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Lectins Labelled with Digoxin as a Novel Tool to Study Glycoconjugates

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Summary

In recent years it has become clear that carbohydrate portions of glycoconjugates are performing numerous vital physiological functions in higher organisms. However, since glycobiology is a relatively new science, and carbohydrate structures are highly complex, the continuous development of novel analytical techniques is necessary to support the process of understanding the intricate nature of glycoconjugate structure and function. The introduction of digoxin as a novel tag for labelling of lectins that are being used to analyse glycoconjugates in immunoassay-like techniques is described. Lectins labelled with digoxin have significant advantages over biotin- or digoxigenin-labelled lectins and will hopefully prove to be a useful addition to the repertoire of glycobiological tools.

Key words: lectins, glycoconjugates, digoxin, immunoassay-like techniques

Introduction

Nearly all membrane proteins are either glycosylated, or in complex with other glycosylated proteins (1). Over a half of all proteins contain N-linked oligosaccharides (2), and other forms of glycosylation are also very abundant (3). Extensive research conducted during the past years clearly demonstrated the importance of carbohydrate portions of glycoconjugates in numerous vital physiological processes, and today it is generally accepted that these processes can not be fully understood without knowing the structure of both protein and carbohydrate parts of the involved molecules (4).

Plant lectins are a simple and versatile tool to analyse glycoconjugates (5). They can be used in a number of immunoassay-like techniques including ELLA (enzyme-linked lectin assay), lectin-western blot or lectin affinity chromatography. However, in contrast with antibodies, lectins do not share common structural features, thus there are no universal secondary antibodies

to lectins. In order to be used in immunoassay-like techniques lectins have to be first labelled with a suitable tag that will enable their subsequent identification and detection. Most frequently used tags today are biotin and digoxigenin and here we describe the introduction of digoxin as a novel tag to label lectins.

Materials and Methods

Digoxin and CNBr were purchased from Aldrich Chem. Co. (Milwaukee, WI), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro-blue tetrazolium (NBT), Concanavaline A and anti-digoxin monoclonal antibodies labelled with alkaline phosphatase from Sigma (St. Louis, MO), PVDF membranes were obtained from Millipore (Bedford, MA). Concanavaline A, WGA and SNA lectins labelled with biotin were purchased from Vector Laboratories (Burlingame, CA).

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Thin-layer chromatography

Thin-layer chromatography (TLC) was performed on 0.2 mm Silica Gel 60 F254 precoated on aluminum sheets (Merck, Darmstadt, Germany). Chloroform:methanol:water (80:20:1, volume fractions) solvent system was used for development and digoxin was detected by »charring« after spraying with 15 % H₂SO₄ in 80 % ethanol.

Activation of digoxin using CNBr

Digoxin (100 µmol) was dissolved to a final concentration of 33 mM in 33 % tetrahydrofuran, 66 % 2 M K-phosphate buffer (pH=12) to form a biphasic mixture. CNBr (10-fold excess) was added to the mixture as 5 M solution in tetrahydrofuran. Reaction mixture was stirred at room temperature for 30–60 min, and the formation of the product was monitored by thin-layer chromatography. »Activated« digoxin appeared as a major product with R_f slightly lower than digoxin. The estimated product yield was usually 40–60 %. If CNBr was excluded from the reaction mixture, TLC analysis revealed no change in digoxin within 60 min of the reaction, suggesting unusual stability of the lactone ring to alkaline conditions.

The reaction mixture was evaporated under reduced pressure, and the dried powder was redissolved in 20 mL mixture of chloroform and 1 M NaCl (1:1, volume fractions). After vigorous shaking, the phases were separated and the water phase was extracted with additional 10 mL chloroform. Virtually all digoxin was found in the combined chloroform phases. The combined chloroform phases were briefly washed with 5 mL water to remove any residual water-soluble material, and dried under reduced pressure. A significant amount of unreacted digoxin remained with the "activated" digoxin, but no steps were performed to remove it since it does not interfere with the subsequent reaction steps. If stored dry, the ratio of "activated digoxin" to digoxin remained constant for months at room temperature.

Labelling of Concanavaline A with digoxin

Pure Concanavaline A (2 mg) was dissolved in 1 mL 50 mM Na-carbonate buffer, pH=9.5 and dialysed against 2 x 1 L of the same buffer at +4 C. CNBr-activated digoxin (2 µmol) was dissolved in 200 µL methanol and rapidly mixed with Concanavaline A solution. After overnight incubation at room temperature, 2 µmol of glycine was added to inactivate any remaining CNBr-activated digoxin. The reaction mixture was dialysed against three 1000 mL changes of 10 mM Na-phosphate buffer, pH=7.5 at +4 C and freeze dried.

Cell and tissue sample preparation

Male Fischer rat was sacrificed and liver, spleen, thymus, cortex and serum were removed. The tissues were homogenised in 10 volumes of ice-cold homogenisation buffer (50 mM Tris buffer, pH=6.5, containing 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 % Triton X-100) and protease inhibitors (Sigma, Ca. #P2714), using a Teflon homogeniser. The protein concentration of the samples was determined by the BCA method (6).

HL60 cells were collected, washed with PBS and lysed by sonication in 1 % Triton X-100, 15 mM NaCl, 5 mM Tris/HCl, pH= 8.0.

Electrophoresis and blotting

Proteins were separated electrophoretically in 12 % SDS-polyacrylamide slab gels as described by Laemmli (7). After electrophoresis, proteins were transferred onto Immobilon PVDF membrane in a semi-dry apparatus according to Towbin (8). Blotting membranes were blocked overnight with 3 % BSA, and incubated in 3 mL 1 µg/mL solution of labelled lectins for 60 min. After washing, blots were developed with anti-digoxin antibodies (for lectins labelled with digoxin) or streptavidin (for lectins labelled with biotin) conjugated with alkaline phosphatase and visualised with 0.02 mg/mL BCIP and 0.04 mg/mL NBT in 50 mM Tris/HCl, pH=8.5, 100 mM NaCl, 5 mM MgCl₂.

Results and Discussion

After using biotin- and digoxigenin-labeled lectins for a number of years (9,10) we have noticed that both, lectins labelled with biotin and lectins labelled with digoxigenin, have significant disadvantages. Primary problem with biotin as a tag is the existence of endogenous biotin in various tissues that can lead to false-positive staining. To assess this problem we have analysed rat cortex, thymus, serum, spleen and liver tissues with streptavidin. As it is clearly shown in Fig. 1A, nearly all tissues have several proteins that contain covalently linked biotin and will turn out positive in any assay system that uses biotin as a label. This is not surprising since biotin is a prosthetic group that is covalently linked to a number of ubiquitously expressed endogenous enzymes (for example pyruvate-carboxylase). Since endogenous biotin cannot be differentiated from biotin that is used as a label, all methods that use biotin-containing conjugates are prone to false-positive results. Sometimes these proteins can be excluded using appropriate negative controls, but this is frequently either not convenient, or even not possible.

An alternative label, which can alleviate the shortcoming of biotin is digoxigenin. Digoxigenin is a deglycosylated form of the cardiac glycoside, digoxin (Fig. 2) that has been used as a drug for centuries. Highly specific and strong-binding monoclonal and polyclonal antibodies have been developed as an antidote to treat digoxin overdose (11,12). When labelled with an appropriate enzyme these antibodies can be used to detect both digoxin and digoxigenin. Digoxigenin was first introduced to glycobiology by Boehringer Mannheim (now Roche Biochemicals), who used it to label lectins (13). It proved to be an excellent label that eliminated problems of false positives due to the presence of endogenous biotin (Fig 1B), but in the recent years this line of products has been significantly downsized and individual digoxigenin-labelled lectins are not commercially available any more.

While the use of digoxigenin is limited by patents awarded to Boehringer Mannheim, high acid sensitivity of the glycosidic bonds between digitoxoses, and the al-

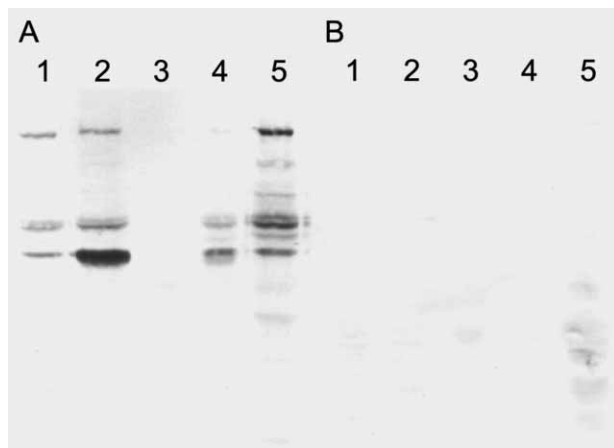


Fig. 1. Endogenous biotin in rat tissues. Homogenates (10 µg protein per lane) of rat cortex (1), thymus (2), serum (3), spleen (4) and liver (5) were separated by reducing SDS-PAGE, blotted onto PVDF membrane and developed with streptavidin-biotinylated alkaline phosphatase complex (panel A) or anti-digoxin antibody labelled with alkaline phosphatase (panel B).

alkaline sensitivity of the lactone ring, impeded chemical derivatisation and use of its parent compound digoxin as a label. By using activation with cyanogen bromide we managed to make digoxin reactive with amino-compounds (14), which enabled us to use it as a tag to label lectins. The procedure is described in detail in Materials and Methods section. The actual product is a mixture of amino-containing compounds linked to 3' or 4' hydroxyl group on the third digitoxose. We have successfully labelled several lectins including those from *Sambucus nigra* (SNA), *Triticum vulgare* (WGA) and Concanavaline A (ConA).

ConA is one of the most widely used lectins. It has broad applicability primarily because it recognises a commonly occurring sugar structure, α -linked mannose. At neutral and alkaline pH, ConA exists as a tetramer of four identical subunits of approximately 26 000 daltons (237 amino acid residues) each. »Native« ConA is a mixture of several forms of the lectin due to »nicks« occurring in the polypeptide chains. Although having little or no effect on the saccharide binding activity, these »nicks« in the sequence are often revealed even in the purest lectin preparations as additional bands in SDS-polyacrylamide gel electrophoresis. These hydrolytic cleavage sites appear to exist in the lectin as it occurs in the seeds and are not a function of isolation procedures.

Concanavaline A was labelled with digoxin as described in Materials and Methods section. The procedures for labelling other lectins were very similar, but since lectins vary significantly in the availability of free amino groups, solubility and stability, it is necessary to optimise labelling conditions for each individual lectin. The addition of a water-miscible organic solvent such as ethanol, methanol or acetonitrile to final concentration of 10–30 % is necessary to keep activated digoxin in solution. Depending upon the lectin being labelled, activated digoxin should be added in 5- to 20-fold molar excess and incubated overnight. Since too many attached digoxin molecules can decrease lectin activity, exact ra-

tio of digoxin and lectin concentrations have to be determined experimentally for each lectin.

The applicability and specificity of digoxin labelled lectins were verified by comparing it to commercially obtained ConA lectin labelled with biotin in a lectin-western blot assay. As it is clearly visible in Fig. 3, lectins that we have labelled with digoxin produced virtually the same staining patterns as commercially obtained lectins. Beside in lectin western-blot, lectins labelled with digoxin can also be used in other applications including histochemistry and enzyme-linked lectin assay (ELLA).

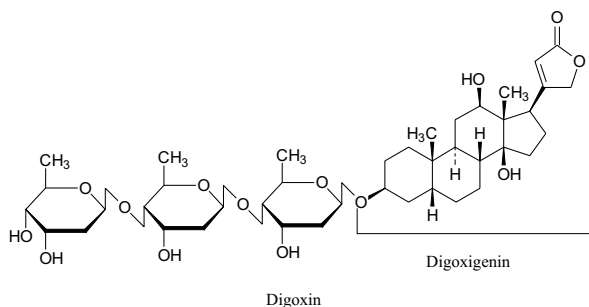


Fig. 2. Structure of Digoxin

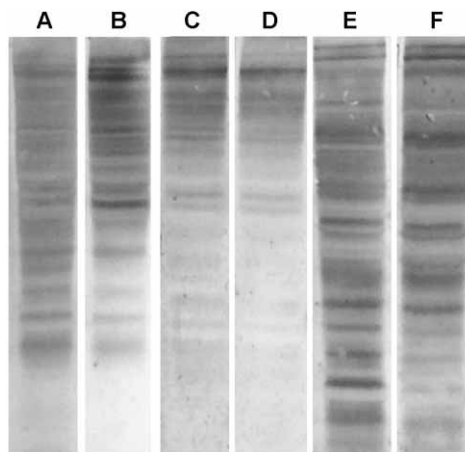


Fig. 3. Glycosylation of analysis of HL60 cells. HL60 cell homogenates were separated by SDS-PAGE (5 µg total protein per lane) and stained with ConA labelled with biotin (A), ConA labelled with digoxin (B), WGA labelled with biotin (C), WGA labelled with digoxin (D), SNA labelled with biotin (E), or SNA labelled with digoxin (F). Antibodies against digoxin or streptavidin labelled with alkaline phosphatase were used to detect digoxin and biotin, and the complex was visualised with BCIP/NBT, as described in Materials and Methods section.

Conclusions

In this paper the introduction of digoxin as a novel tag to label lectins has been described. Contrary to biotin, digoxin does not exist as a covalently linked cellular component, and it is thus much more reliable and/or convenient label. Lectins labelled with digoxin can be easily prepared in any laboratory, and since they can be used interchangeably with digoxigenin-labelled lectins,

and detected with antibodies against digoxin or digoxigenin, hopefully they will help to fill the current gap in the availability of reliable labelled lectins.

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Digoksinom označeni lektini kao novo sredstvo za istraživanje glikokonjugata

Sažetak

Istraživanja provedena tijekom posljednjih godina nedvojbeno su pokazala da šećerni dijelovi glikokonjugata imaju mnoge bitne fiziološke funkcije u viših organizama. Budući da je glikobiologija razmjerno nova znanost, a strukture šećera vrlo složene, potrebno je trajno razvijati nove analitičke postupke koji će unaprijediti spoznaje o zamršenoj strukturi i funkciji glikokonjugata. U ovom je radu opisano uvođenje digoksina kao novog biljega za lektine radi njihove primjene u analitičkim postupcima, kao što je »lectin-western blot« ili histokemija. Lektini obilježeni digoksinom imaju bitne prednosti pred onima obilježenim biotinom ili digoksigeninom i trebali bi biti korisni u glikobiološkim istraživanjima.