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Gastric lysozyme as a digestive enzyme in the hoatzin *(Opisthocomus hoazin)*, a ruminant-like folivorous bird

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Abstract. The hoatzin is the only bird known to have pregastric fermentation in the crop. This digestive strategy is supported by morphological and microbiological adaptations analogous to those present in ruminants and ruminant-like mammals. The hoatzin expresses a lysozyme-like bacteriolytic activity in its foregut. The enzyme has a high activity, and its low pH optimum, pepsin resistance and localization to the proventriculus allow it to be active for digestion in the stomach. The hoatzin enzyme and the ruminant gastric lysozyme present similar biochemical characteristics. The lysis of bacterial cells may be of significance for the nutrition of the hoatzin. We propose that the hoatzin expresses a lysozyme which has been recruited to function as a digestive enzyme, representing a unique case of evolutionary convergence of digestive adaptations in this bird and foregut fermenter mammals.
Key words. Lysozyme; birds; herbivory; foregut fermentation.

Lysozyme is a glycosidase displaying an endo-N-acetylmuramyl hydrolase activity, able to degrade chitin and bacterial cell walls. Its presence in tears, nasal mucus, saliva, milk, blood serum and egg white, and in a great number of tissues of vertebrates and invertebrates, has been associated with a protective role against bacterial infections[1]. Herbivorous mammals with digestive fermentation in the foregut, such as ruminants and colobine monkeys, have high amounts of lysozyme in their stomachs, which may function as a digestive enzyme[2–4].

The bacterial populations from the rumen or other gastric fermentative compartments in herbivores allow the digestion of dietary plant cell walls, for which the host lacks digestive enzymes. Bacteria constitute the main source of protein, carbon and phosphorus in these animals[5]. Since the glycoside moieties of bacterial cell walls are poorly degraded by conventional mammalian digestive enzymes[6], the increased expression of gastric lysozyme in these animals has an important function.

The hoatzin is the only known leaf-eating bird to possess foregut fermentation in the crop[7]. Its crop is unusually large, and contains a rather rich population of symbiotic bacteria and protozoa[8]. These morphological and microbiological adaptations support a digestive strategy in this bird analogous to that of ruminants and ruminant-like mammals[7–9]. Bacteria are an important component of the digesta when it enters the proventriculus (bacterial densities are 10^9 per gram of crop contents)[8,9]. The question may be asked as to whether, as in mammal foregut fermenters, there is an increased expression of gastric lysozyme in amounts such that it could function as a digestive enzyme.

Materials and methods

Wild hoatzins (*Opisthocomus hoazin*) were caught at Hato Piñero in the central Venezuelan plains. The birds were killed, and the organs excised, weighed, and immediately frozen in liquid nitrogen. Tissue extracts were prepared by homogenization in 5 volumes of 2% acetic acid (v/v) in a teflon/glass homogenizer[2], and stored at −20 °C. Bacteriolysis by tissue extracts was recorded continuously as a decrease in light scattering of a bacterial suspension of *Micrococcus luteus*[10] or a hoatzin crop mixed culture. The suspension of lyophilized *Micrococcus luteus* (0.25 mg/ml, from Sigma) was prepared in a buffer containing 0.022 M sodium acetate and 0.177 M NaCl at pH 5.0.

For the mixed bacterial culture suspension, 4 g of crop contents was inoculated into 45 ml of medium M2[11] and the bacteria grown at 38 °C for 24 h. Bacteria were harvested by centrifugation at 10,000 *g* for 10 min and washed several times in assay buffer. The concentration was adjusted at the start to give the same optical density as that of a 0.25 mg/ml suspension of *Micrococcus luteus*. Scattering measurements were performed in the cuvette of a spectrofluorometer (Aminco Bowman) containing 1.5 ml of the *Micrococcus luteus* or mixed bacterial culture suspensions. The apparatus was equipped with a magnetic stirrer, and the excitation and emission monochromators were set at 540 nm. The reaction was started by the addition of 5–50 µl of tissue extract which contained approximately 200 µg wet weight/ml. Enzyme activity was estimated from the initial slopes of the traces. The activity was found to be linearly related to enzyme concentration. A standard curve was performed in the same manner with commer-

cial lysozyme (egg-white; Sigma, St Louis, Missouri, USA) for each experimental day.

Lysozyme activity was also detected after separation of proteins on polyacrylamide gels, using the overlay method[2,12,13]. First, a non-denaturing electrophoresis was performed, and then an overlay gel in which bacteria were embedded was applied. The zones of bacteriolysis were seen as translucent areas. Electrophoresis of tissue extracts in non-denaturing gels was performed at pH 4.3 in vertical slaps of 10% polyacrylamide in the cathodal direction[2,12]. Tissue samples of cow abomasum (15 µl) and hoatzin, pigeon or chicken stomach (30 µl), diluted 1/2 with glycerol, were loaded on to the gel. For chicken egg-white 2 µg was used. The overlay gel to test bacteriolytic activity was prepared by embedding *Micrococcus luteus* (1 mg/ml) in the 10% polyacrylamide gel at pH 5.0 in NaCl-Na acetate buffer[12,13]. Overlays were placed on the original slab gel for 30 min at 37 °C. Bacteriolysis was detected by a decrease in the opacity of the gel (areas which appear black in the photograph, as a negative is used). After incubation with the overlay gel, the original gels were stained with Coomassie Brilliant Blue to develop protein bands.

Results and discussion

Lysozyme was expressed in the hoatzin foregut. Extracts of adult hoatzin proventriculus induced bacteriolysis of *Micrococcus luteus* in liquid culture measured as a decrease in light scattering (fig. 1A), at pH 5, and in the same range of activity as that shown by cow abomasum (table). Extracts of hoatzin intestine, pancreas and heart had no lytic effect whatsoever (table).

The expression of stomach lysozyme in the hoatzin does not appear to be induced by the bacterial substrate. Lysozyme activity was present in the hoatzin stomach even before eclosion (table), at a time when the foregut is sterile, as innoculation with regurgitated food by the parents has not yet occurred (Domínguez-Bello et al., submitted). However, higher levels of activity found in chick proventriculus, as compared to that of the adult, coincide with higher bacterial counts in the chick crop (10^{12} bacteria/gram crop contents in chicks and 10^9 in adults[8,9]). This may suggest that some degree of regulation of expression and/or activity by the bacterial contents of the digesta may be present. Other birds, like pigeons and domestic fowl, which have bacteria in their crop[14–16] did not express lysozyme in the stomach (table). However, in these birds, the presence of crop bacteria does not appear to be nutritionally significant.

The hoatzin proventriculus showed lytic activity against mixed bacterial cultures which originated in the hoatzin crop, whereas chicken egg-white lysozyme had almost no effect (fig. 1B). Identical results were obtained when a pure culture of a bacterium from the crop (H271, a gram negative rod) was used as a substrate (not shown). However, the hoatzin lysozyme appears to be less active in lysing crop bacteria than in lysing *Micrococcus luteus*.

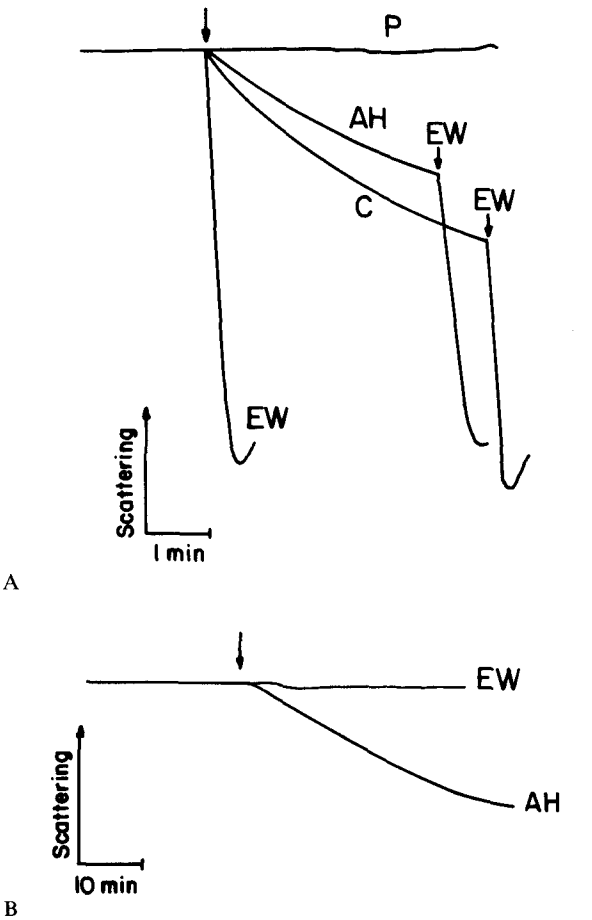

Figure 1. Bacteriolysis by gastric extracts of adult hoatzin (AH), pigeon (P) and cow (C), and chicken egg-white (EW), measured as a decrease in light scattering. Volumes (approximately 25 µl) were added to give 15 µg of the original wet tissue extracted, or 100 µg of chicken egg-white lysozyme. The substrate for the lysozyme activity assay was a suspension of *Micrococcus luteus* in A or a mixed bacterial culture from the hoatzin crop in B.

The gastric lysozyme of the hoatzin was characterized by a low pH optimum and resistance to peptic cleavage. The activity actually increased as the pH of the assay medium was dropped from 5 to 3.5, whereas bacteriolysis by chicken egg-white lysozyme decreased by 50% under these conditions. After treatment with pepsin (100 µg/ml, 37 °C, pH 2.0, 3 h), the activity of the hoatzin lysozyme remained as high as 60%, whereas this treatment would inactivate egg-white lysozyme[2]. These biochemical characteristics of the gastric bacteriolytic enzyme of the hoatzin are compatible with its being able to function in the stomach, where we found an acidic pH and high peptic activity (data not shown). The enzyme responsible for the bacteriolytic activity has recently been purified and identified as a true lysozyme by Kornegay[17]. The adaptations it shows at the biochemical level are similar to those present in gastric lysozymes of foregut fermenter mammals[2].

Lysozyme activity of hoatzin and other animals

Animal/tissue	Activity (units/g tissue)	n experiments (animals)
Hoatzin proventriculus:		
adult	63 ± 4.0	18(10
chick	105 ± 6.0	3(3)
unhatched chick	133 ± 4.0	2(1)
Hoatzin small intestine	0	2(1)
Hoatzin pancreas	0	2(1)
Hoatzin heart	0	2(1)
Cow abomasum	139 ± 0.5	2(1)
Pigeon proventriculus	0	2(1)
Domestic fowl proventriculus	0	2(1)

Lysozyme activity of tissue extracts of adult hoatzin, hoatzin chick, unhatched chick, cow, pigeon and domestic fowl. Tissue extracts and scattering measurements were performed as described in Materials and methods. The activity expressed (units/g tissue; average ± se) corresponds to the relation between the slope values obtained with 1 μg of egg-white lysozyme and those obtained with tissue extracts.

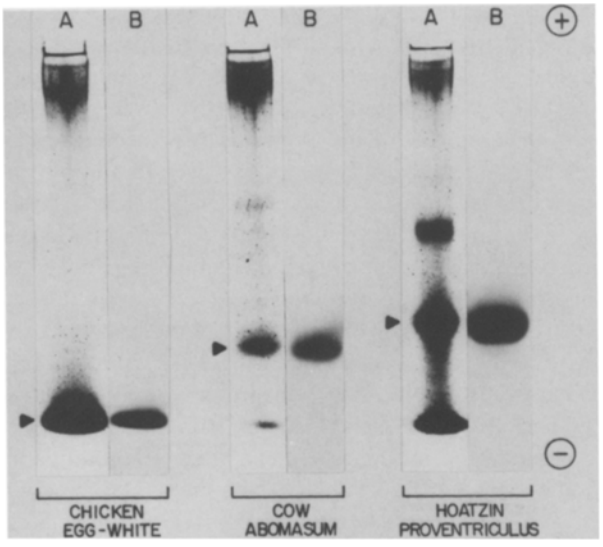

Figure 2. Non-denaturing gel electrophoresis of lysozymes from proventriculus of adult hoatzin, cow abomasum and commercial chicken egg-white and activity overlay of lysozyme. Native gel stained with Coomassie Brilliant Blue (A) and overlay (B) lanes are juxtaposed for comparison for each different lysozyme.

The presence of gastric lysozyme was confirmed by electrophoresis in non-denaturing gels. Protein bands showed bacteriolytic activity on overlay gels containing cells of *Micrococcus luteus* (fig. 2). These lytic bands were found in hoatzin proventriculus, cow abomasum and chicken egg-white. The mobility of the hoatzin proventriculus lysozyme was less than that of the cow enzyme and much less than that of chicken egg-white in these conditions. Pigeon and domestic fowl preventriculi, which did not show activity in the light-scattering assay, also did not show bacteriolysis in the overlay (not shown).

Bacteria and semi-digested plant material leave the hoatzin crop and posterior esophagus and enter the proventriculus, the acid-secreting stomach of the bird, where the environmental pH drops from neutral to very acid values. Lysozyme secreted in the stomach would have to be active in the presence of pepsin and in an acid environment. The high activity of lysozyme in the stomach, the biochemical characteristics of this enzyme, and the peculiar alimentary regime of the bird, with a high bacterial content in the digesta, lead us to consider the gastric lysozyme of the hoatzin as a digestive enzyme. The hoatzin lysozyme is probably a case of functional evolutionary convergence with that of ruminants and ruminant-like mammals. This extraordinary feature found in this bird may throw light into the mechanisms of evolutionary adaptation to new diets that took place after the appearance of the angiosperms and the radiation of foregut fermenter vertebrates.

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