Kasetsart J. (Nat. Sci.) 33 : 243 - 250 (1999)

Histochemical Detection of Glycoconjugates in the Anterior Lingual Salivary Glands of the Domestic Fowl

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ABSTRACT

Glycoconjugates in secretory epithelium of the anterior lingual salivary glands of chicken have been studied by means of light microscopic histochemical methods. A series of staining procedures were employed including peroxidase - labeled lectin - diaminobenzidine methods and digestion techniques with neuraminidase or α - amylase. The terminal portion of the anterior lingual gland consisted of mucous and seromucous cells. All the mucous cells in the glands possessed a mixture of glycoconjugates of different nature, but they could be divided into three types, on the basis of the principle type of glycoconjugates involved, namely primarily sulfated, carboxylated and neutral glycoconjugate-containing cells. The first mentioned type of cells was predominant. The presence of sialic acid, α - D - mannose, α - D - glucose and β - D - galactose in these glycoconjugates was confirmed. The seromucous cells found in the anterior lingual gland contained a very small amount of comparable carbohydrates predominantly in the apical cytoplasm. Possible functional activities of the glands were discussed.

Key words : lingual salivary gland, glycoconjugates, domestic fowl

INTRODUCTION

In vertebrates, one of the main functions of the salivary glands is known to be production of protective and lubricant mucins. The nature of such mucins can be analyzed by means of histochemistry.

In mammalian species, numerous histochemical studies have been made on complex carbohydrates of different salivary glands (Laden *et al*., 1984; Schulte and Spicer, 1985; Spicer and Schulte, 1992). In the chicken, however, the salivary glands have not received much attention, and the results obtained by a few histochemical studies in the glands have been very controversial (Saito, 1966 a, 1966 b; Fujii and Tamura, 1966; Rangel *et al*., 1968; Nalavade and Varute, 1977). According to Saito (1966 a, 1966 b), the chicken anterior lingual gland consisted of seromucous cells which could be grouped into two types, light mucoid cells containing only acidic carbohydrates and dark seromucous cells containing both neutral and acidic carbohydrates. Fujii and Tamura (1966) substantiated that the terminal portions of all the salivary glands from the chicken were mucous in type and that the secretory substances of the anterior lingual gland were primarily non-sulfated acid glycoprotein. Rangel *et al*. (1968) studied histochemically the glands and reported the absence of glycogen and ester sulfates and the presence of neutral group and sialic acid in their terminal portions. A subsequent histochemical study by

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Nalavade and Varute (1977) on the terminal portions of the anterior gland has disclosed three distinct cell types elaborating and releasing separate sulfomucins, sialomucins and neutral mucosubstances respectively.

In view of such controversies in previous histochemical studies on the chicken lingual salivary glands, attempts have been made to investigate histochemistry of glycoconjugates involving in the secretory portions of the anterior lingual glands of the chicken. The results obtained here are believed not only to dissolve the controversies mentioned but also to give an important baseline for physiological functions of avian oral glands in general.

MATERIALS AND METHODS

Twenty-four Brown Leghorn chicken aged 1-6 months were sacrified by intravenous administration with overdose of sodium pentobarbiturate. Anterior lingual glands were removed and immediately fixed at 4º C in either 2% calcium acetate in 10% formalin or Rossman's fluid for 12-24 hours. After dehydration in graded ethanol series, the tissue pieces were embedded in paraffin wax. On a sliding microtome, sections were cut at a thickness of 4-6 μm and subjected to the following staining procedures.

For general observation of histological structures, sections were stained with hematoxylin and eosin.

For histochemical detection of glycoconjugates, the following staining procedures were employed.

1. Alcian blue (AB) pH 1.0 method for sulfated glycoconjugates (Lev and Spicer, 1964).

2. High iron diamine (HID) method for sulfated glycoconjugates (Spicer, 1965).

3. Aldehyde fuchsin (AF) method for sulfated glycoconjugates (Gomori, 1950).

4. AB pH 2.5 method for acidic glycoconjugates (Spicer *et al*., 1967).

5. Low iron diamine (LID) method for acidic glycoconjugates (Spicer, 1965).

6. Periodic acid-Schiff (PAS) method for glycoconjugates with vicinal glycol groupings (Pearse, 1968).

7. HID - AB method to differentiate sulfated and carboxylated glycoconjugates (Spicer, 1965).

8. AB pH 2.5 - PAS method to differentiate acidic and neutral glycoconjugates (Spicer *et al*., 1967).

9. Concanavalin A - Peroxidase - diaminobenzidine (Con A - PO - DAB) method for α - D - mannose and α - D - glucose residues of glycoconjugates (Yamada and Shimizu, 1976).

10. Peroxidase labeled - Ricinus communis agglutinin - I - diaminobenzidine (PO - RCA - I - DAB) method for β - D - galactose residues of glycoconjugates (Yamada and Shimizu, 1977).

In addition to these procedures, the following confirmation and control experiments were performed.

1. Enzyme digestions : Some sections were incubated with 1 u/ml. neuraminidase (from *Arthrobacter ureafacien*) in 0.1 m. acetate buffer (pH 5.3) containing $CaCl_2$ at 39-41ºC for 12-17 hours (Spicer *et al*., 1967) prior to staining with AB pH 2.5; other sections were subjected to digest with 1 mg/ml α - amylase (from *Bacillus subtilis*) in 0.1 M buffered saline (0.8% NaCl, 0.08% NaH_2PO_4, H_2O 0.13% Na_2HPO_4) 37º C for 1 hour (Casselman, 1959) prior to staining with PAS. The neuraminidase and α - amylase preparation were purchased Seikagaku Kogyo Co. Ltd. and Marukinshoyu Co. Ltd. respectively. Two types of control procedures were performed : (a) some sections were incubated in respective buffer solutions without enzymes under identical condition of temperature and duration; (b) other sections

were kept intact without any incubation procedures.

2. Lectin controls : Sucrose and β - D - galactose were added at a final concentration of 0.1 M to the Con A and RCA - I solutions respectively.

In order to detect endogenous peroxidase activity in tissues, some control sections were reacted with DAB only

RESULTS

Histology

The anterior lingual gland was a compound tubular gland locating on both sides of os entoglossum. Its secretory portion consisted of both mucous and seromucous cells. The mucous portion was confined to the medial part of posterior one third of the whole gland. The majority of the gland, seromucous portion, therefore, situated anterolateral to the mucous portion. Glandular epithelium of the mucous parts consisted of a single layer of high columnar cells containing a compressed nucleus in the basal cytoplasm (Figure 1). In contrast, low columnar cells containing a round nucleus were found in the glandular epithelium of the seromucous parts. However, there were occasional basal cells subjacent to the secretory cells of both parts. The cytoplasm of the mucous cells appeared clear whereas that of the seromucous cells was somewhat dark with hematoxylin and eosin staining procedures (Figure1).

Histochemistry

The AB pH 1.0 staining procedure resulted in strong positive reaction of most of the mucous cells, whereas relatively mild positive AB pH 1.0 reaction was exhibited by the apical cytoplasm of the seromucous cells (Figure 2). The staining patterns obtained by HID (Figure 3) and AF (Figure 4) methods were nearly comparable to those obtained by AB pH 1.0 staining method. Throughout the gland, however, the connective tissue elements were stained rather mildly by these staining procedures.

Both the mucous and seromucous cells gave vividly positive AB pH 2.5 reaction (Figure 5). Likewise, the LID (Figure 6) staining procedure resulted in staining patterns nearly comparable to those obtained by the AB pH 2.5 procedure. When the epithelial cells lining the anterior lingual gland were stained by PAS, the mucous cells were positively reacted in a magenta shade of relatively strong intensity (Figure 7). A moderately positive PAS reaction was shown at the apical cytoplasm of the seromucous cells and the elements of connective tissues.

The staining patterns obtained by the AB pH 2.5 - PAS procedures could be taken as showing the addition of the staining images obtained by the AB pH 2.5 and PAS methods respectively. On the basis of these staining results, two types of mucous cells could be differentiated : the majority of mucous cells were vividly stained predominantly with AB pH 2.5, whereas a minority of the cells stained predominantly with PAS. All the seromucous cells were loaded with small amounts of mucins at the apical cytoplasm which reacted more strongly for AB pH 2.5 than for PAS. In the chicken anterior lingual gland, the HID - AB pH 2.5 procedure revealed two types of secretory cells : predominantly HID reactive cells and predominantly AB pH 2.5 reactive cells. All of the seromucous cells were moderately stained by either HID or AB pH 2.5.

The Con A - PO - DAB procedure stained the mucous cells strongly whereas it did the seromucous cells moderately (Figure 8). In tissue sections stained with PO - RCA - I - DAB, the interlobular connective tissue fibers were found to react strongly. The PO - RCA - I - DAB staining patterns of both the mucous and seromucous elements were distinguished from those obtained by the Con A - PO - DAB procedure (Figure 9).

Digestion with neuraminidase diminished

moderately the intensity of AB pH 2.5 reaction in mucous and seromucous cells of the gland. Digestion with α - amylase virtually failed to alter the positive PAS reaction of both the mucous and seromucous cells. The two control procedures for neuraminidase and α - amylase produced no significant changes in intensities of the AB pH 2.5 and PAS reaction respectively.

According to the control experiments for the PO - labeled lectin - DAB procedures, the

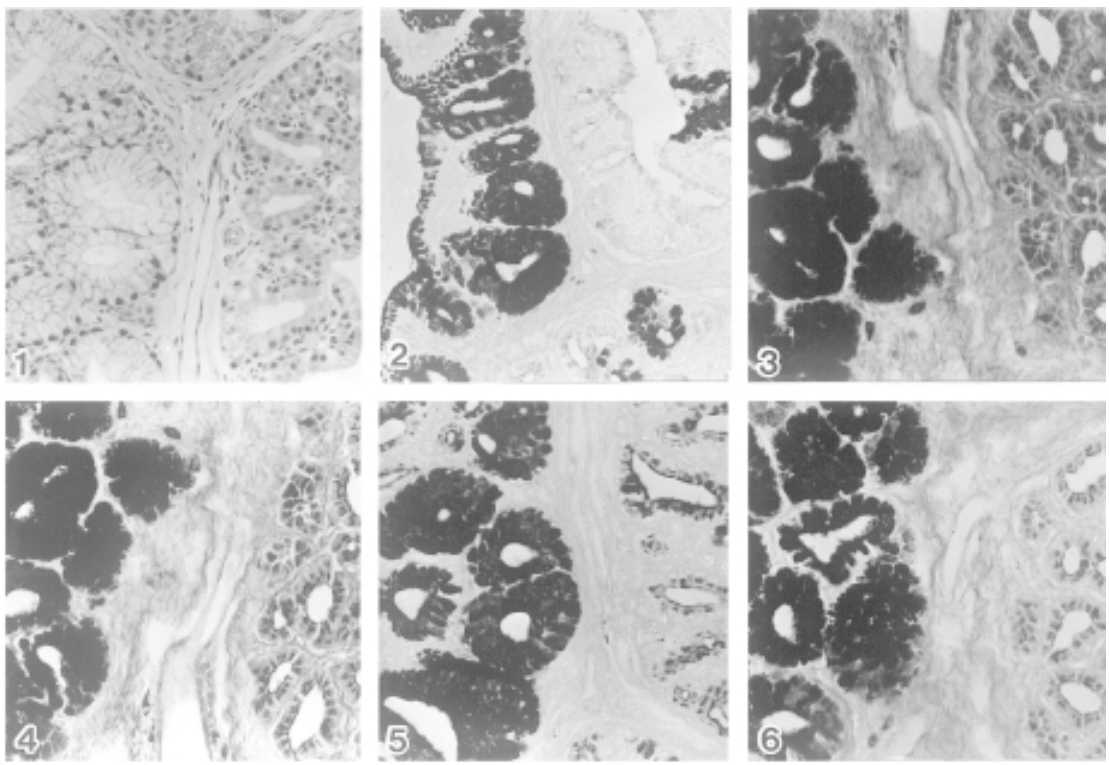

Figure 1 Anterior lingual gland of a chick (1 month). The terminal glandular portion consists of mucous (left) and seromucous (right) tubuli, which are stained light and dark respectively. Hematoxylin - eosin - stained, X 264.

Figure 2 Anterior lingual gland of a chick (1 month). The mucous tubuli reacted vividly, whereas the seromucous ones being coloured weakly. AB pH 1.0 stained, X 200.

Figure 3 Anterior lingual gland of a chick (3 month) The mucous tubuli and seromucous tubuli reacted strongly and moderately respectively HID stained, X 264.

Figure 4 Anterior lingual gland of a chick (1 month). The stainabilities of mucous and seromucous tubuli are nearly comparable in intensity to those illustrated in Fig. 4. AF stained, X 264.

Figure 5 Anterior lingual gland of a chick (1 month). The mucous tubuli reacted strongly and the seromucous ones are coloured less intensely. AB pH 2.5 stained, X 264.

Figure 6 Anterior lingual gland of a chick (1 month). The reactions of both the mucous and seromucous tubuli are nearly similar in intensity to their AB reactions illustrated in Fig. 5 LID stained, X 264.

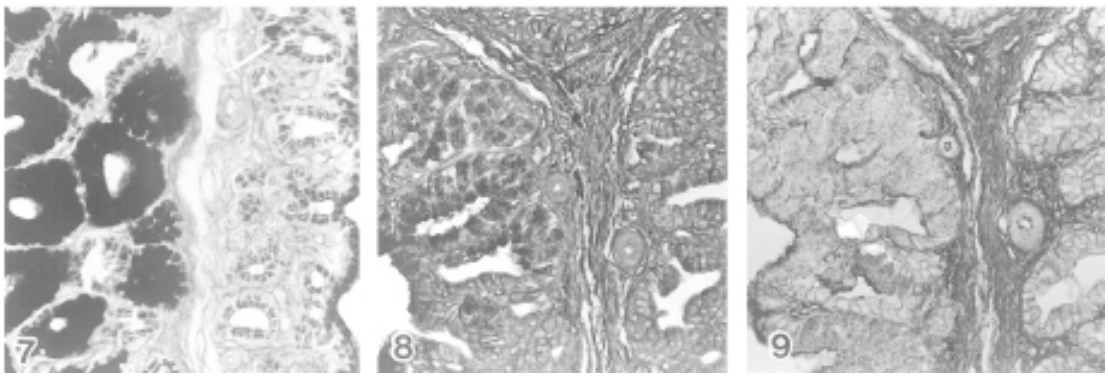

Figure 7 Anterior lingual gland of a chick (3 month). The mucous tubuli reacted strongly, whereas the seromucous tubuli are stained either vividly of moderately. PAS stained, X 264.

Figure 8 Anterior lingual gland of a chick (1 month). The mucous tubuli reacted strongly, whereas the seromucous tubuli exhibit weak positive reaction. Con A - PO - DAB stained, X 264.

Figure 9 Anterior lingual gland of a chick (1 month). The reactions of both the mucous and seromucous tubuli are not similar to their Con A - PO - DAB reactions, however the connective tissues surrounding the tubuli exhibit pronouncedly positive reaction. PO - RCA - I - DAB stained, X 264.

addition of appropriate saccharides to the lectin solution greatly diminished or even abolished the lectin bindings in all of the histological structures tested. In the salivary gland, the sole evidence of endogenous peroxidase activity was demonstrated in erythrocytes within the lumen of blood vessels.

DISCUSSION

In the secretory endpieces of the salivary gland, three types of cells have been recognized, namely serous, mucous and seromucous cells. In the present study, secretory cell types of chicken salivary gland have been classified on the basis of the histochemical reactions of mucosubstances involved, as reported previously (Shackleford and Wilbor, 1968). According to this classification, the secretory endpieces of chicken anterior, lingual gland consisted of mucous and seromucous cells, contrary to the previous authors report except that of Saito (1966 a, b).

In the mucous cells of the chicken anterior lingual gland, glycoconjugates exhibited positive reaction to AB pH 1.0, AB pH 2.5, AF, LID, HID, PAS. The AB pH 2.5 reactive substances tended to diminish in intensity following prior digestion with neuraminidase. However, their positive reaction to PAS was hardly or not altered at all by prior digestion with α - amylase. If the staining selectivities of various procedures are taken into consideration, the present histochemical results imply the presence of acidic and neutral glycoconjugates with sulfate, carboxylate and vicinal glycol groupings together with sialic acid residues in the mucous cells and their secretions. Glycogen was absent in the mucous cells, in accordance with the findings of Fujii and Tamura (1966) and Rangel *et al*. (1968).

In the dual staining, AB pH 2.5 - PAS, acidic glycoconjugates are known to be coloured blue with AB pH 2.5 and neutral ones magenta with PAS (Spicer *et al*., 1967). According to the results

obtained by the dual staining procedure, HID - AB, sulfated glycoconjugates were thought to be coloured black with HID, whereas carboxylated ones blue with AB pH 2.5 (Spicer, 1965). In view of the present results, the mucous cells in the anterior lingual glands elaborate a mixture of sulfated, carboxylated and neutral glycoconjugates, but can be grouped into three types.

1. With HID - AB pH 2.5 predominantly HID reactive cells (primarily sulfated glycoconjugate - containing cells). These cells are the majority among three types of cells.

2. With HID - AB pH 2.5 predominantly AB reactive and with AB pH 2.5 - PAS predominantly AB reactive cells (primarily carboxylated glycoconjugate - containing cells).

3. With AB pH 2.5 - PAS predominantly PAS reactive and with HID - AB pH 2.5 moderately both HID and AB reactive cells (primarily neutral glycoconjugate - containing cells).

The results obtained from the present study were not necessarily in keeping with those in the previous studies on the mucous cells lining the anterior and posterior lingual glands of the chicken. According to Saito (1966 b), the mucous cells contained only acid carbohydrates. Fujii and Tamura (1966) reported that the mucous cells in the anterior lingual glands contained mainly nonsulfated acid carbohydrates. They also demonstrated a small quantity of neutral carbohydrates but not sialomucins in the glands. Rangel *et al*. (1968) reported in the mucous cells of both glands the presence of the neutral groups and sialic acid residues, but not of sulfate groups. Nalavade and Varute (1977) disclosed three distinct cell types containing exclusively sulfomucins, sialomucins (both mucous type of cells) and neutral mucosubstances (serous type) in the anterior lingual gland. On the contrary, they identified two types of mucous cells, one secreting sialomucins and the other sulfomucins respectively. Such conflicting results may be due to the different histochemical staining methods employed in the previous and present studies. In the present study, mucous cells in both glands could be grouped into three types depending on the major type of glycoconjugates involved, but have never been found to release each glycoconjugates seperately.

Identification of various monosaccharides residues in the lingual glands have not yet performed in avian species, although a lot of works have been published in mammals (Laden *et al*., 1984; Schulte and Spicer, 1985). The present study using con A - PO - DAB (Yamada and Schimizu, 1976) and PO - RCA - I - DAB (Yamada and Schimizu, 1977), α - D - mannosyl, α - D - glucosyl and β - D - galactosyl residues in the glycoconjugates of the chicken lingual glands was confirmed. The staining intensity was much stronger with Con A - PO - DAB procedure than with PO - RCA - I - DAB procedure.

In the chicken anterior lingual gland, Saito (1966 a) differentiated two types of cells and named them dark and light cells with the former being the majority. Subsequently the same author (1966 b) reported that the seromucous cells contained varying amounts of neutral and acidic carbohydrates and composed about 55% of the total secretory cells. The present study pointed out that the mucous cells occupied posterior one third of the medial part of the gland, while the seromucous cells located anterolateral to the mucous portion. In the seromucous cells, the reactive substance was found to consist of a mixture of glycoconjugates of the same nature as was in the mucous cells, but their staining intensity was much weaker than in the mucous cells and reacting site was confined to the apical portion of the cytoplasm. Although the staining patterns somewhat different each other, it is likely that the seromucous cells in the present study corresponds to dark seromucous cells of Saito (1966 a, b). However, it seemed that Fujii and

Tamura (1966), Rangel *et al*. (1968) and Nalavade and Varute (1977) did not refer to such a type of seromucous cells at all.

The presence of glycoconjugates with sulfated and sialic acid residues in the secreting epithelium lining anterior lingual glands of the chicken, deserves specific comment. The abundance of highly sulfated glycoconjugates in secretory granules and luminal surface of the glandular epithelial cells appears to play certain parts for protection against toxic agents. In addition, the results of recent electrophoretic identification and quantitation studies on various tissues of mammals lead to the hypothesis that sulfated glycoconjugates might be involved in the process of cytodifferentiation (Cassaro and Dietrich, 1977; Toledo and Dietrich, 1977). Furthermore, the presence of sialic acid residues in secretion of the epithelial cells lining the anterior lingual glands bespeaks that it coats the mucosal surface providing a hydrophilic environment and preventing dehydration, as well as protection against pathogenic organism (Montreil, 1980). If sialic acid is present at the cell surface, it imparts net negative charges to the cell surface at physiological pH level and possible plays a role in several functional acitvities such as the protection of cells against phagocytosis and dehydration, recognition of one cell to another cell, binding hormone and mitogen to cells and transportation of ion across the plasma membrane (Jeanloz and Codington, 1976). However, the morphology of the sulfated glycoconjugates and sialic acid residues at the cell surface of lingual glands to be elucidated further by means of ultrastructural histochemistry.

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Received date : 9/12/98
Accepted date : 8/06/99