2.43 ± 0.21</td>
</tr>
<tr>
<td>Turkey</td>
<td>0.94 ± 0.06</td>
<td>1.20 ± 0.34</td>
</tr>
<tr>
<td>Duck</td>
<td>0.52 ± 0.07</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>Goose</td>
<td>0.14 ± 0.03</td>
<td>1.22 ± 0.21</td>
</tr>
</tbody>
</table>

1 pH optimum of c-type lysozyme (Gallus gallus).
2 pH optimum of g-type lysozyme (Anser anser).

Figure 2. Lysozyme activity of chicken and goose albumen in buffered 10 mM sodium phosphate at various pH. Samples of chicken and goose albumen were added to Micrococcus lysodeikticus cell wall suspension in 10 mM sodium phosphate buffer (pH 5-0 to 8-0) and optical density at 600 nm was recorded over time. Experiment was conducted on two separate occasions with triplicate readings.
activity, in which cell death results in the reduction of bacterial populations. These two possible antimicrobial mechanisms were evaluated separately.

The experiment depicted in Figure 3(a) was conducted under conditions designed to detect ovotransferrin-mediated bacteriostasis. In the presence of 10 mM sodium citrate and 50 mM sodium bicarbonate, *B. subtilis* was completely inhibited by all egg white samples while *S. aureus* showed significant inhibition in the presence of 10 mg/ml chicken or turkey egg white (Figure 3(a)). In the presence of 50 mM sodium bicarbonate, all egg white samples inhibited the growth of *P. aeruginosa* while chicken, turkey and duck egg white inhibited *E. coli* D31 (Figure 3(a)).

In contrast, in the absence of salts, none of the egg white samples inhibited *E. coli* D31 while only turkey egg white caused significant inhibition of *S. aureus* and *P. aeruginosa* (Figure 3(b)). At a concentration of 10 mg/ml, chicken, turkey, duck and goose egg white completely inhibited the growth of *B. subtilis* regardless of the presence of salts (Figure 3(a), (b)) suggesting the action of a protein with an antimicrobial mechanism independent of the iron-binding activity of ovotransferrin.

The bactericidal activity of egg white was evaluated in a different assay (Figure 4) using a low egg white concentration in order to distinguish species differences. At a concentration of 300 μg/ml, avian egg white did not significantly reduce the populations of *S. aureus, P. aeruginosa* or *E. coli* D31 (Figure 4). As can be seen in Figure 5, *S. aureus, P. aeruginosa* and *E. coli* D31 are insensitive to the action of lysozyme while *B. subtilis* is highly sensitive. Chicken egg white,
followed by turkey, duck and goose egg white, showed significant reductions of *B. subtilis* (Figure 4). Bactericidal activity of egg white against *B. subtilis* (Figure 4) and lysozyme activity at pH 7.3 (Table) appear to correlate, because both lysozyme activity and bactericidal activity were highest in chicken followed by turkey, duck and goose egg white samples.

**DISCUSSION**

In avian species, the albumen provides the developing embryo with nutrients and water while limiting microbial proliferation. Egg white proteins act synergistically to contribute to antimicrobial activity. Ovotransferrin is implicated in iron-dependent bacterial inhibition (Valenti et al., 1981; von Hunolstein et al., 1992). Enhanced binding of iron to ovotransferrin is favoured in the presence of sodium citrate and/or sodium bicarbonate and leads to inhibition of bacterial growth. During the current study, we observed enhanced inhibition in the presence of these salts for albumen samples from all species; this difference corresponds to the activity of ovotransferrin.

The ovotransferrin activity of turkey albumen showed the greatest inhibition of bacterial
growth during the bacteriostatic assay, followed by that of chicken egg white (Figure 3(a)). Within the Anseriformes, duck ovotransferrin was more active than goose ovotransferrin against S. aureus, B. subtilis and E. coli D31 than that of goose albumen ovotransferrin, while the opposite was observed against P. aeruginosa. SDS-PAGE and Western blot analysis (Figure 1(a); 1(b)) demonstrated that ovotransferrin is present at higher concentrations in Galliform albumen than in Anseriform albumen.

Regardless of the presence of salts, B. subtilis was completely inhibited by high concentrations of albumen from all of the species examined using the bacteriostatic assay (Figure 3(b)), indicating the presence of an antimicrobial protein which acts by another mechanism. In both the bacteriostatic (Figure 3) and bactericidal (Figure 4) assays, Gram-positive bacteria were affected by egg white samples to a greater extent than Gram-negative bacteria. Avian albumen is known to be a rich source of lysozyme. Western blotting revealed that the intensity of the 14 kDa c-type lysozyme varied between species (Figure 1(b)). Moreover, SDS-PAGE analysis (Figure 1(a)) revealed that chicken albumen showed a most intense Coomassie Blue stained band at 14 kDa, followed by that in the turkey and duck; no 14 kDa band was detected in the goose albumen sample. This pattern was confirmed by determining lysozyme enzymatic activity (Table).

Lysozyme effectively breaks down the cell walls of some Gram-positive bacteria. S. aureus, P. aeruginosa and E. coli D31 are insensitive to lysozyme while B. subtilis is highly sensitive as demonstrated in Figure 5. In the bacteriostatic assay, the albumen concentration of 10 mg/ml tested corresponded with approximately 0-01 (goose), 0-05 (duck), 0-1 (turkey) and 0-24 mg/ml (chicken) lysozyme, by extrapolation using lysozyme activity determined at pH 7.3 (Table). This high lysozyme concentration would completely kill B. subtilis populations and prevent their further growth in the bacteriostatic assay. Utilising lower levels of the albumen samples, as tested in the bactericidal assay (sample concentration corresponds to a 300-fold dilution of natural egg white), would not have completely killed the bacilli (in contrast to the bacteriostatic assay) and allows detection of species differences in the ability of albumen to kill bacteria. Chicken egg white lysozyme is highly active against Micrococcus and Bacillus species while demonstrating lower activity against Gram-negative bacteria (Burley and Vadehra, 1989; Bera et al., 2005). In our study, the bactericidal activity of egg white samples against B. subtilis was strongly correlated with lysozyme activity. This relationship, as well as the fact that only a bacterial species sensitive to lysozyme showed significant sensitivity, is consistent with the notion that lysozyme is a major bactericidal egg white protein although it demonstrates limited specificity.

Two types of lysozyme activity: c-type (chicken-type) and g-type (goose-type) have been described. These two lysozymes have similarities in their tertiary structures although their amino acid sequences are entirely different and antibodies directed against c-type lysozyme do not cross-react with g-type lysozyme, and vice versa (Hemmen et al., 1992; Irwin and Gong, 2003; Pooart et al., 2005). C-type lysozyme has been demonstrated in the albumens of Galliformes and Anseriformes, whereas the g-type is found in the albumen of Anseriformes, Struthioniformes, Rheiformes, Apterygiformes, Tinamiformes, Podicipediformes, Sphenisciformes, Casuariformes and Charadriiformes. Simultaneous expression of both c-type and g-type lysozyme is observed in the albumens of some Anseriformes (Florkin and Scheer, 1978; Hemmen et al., 1992). G-type lysozyme is up to 3 times more active at pH 6.2 than equimolar amounts of c-type lysozyme, although it has much less activity at neutral pH (Canfield and McMurry, 1967; Hindenburg et al., 1974). Consistent with this, we observed that goose egg white exhibited a 9-fold increase in lysozyme activity at pH 5-3 (Table) in the absence of immunoreactive c-type lysozyme as demonstrated by Western blotting (Figure 1(b)). The pH of non-incubated chicken albumen rises from about 7.6 to 9.5 within a few days after oviposition (Lapao et al., 1999). Non-incubated Galliform eggs may therefore represent a safer food for human consumption, because these eggs should remain free of lysozyme-sensitive micro-organisms for longer periods during storage than g-type lysozyme-containing Anseriform eggs. Gram-positive bacilli are major contaminants on the eggshell surface (Cook et al., 2003) and are associated with food-borne illness. Other lysozyme-sensitive egg contaminants include Micrococcus spp., a Gram-positive cocci associated with embryonic death (Deeming, 2002; Cook et al., 2003).

The lower defensive capabilities of eggs against Gram-negative bacteria represent a potential hazard to human health. Gram-negative bacteria, such as E. coli, Salmonella enterica and Pseudomonas spp. are known to penetrate the eggshell and potentially produce embryonic mortality (Deeming, 2002; Messens et al., 2006). For this reason, eggs destined for human consumption are sanitised and kept in low humidity conditions in order to prevent the proliferation of Gram-negative pathogens. Considering that 92% of fowl eggshell contaminants from commercial duck hatcheries are...
Gram-positive cocci, mainly Micrococcus spp. and Staphylococcus spp. (Seviour and Board, 1972), it is likely that evolution (and possibly human selection) has led to more potent avian egg white antimicrobial defences against the attack of Gram-positive micro-organisms.

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REFERENCES


