CHAPTER 2

RESEARCH DESIGNS AND METHODS

Animals

Male Sprague Dawley rat including ages 2 weeks (n=5), 1 month (n=5), 3 months (n=5), and 14 months (n=5) were obtained from the Laboratory Animal House, Faculty of Medicine, Chiang Mai University.

Tissue preparation

The rats were killed by an overdose of pentobarbital sodium. The prostate were exposed by a lower abdomen incision and removed by careful dissection. They were fixed in 10% neutral buffered formalin overnight and separations of each lobe under a dissecting microscope (Olympus, SZ30). Each prostatic lobe can be separated by their location, which was shown in Figure 6. The ventral lobe was immediately below the bladder. The dorsal lobe was located below and behind the attachment of the seminal vesicle. The lateral lobe was just below the seminal vesicle and coaglulating gland, which extended ventrally overlap the ventral lobe and blended dorsally with the dorsal lobe. The anterior lobe (coaglulating gland) attached to the inner concave surface of the seminal vesicle. Then tissue samples for all lobes were dehydrated in graded ethanol series and embedded in paraffin.

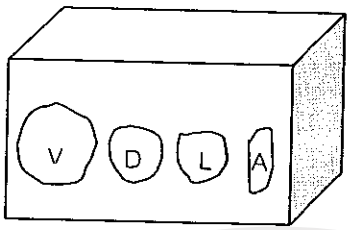

Figure 10 Diagram showing the embedding orientation in a tissue block. V, ventral prostate; D, dorsal prostate; L, lateral prostate; A, anterior prostate.

Section preparation

1. All excess paraffin was trimmed off the blocks.
2. Blocks were incubated at 4°c for 20-30 minutes.
3. Tissue blocks were cut into sections with microtome at 5 micrometre of thickness.
4. The sections were then floated in the water bath (40-45°c) with gelatin added (5% solution) to serve as section adhesive.
5. Floating sections were picked up and placed on glass slides.
6. Slide sections were dried up and deparaffied in an oven at 60°c for 30 minutes. Then the sections were ready to be further processed for tissue staining.

Tissue staining

1. Harris's hematoxylin and eosin staining.

See the appendix for making up the stain and solutions.

Procedure

1. Deparaffinized slides and hydrate to distilled water
2. Stained in freshly filtered Harris' hematoxylin for 6 minutes.
3. Washed in running tap water for 5 minutes.
4. Differentiated in 1% acid alcohol, 1 to 2 dips.

5. Washed briefly in tap water.
6. Place in weak ammonia water or saturated lithium carbonate solution until sections are bright blue.
7. Washed thoroughly in running tap water for 10 minutes.
8. Placed in 80% ethyl alcohol for 1 to 2 minutes.
9. Counterstained in eosin-phoxine solution for 2 minutes.
10. Dehydrated and clear through 2 changes each of 95% ethyl alcohol, absolute ethyl alcohol, and xylene, 2 minutes each.
11. Mounted with mounting medium.

2. Lectin histochemistry

Identification of the glycoconjugates was performed by lectin technique. Seven biotin conjugate lectin, *Dolichos biflorus* (DBA), *Canavalia ensiformis* (Con A), *Maclura pomifera* (MPA), peanut agglutinin *Arachis hypoalgaea* (PNA), *Pisum sativum* (PSA), *Ulex- europaeus* (UEA-I), and wheatgerm agglutinin *Triticum vulgaris* (WGA) (Sigma, USA) were used in this study. The binding specificity of the lectins employed in this study was shown in Table 1. Each lectin was made up in to a lectin solution at concentration of 20 μg/ml in PBS. The reactivity of the lectin with its specific sugar residues was detected using Avidin-Biotin Complex technique following the procedure below.

Procedure

1. Tissue sections were deparaffinized by xylene and hydrated by ethanol series.
2. Endogenous peroxidase in the tissue was blocked by incubation with 3% H_2O_2 for 60 minutes.
3. Washed in PBS three changes, then incubated with lectin solution overnight.

4. Washed in PBS three changes, then incubated with avidin-biotin-peroxidase complex (ABC) for 90 minutes, and then washed thoroughly with PBS.
5. Chromed with diaminobenzidine (DAB) in the presence of H_2O_2 for about 1 minute, and washed well in PBS.
6. Dehydrated slides in graded alcohols, cleared in xylene and mounted with Permount.

Table 1 List of lectins used in this study and their binding specificity. (Castell *et al.*, 1991; Brooks *et al.*, 1997; Mcneal *et al.*, 1998; Chan and Ho, 1999)

Lectin	Source	Specific glycoconjugates group
DBA	*Dolichos biflorus*	α-D-GalNAc
Con A	*Canavalia ensiformis*	α-D-Man, α-D-Glc, GlcNAc
MPA	*Maclura pomifera*	α-D-GalNAc, α-D-Gal
PNA	*Arachis hypoalgaea*	β-D-Gal(1-3)-D-GalNAc
PSA	*Pisum sativum*	α-Man,α-Glc,α-GlcNAc
UEA-I	*Ulex europaeus*	α-L-Fuc
WGA	*Triticum Vulgaris*	$(\beta$-(1-4)-D-GlcNAc$)_2$NeuAc

Abbreviation: Fuc; fucose, Gal; galactose, GalNAc; *N*-acetyi galactosamine, Glc; glucose, GlcNAc; *N*-acetyl glucosamine, Man; mannose, NeuAc; neuraminic acid (sialic acid)

Data collection

1. Ten prostatic acini with markedly high intensity of lectin labeling were chosen from five sections in each age group.
2. Black and white photographs were taken through a 40 objective lens of Zeiss Axiolab microscope.

3. Ten epithelial cells in each picture were measured for their lectin binding intensities. Points of measurement were concentrated on the rER-Golgi area of the epithelial cells, which located in the supranuclear area. The image analysis software used for data analysis was the *Axiovision (Zeiss) program.*

4. By the *Axiovision software,* the intensity of lectin labeling was evaluated and scored in gray scale's pixel value. The scale ranged from 0 to 255 pixels as shown in Figure 11.

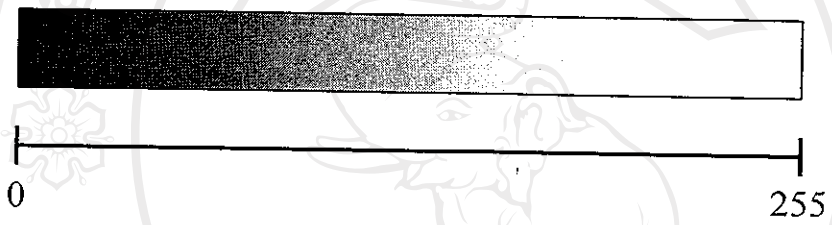

Figure 11 Diagram showing the palette of gray scale and its range of pixel values.

Evaluation of lectin staining

The staining intensity of the chosen prostatic secretory cells was scored. A Mean ($\overline{X}$) was calculated from summation of the whole intensity scores. Four intervals were divided among all score values as shown by the scale bar in Figure 12. The intensity of lectin labeling was then graded into four levels including strong (+++), moderate (++), weak (+), and very weak (±) according to the score intervals.

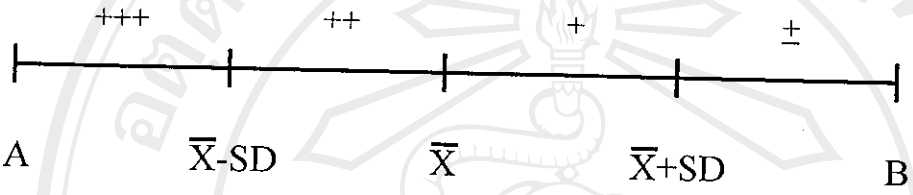

Figure 12 The scale bar showing four score intervals to grade the lectin staining intensity.

±	Very weak staining
+	Weak staining
++	Moderate staining
+++	Strong staining
A	A maximum of gray scale's pixel value of the whole intensity scores.
B	A minimum of gray scale's pixel value of the whole intensity scores.
$\overline{X}$	A Mean of gray scale's pixel value of the whole intensity scores.
S.D.	A standard deviation of gray scale's pixel value of the whole intensity Scores.