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SIMULTANEOUS VISUALIZATION OF TWO HORSERADISH PEROXIDES CONJUGATED LECTINS IN SEMI-THIN SECTIONS

(With 1 Table and 6 Figures)

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الإظهار التتابعي لاثنين من اللكتينات المقترنة مع إنزيم الهورس راديش بيراوكسيديز في قطاعات شبه رقيقة

مسعود حسن فايد

في هذه الدراسة تم تطبيق طريقة ملائمة جديدة للإظهار التتابعى لاثنين من اللكتينات المقترنة مع إنزيم الهورس راديش بيراوكسيديز في قطاعات شبه رقيقة من عينات معروف قابليتها للكتينات المستخدمة ، وكانت تلك العينات مطمورة في راتنج الايبون ٨١٢. وقد تم إزالة الايبون من القطاعات بواسطة محلول ١٠% من هيدروكسيد الصوديوم. وتم إظهار اللكتين الأول في محلول ٣ أمينو-٩ اثيل كربازول مع ماء الأوكسجين والثانى في محلول ٣.٣ ثنائى أمينوبنزيدين رباعى هيدروكلوريد مع ماء الأوكسجين.

SUMMARY

A convenient method for simultaneous visualization of two horseradish peroxides (HRP) conjugated lectins was applied to routinely prepared semi-thin sections of epon embedded specimens of tissues of well known of their lectin staining affinity. The epoxy resin was removed by 10 % sodium hydroxide in ethyl alcohol (Na-ethoxide). The first lectin was visualized by 3-amino 9-ethylcarbazole/hydrogen peroxide medium (AEC/H_2O_2) and the second was developed in 3,3′ diaminobenzidine tetrahydrochloride /hydrogen peroxide medium (DAB-H_2O_2).

Key words: *Lectin, Semithin section, Epon 812*

INTRODUCTION

Since the introduction of lectins to the field of the biochemistry [Goldstein and Hayes, 1978; Hammarstrom and Kabat, 1971; Hammarstrom, Westoo and Bjork, 1972; Howard and Batsakis, 1982; Lotan, Skutelky, Danon and Sharon,1975; Nagata and Burger,1974 and Sharon and Lis, 1972] some of them such as Concanavalin-A [Kirenan, 1975; Yamada and Shimizu,1976] and Ricinus communis [Yamada and Shimizu,1977] have been successfully utilized for in situ histochemical detection of particular saccharide residues in complex- carbohydrate. The visualization of the color of HRP reaction products depends upon the type of the medium used for this purpose. If the HRP enzyme is developed in different media the reaction product will exhibit varying colors. Utilizing such characters, with the less shrinkage artifacts of the plastic embedding media, we attempted to develop a dual staining method for detection of two lectin binding sites in the same tissue section of epon embedded specimen.

MATERIAL and METHODS

Tissue preparation:

A tissue specimen, their lectin staining affinity well known from our previous studies [Fayed and Makita, 1996 ; 1997 a and 1997 b], were used. A pieces of tissue from the glandular part of the stomach and from the proximal part of the duodenum of the one humped camel (Camelus dromedarius) were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4.) for 72 hours. After fixation the samples were throughly rinsed in the same buffer as used with the fixative and then post fixed in 1% osmium tetroxide (Os O_4). Specimens were then dehydrated in ascending grades of ethanol and embedded in poly/Bed 812 (polysciences, Warrington, PA). Semi-thin sections of one µm thickness were mounted on poly-L-lysine coated glass slides.

Epon solvent:

Different concentration of Na-ethoxide solution (1%, 2%, 3%, 10% and full saturation) was used for 30, 60, 90 and 120 min.

Staining procedure:

1- Sections were immersed in covered coupling jar containing Na-ethoxide solution for 30, 60, 90 and 120 min. The slide must be

completely immersed in the solution to avoid formation of NaOH crystals since evaporation of the solution is rapid.

2- Sections were removed and drained well on a filter paper.

3- Sections were washed well in absolute alcohol for 5 min.

4- Sections were drained again on a filter paper. Washing in absolute alcohol and draining on a filter paper was repeated six times 5 min. each to assure that all Na-ethoxide was completely removed.

5- Sections were washed two times 5 min. each in phosphate buffer saline (PBS) [0.05 M phosphate (Na_2HPO_4 / NaH_2HPO_4) buffer of pH 7.2 containing 0.2 M NaCl].

6- The endogenous peroxide reactions were blocked by incubation of the sections in O.3 % hydrogen peroxide (H_2O_2) for 5 min.

7- Sections were incubated for 18 hr. at 4 °C with the first peroxides-conjugated lectins : Arachis hypogea agglutinin (PNA) (12µg/ml) or Concanavalia esiformis agglutinin (Con-A) (20 µg/ml). [*PNA was diluted with O.1 M PBS while Con-A was diluted in 0.05 M Tris-HCl buffer*].

8- Sections stained with PNA were rinsed in three changes of PBS for 5 min. each followed by three changes in 0.05 M Tris-HCl buffer (pH 7.6) for 5 min. each, while Sections stained with Con-A were rinsed three changes of 0.05 M Tris-HCl buffer (pH 7.6) for 5 min. each.

9- Peroxides reaction was developed in DAB-H_2O_2 / Tris-HCl buffer solution [a mixture of 4.9 ml of 0.05 M Tris-HCl buffer pH 7.6, 0.1 ml of 3% H_2O_2 and 2.5 mg of 3,3′-diaminobenzidine tetrahydrochloride] for 10 min.

10- Sections were rinsed well in PBS two times 5 min. each.

11- The residual of the endogenous HRP reaction was blocked by incubation in O.3 % hydrogen peroxide (H_2O_2) for 5 min.

12- Sections were incubated for 18 hr. at 4 °C with the second HRP-conjugated lectins: Triticum vulgaris agglutinin (WGA) (6 µg/ml) or Helix pomatia agglutinin (HPA) (6 µg/ml). [WGA and HRA was diluted in O.1 M PBS].

13- The second peroxide reaction was visualized in AEC-H_2O_2 media [10 mg of 3-amino 9-ethylcarbazole was dissolved via 1 ml dimethyl formamide in 50 ml acetate buffer pH 5.2, containing 0.001% H_2O_2] for 30 min..

14- Sections were rinsed well in PBS two times 5 min. each.

15- Sections were rinsed in distilled water two times 5 min. each.

16-Counter stain in Mayor's hematoxline or Alcian blue was available if necessary.

Since the 3-amino 9-ethylcarbazole are soluble in organic solvent, dehydration was avoided and the sections were directly covered by water base media [glycerol].

Control Staining:

The following control experiment was used

1- Conjugated lectins were substituted by unconjugated ones
2- The sections were incubated in the peroxides alone.
3- The haptten sugar inhibitors of each lectins were used as control

The used lectin's scientific and common name, abbreviations, concentrations major sugars specifications and inhibitors were listed in Table 1.

RESULTS and DISCUSSION

Single staining with PNA-DAB (Fig 1), Con A- DAB (Fig 2), WGA-AEC (Fig 3) and HPA-AEC (Fig 4) were shown intense positive reaction indicating the existance of Gal-β-(1-3)-GalNAc, α-D-Man, α-D-Gal, $(\beta\text{-}(1\text{-}4)\text{-D-GlcNAc})_2$ and NeuNAc, and α-D-GalNAc respectively. For similtanous visualization of two HRP-conjugated lectins the present double lectins staining, PNA-DAB/WGA-AEC or Con A-DAB/HPA-AEC were applied. In all tissues subjected to this method clear differentiation were obtained (Fig 4 & 5) emphasising the existance of two different sugar residues in the same tissuse. The deep brown color of the reaction product of the first lectin can be easily differentiated from the red brillant color of the staining with the second lectin. In double staining methods for detection of two lectins, the different binding sites are to be clearly differentiated from each other and the staining methods preferably be simple.

Some procedures for deplastisizing epon embeded sections have been reported [Lane and Europa, 1965 and Mayer, Hampton and Rosario, 1961]. Others satisfactory stained the epoxy resin embedded sections without deplastisizying it [Cardno and Steiner, 1965; Munger, 1961 and Trump, Smuckler, and Benditt, 1961]. The cellular details are superior in epon free sections because of more epitopes are exposed when the epoxy section are deplastised [Brorson and Skjorten,1995]. Deplastiszing of epoxy resins with Na-ethoxide is based on the fact that,

ester bonds in the polymarized epoxy resins are senistive to strong alkaline solutions [Baigent and Muller, 1990]. In the present study 10% of Na-ethoxide was enough to remove all epon from sections at 1 hr. and produce intense staining. Lane and Europa 1965 recommended saturated solution of NaOH for removal of epon and leaving it for 2-3 days before use [Lane and Europa, 1965]. The present study showed that, the time factor, not the strength of Na-ethoxide, was critical since it might result in detachment of the section if the time incresed than one hour.

Although only four types of lectins were used in this study, other HRP-conjugated lectins could be utilized similar to those four lectins.

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LEGENDS

Fig. 1: A camel's glandular stomach mucosa stained with PNA-DAB. Surface mucous cells and foveolar mucous cells (large arrows) were shown intense deep brown color of the staining reaction indicating the existence of Gal-β-(1-3)-GalNAc. bar = 100 μ m.

Fig. 2: A camel's glandular stomach mucosa stained with Con A- DAB. glandular mucous cells (small arrows) were shown intense deep brown color of the staining reaction indicating the existence of α-D-Man, α-D-Gal. bar = 100 μ m

Fig. 3: A camel's glandular stomach mucosa stained with WGA-AEC. glandular mucous cells (small arrows) were shown red brilliant color of the staining reaction indicating the existence of $(\beta\text{-}(1\text{-}4)\text{-D-GlcNAc})_2$ and NeuNAc. bar = 100 μ m.

Fig. 4: A camel's glandular stomach mucosa stained with HPA-AEC. Glandular mucous cells (small arrows) were shown red brilliant color of the staining reaction indicating the existence of α-D-GalNAc. bar = 100 μ m.

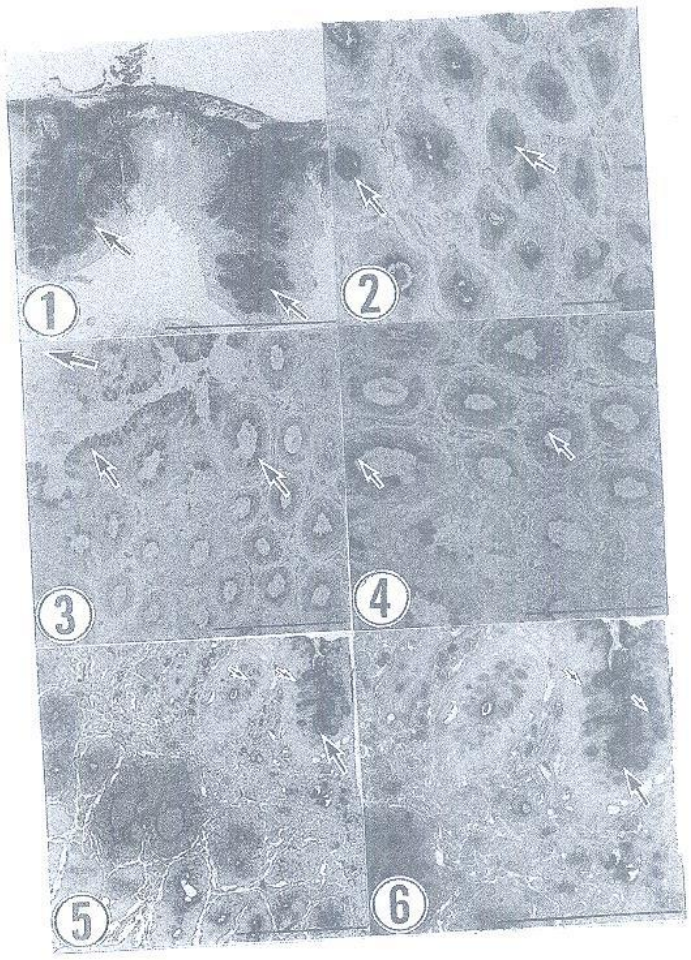
Fig. 5: A camel's glandular stomach mucosa stained with double lectins staining (PNA-DAB/WGA-AEC). The deep brown color of the reaction product of the first lectin (large arrows) can be easily differentiated from the red brilliant color of the staining with the second lectin (small arrows) emphasizing the existence of the two different sugar residues in the same tissues. bar = 100 μ m.

Fig. 6: A camel's glandular stomach mucosa stained with double lectins staining (Con A-DAB/HPA-AEC). The deep brown color of the reaction product of the first lectin (large arrows) can be easily differentiated from the red brilliant color of the staining with the second lectin (small arrows) emphasizing the existence of he two different sugar residues in the same tissues. bar = 100μm.

Table 1 : Carbohydrate binding specificty of lectins used in this study

Taxonomic name	Abbreviation	Common name	Concentration µg/ml	Major sugar specification	Sugar binding inhibitors
Arachis hypogaea	PNA	Peanut	100	β-(1-3)-GalNAc, Gal	Gal
Concavalin ensiform	Con A	Jak bean	20	α-D-Man, α -D-Glc	α-D-methyel Man.
Helix pomatia	HPA	Roman snail	6	α-D-GalNAc	α-D-GalNAc
Triticum vulgaris	WGA	Wheat germ	6	$(\beta$-(1-4)-D-GlcNAc$)_2$, NeuNAc	NeuNAc

Symbols : Gal = Galactose ; Glc = Glucose ; GalNAc = N - acetygalactoseamine ; GlcNAc = N - acetyelglucosamine ; Man = Mannose ; NeuNAc = N-acetyl neuraminic acid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