

RESEARCH ARTICLE

Glycoconjugates in the Secretory Epithelium of the Mandibular Salivary Gland of Malayan Pangolin (*Manis javanica*)

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Abstract

Objective — To reveal the distribution of glycoconjugates in the mandibular salivary gland of the Malayan pangolin (*Manis javanica*).

Materials and Methods — The histology and glycoconjugates histochemistry of the mandibular salivary gland in Malayan pangolin (*Manis javanica*) were examined by conventional histology and lectin histochemistry in combination with enzymatic (neuraminidase) digestion. Staining for conventional histology included hematoxylin and eosin (H&E), alcian blue (AB), and periodic acid Schiff (PAS). For lectin histochemistry, we used 7 lectins including *Glycine max* (SBA), Wheat germ agglutinin (WGA), *Dolichos biflorus* agglutinin (DBA), Concanavalin A (Con A), *Ulex europaeus* agglutinin-I (UEA-I), *Ricinus communis* agglutinin-I (RCA-I) and Peanut agglutinin (PNA). Intensities of staining were evaluated.

Results — Morphology of the mandibular salivary gland appears as a tubuloacinar gland with its secretory endpieces contained exclusively mucous acinar cells. A large number of acidic and neutral glycoconjugates were observed in the sections stained with AB and PAS. In the sections stained with lectins, glycoconjugates with N-acetylgalactosamine, N-acetylglucosamine, Galactosyl ($\beta 1 \rightarrow 4$) N-acetylglucosamine and mannose were present; conversely, fucose residues were not found in mucous cells of the gland. Neuraminidase digestion revealed the presence of sialic acid residues linked to Galactosyl ($\beta 1 \rightarrow 3$) N-acetylgalactosamine (PNA staining). In intercalated duct of the gland, the apical surface bordering the lumen was heavily to moderately stained with lectin SBA, WGA, DBA, UEA-I and Con A and weakly to negatively stained with AB pH 2.5, PAS, AB pH 2.5 - PAS and RCA - I.

Conclusion — This study demonstrated a remarkable variety of glycoconjugates in the mandibular salivary gland of Malayan pangolin. The variety may reflect different functions.

KKU Vet J. 2009;19(2):162-170

<http://vet.kku.ac.th/journal/>

Keywords: Mandibular salivary gland; Malayan pangolin; Glycoconjugates; Lectin histochemistry

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Introduction

The Malayan pangolin (*Manis javanica*) or a scaly anteater, found in Thailand and other countries in Southeast Asia, is a unique and interesting mammal because of at least 2 reasons. First, Chinese people believe that scales of pangolins can reduce swelling, promote blood circulation, and help breastfeeding women to produce milk. For this belief, Thailand has become a major transit hub for pangolins smuggled from Malaysia and Indonesia to China. Second, the pangolin eats insects and ants; therefore, around the forest area where it lives, it may play an important role for controlling the population of those animals. The pangolin also has a distinct gastrointestinal tract. It has no teeth but uses a sticky tongue to eat its prey. In addition, its stomach shows peculiar structures, a thick wall with a lumen often containing stones. This may indicate adapting for its toothlessness, eating habit [1] and triturating function [2].

Histochemistry can be defined as the chemistry of tissue components and its relation to tissue morphology. In histochemical study, lectins have extensively been used as probes in studying the cell surface interaction and carbohydrate composition in many tissues because lectins, naturally polypeptides, can bind specifically to carbohydrate residues in term of glycoconjugates [5]. Lectin histochemistry can be used in combination with enzyme neuraminidase digestion procedure because neuraminidase cleaves terminal sialic acid residues from carbohydrate moieties on the surface cell. Many authors have focused on the importance of glycoconjugates in salivary glands of mammalian species and correlated them with body functions such as, transporting of macromolecules for digestive efficiency, preventing proteolytic damage on epithelium, and defending against bacteria [3,4].

Glycoconjugates histochemical data on mandibular salivary gland of the Malayan pangolin, however, are not available. Therefore, in this study we conducted conventional staining for histological analysis and lectin histochemical methods for detecting sugar residues in the glycoconjugates.

Material and Methods

Tissue Collection

The Malayan pangolins (*Manis javanica*) were collected from Khao Prathup Chang Wildlife Breeding Research Station, Ratchaburi province in Thailand. One male Malayan pangolin was deeply anesthetized with sodium pentobarbital by abdomen injection. The mandibular salivary gland was dissected; then, tissue samples of the gland were quickly removed. These tissues were

fixed in 10 % neutral buffered formalin, dehydrated in ethanol, and embedded in paraffin wax. Serial sections (3–4 μm) of the tissue samples were mounted on albumin coated slides. After that, the sections were stained with either conventional or lectin histochemical methods in combination with enzyme neuraminidase digestion.

Tissue Staining

As a detail in **Table 1**, we used both conventional staining (H&E, AB, and PAS) and lectin (7 types of lectins) histochemical staining methods.

Table 1. Summary of Staining for Conventional Histology and Lectin Histochemistry

Conventional Staining Procedures	Lectin Histochemical Staining Procedures
1. Hematoxylin and Eosin (H&E) for the general observation of histological structures.	1. <i>Glycine max</i> (SBA) for N - acetylgalactosamine residues.
2. Acian Blue pH 2.5 (AB pH 2.5) for detection acidic glycoconjugates [6].	2. Wheat germ agglutinin (WGA) for N - acetylglucosamine residues [9].
3. Periodic acid - Schiff (PAS) for vicinal - diol containing glycoconjugates [7].	3. <i>Dolichos biflorus</i> agglutinin (DBA) for N - acetylgalactosamine residues.
4. AB pH 2.5 - PAS for demonstrating acidic and neutral glycoconjugates [8].	4. Concanavalin A (Con A) for mannose residues of glycoconjugates [10].
	5. <i>Ulex europaeus</i> agglutinin -I (UEA-I) for fucose residues [9].
	6. <i>Ricinus communis</i> agglutinin -I (RCA-I) for Galactosyl ($\beta 1 \rightarrow 4$) N- acetylglucosamine residues.
	7. Peanut agglutinin (PNA) for Galactosyl ($\beta 1 \rightarrow 3$) N - acetylgalactosamine residues.

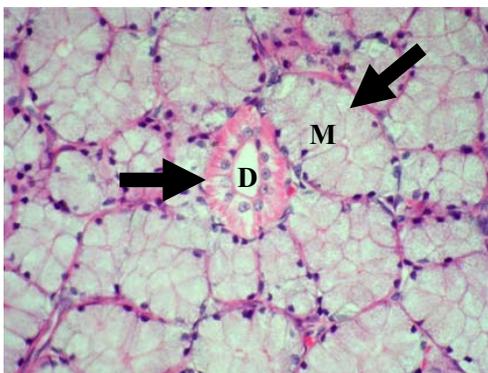
Enzyme Digestion

We used neuraminidase (from *Vibrio cholerae*) for enzymatic digestion procedures. Before stained with AB pH 2.5, AB pH 2.5 - PAS and lectin PNA, the sections were incubated in 0.1 M sodium acetate buffer (pH 5.5) containing 1 unit/ml of the enzyme and 0.04 M CaCl_2 at 39–41 °C for 12–16 h [6]. The controls for enzyme digestion were exposed to neuraminidase-free buffer under the same experimental conditions. The section were observed and photographed under light microscope (LM). To determine intensities of each staining, each stained slide was evaluated subjectively under 4 categories (0=unstained, 1=weak stained, 2=moderate stained, 3=strong stained, and 4=very strong stained).

Results

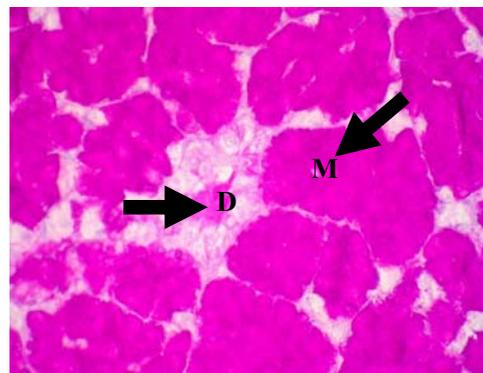
The mandibular salivary gland of Malayan pangolin (*Manis javanica*) showed tubuloacinar gland with its secretory endpieces containing exclusively mucous cells (**Figure 1**). The intensities of staining of mucous cells of the gland were listed in **Table 2**. The secretory granules of the mucous cells were stained strongly with AB pH 2.5 and PAS (**Figure 2**). In combined staining, the mucous cells were in deep blue with AB pH 2.5-PAS (**Figure 3**). With lectin staining procedure, the mucous cells showed strong to very strong staining with SBA, WGA and DBA (**Figure 5**), moderate staining with lectin RCA- I, and weak to negative staining with Con A and UEA-I (**Figure 6**). Under AB pH 2.5-PAS staining, color of numerous mucous cells changed from deep blue to light blue after neuraminidase digestion (**Figure 4**). The mucous cells were reacted very strongly with AB pH 2.5 staining but reacted weakly after treatment with neuraminidase. The mucous cells were unstained with lectin PNA; however, they were stained weakly with this lectin after neuraminidase digestion. In an intercalated duct of the gland, the apical surface bordering the lumen was positively stained with SBA, WGA, DBA, UEA - I and Con A , with the exception of AB pH 2.5, PAS, AB pH 2.5-PAS and lectin RCA - I.

Figure 1. The Mandibular Salivary Gland of Malayan pangolin (*Manis javanica*) with H&E Staining



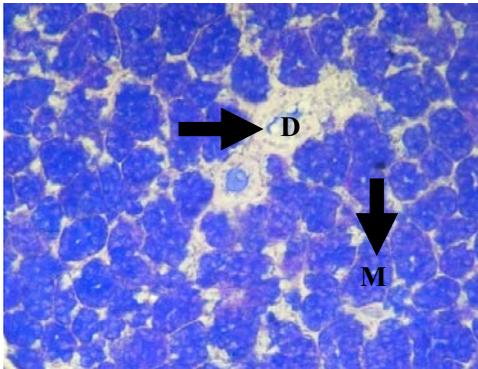
A tubuloacinar gland was observed with its secretory endpieces containing exclusively mucous cells [M]. The intercalated duct [D] was stained moderately with H&E. Image magnification=400X

Figure 2. The Mandibular Salivary Gland of Malayan pangolin (*Manis javanica*) with PAS Staining



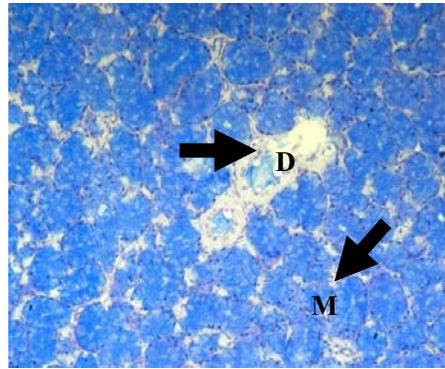
With PAS staining, mucous cells [M] of the gland were strongly positive, but the intercalated duct [D] was weak to negative. Image magnification=400X

Figure 3. The Mandibular Salivary Gland of Malayan pangolin (*Manis javanica*) with AB pH 2.5-PAS Dual Staining



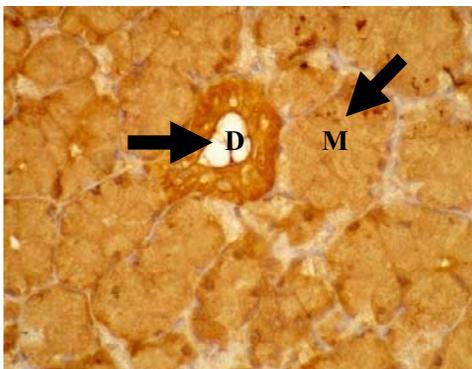
A large number of mucous cells [M] were in deep blue, but the intercalated duct [D] was not stained. Image magnification=200X

Figure 4. The Mandibular Salivary Gland of Malayan pangolin (*Manis javanica*) with AB pH 2.5-PAS Dual Staining Plus Neuraminidase Digestion



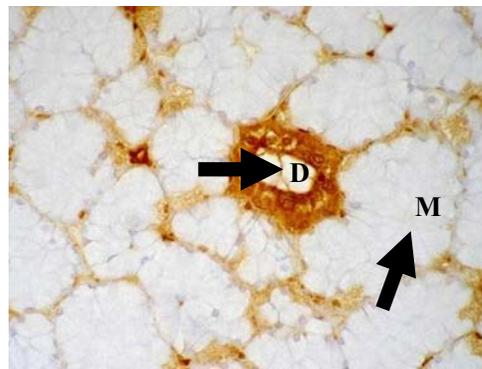
The staining of the mucous cells [M] changed from deep blue to light blue. The intercalated duct [D] remained unreactive. Image magnification=200X

Figure 5. The Mandibular Salivary Gland of Malayan pangolin (*Manis javanica*) with lectin DBA Staining



The mucous cells [M] and the intercalated duct [D] were strongly to very strongly positive. Image magnification=400X

Figure 6. The Mandibular Salivary Gland of Malayan pangolin (*Manis javanica*) with lectin UEA - I Staining



The mucous cells [M] were unstained; conversely, the intercalated duct [D] exhibited strongly reaction. Image magnification=400X

Table 2. Intensities of Conventional and Lectin Histochemical Staining in the Mandibular Salivary Gland of Malayan Pangolin (*Manis javanica*)

Staining Methods	Intensities in Mucous Cells
AB pH 2.5	4
Neu - AB pH 2.5	1 - 2
PAS	4
AB pH 2.5 - PAS	3 - 4
Neu AB pH 2.5 - PAS	1 - 2
SBA	3 - 4
WGA	3 - 4
DBA	3 - 4
Con A	1
UEA - I	0
RCA - I	2
PNA	0
NEU - PNA	1

Abbreviations: AB, Alcian blue; Neu, Neuraminidase; PAS, Periodic acid Schiff; SBA, *Glycine max*; WGA, Wheat germ agglutinin; DBA, *Dolichos biflorus* agglutinin; Con A, Concanavalin A; *Ulex europaeus* agglutinin-I, UEA-I; RCA-I, *Ricinus communis* agglutinin -I; PNA, Peanut agglutinin.

Staining interpretations: 0 = Unstained, 1 = Weak, 2 = Moderate, 3 = Strong, 4 = Very strong

Discussion

As a major salivary gland of the body, a mandibular salivary gland of different mammals shows a great variety in the form of its secretory endpieces. In cats [11,12] and dogs [11,13,14], the secretory endpieces showed mucous cells with seromucous cells. However, in goats, the secretory endpieces are mainly composed of mucous cells with serous demilunes, but some serous cells were also found. In contrast, the mandibular salivary gland of mice revealed the presence of seromucous cells. [15,16]. In the present study, the mandibular salivary gland of Malayan pangolin showed tubuloacinar gland with its secretory endpieces containing exclusively mucous cells. Similar results were found in the chicken [17]. Histochemical methods have been developed for qualitative and quantitative analysis of virtually all cellular components including proteins, carbohydrates, and lipids [18,19]. The histochemical results in a mandibular salivary gland of the mouse demonstrate the presence of neutral glycoconjugates [15,16].

In this study, vicinal diol group and acidic glycoconjugates were observed in a numerous mucous cells of the mandibular salivary gland of Malayan pangolin. Lectin histochemistry demonstrated a remarkable variety of glycoconjugates in the tissue. Lectin staining showed the presence of N-acetylgalactosamine (SBA and DBA labeling), N-acetylglucosamine (WGA labeling) and Galactosyl ($\beta 1 \rightarrow 4$) N-acetylglucosamine (RCA-I labeling) in a large number of the mucous cells. Conversely, the failure of mucous cells to staining with lectin UEA-I and Con A indicated the absence or the presence at only low levels of fucose and mannose residues. This basic study shows that the mucous cells of Malayan pangolin is characterized by a rich supply of glycoconjugates. Acid glycoconjugates prevent damage to the gut epithelium [20] and the presence of neutral glycoconjugates could be evidence of absorption [21]. The mucus secreted by mucous cells aids lubrication of the food during passage and may provide cofactors required for enzymatic degradation of the food [22]. It is well known that salivary mucins serve as a masticatory lubricant, contributing to the formation of a thin film, which maintains the mucosa integrity of the first tract of the digestive system.

In addition, the acidic glycoconjugates are thought to contain terminal sialic acid residues since AB pH 2.5 reaction decreased in intensity after digestion with neuraminidase [23]. Furthermore, the present effects of digestion of this enzyme upon the AB pH 2.5-PAS reaction of the mucous cells indicate the presence of the sialic acid residues. Intensity of the lectin PNA staining in the mucous cells was increased after neuraminidase digestion, this indicated the presence of sialic acid residues linked to Galactosyl ($\beta 1 \rightarrow 3$) N - acetylgalactosamine in the terminal position. Predominant glycoconjugates with terminal sialic acid in mucous cells may coat the mucosal surface so as to provide an environment designed to preserve hydration [24,25] and to protect the cell from pathogenic organisms [26].

Acknowledgement

This research was supported by the Graduate School, Kasetsart University. We thanked the Department of Anatomy, Faculty of Veterinary Medicine, for Kasetsart University for providing some of experimental facilities.

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