

Structural Characterization of Glycoprotein Carbohydrate Chains by Using Digoxigenin-Labeled Lectins on Blots

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The carbohydrate structures of blotted glycoproteins can be analyzed by probing them with lectins. Here we describe a method where lectins conjugated with digoxigenin are used in combination with an anti-digoxigenin antibody AP conjugate as a very sensitive detection system for this type of analysis. The specificity of the lectins used, and the sensitivity of the detection system, provide valuable conclusions on the glycan structures. Only small amounts of glycoproteins are required for the analysis. The binding specificity of a set of lectins is demonstrated with various glycoproteins of defined carbohydrate structure. The application of these labeled lectins in combination with specific glycosidases for the characterization of the carbohydrate chains of recombinant tissue plasminogen activator and erythropoietin is presented. © 1990 Academic Press, Inc.

Lectins have become very important tools in glycoconjugate research (1). One valuable application is their use for the analysis of glycoproteins on blots. Several different methods for visualizing the bound lectins have been described (2-5).

Recently we showed that the labeling of glycoproteins with the steroid hapten digoxigenin in combination with an immunological detection procedure is a very sensitive method for the detection and analysis of glycoproteins on blots (6). In order to utilize the high sensitivity and suitability of this immunological detection system with lectins, digoxigenin was covalently attached to various lectins for probing glycoproteins on blots. Since we wished to obtain information as precisely as possible on the structures of the carbohydrate chains, only lectins with well described and rather narrow binding specificities were employed. The lectins from elderberry

(*Sambucus nigra* (SNA)),¹ *Maackia amurensis* (MAA), snowdrop (*Galanthus nivalis* (GNA)), *Aleuria aurantia* (AAA), *Datura stramonium* (DSA), and peanut (*Arachis hypogaea* (PNA)), among others, fulfill these criteria. Herein we demonstrate the binding specificity and the sensitivity of this detection method using a set of glycoproteins. In addition, data concerning the characterization of the carbohydrate chains of two recombinant glycoproteins produced in chinese hamster ovary cells, tissue plasminogen activator (tPA) and erythropoietin (EPO), will be presented. Finally advantages of this detection system compared to others will be discussed.

MATERIALS

Glycoproteins. Carboxypeptidase Y, erythropoietin (recombinant from CHO cells), tissue plasminogen activator (recombinant from CHO cells), and transferrin (human) were from Boehringer Mannheim. Fetuin (fetal calf serum) and lactoferrin were obtained from Sigma (Deisenhofen); asialofetuin was prepared from fetuin by chemical desialylation as described (7).

Lectins. The lectins PNA, AAA, DSA, GNA, MAA, and SNA were from Boehringer Mannheim. The lectin-digoxigenin conjugates were prepared by chemical coupling of digoxigenin-carboxymethyl-*N*-hydroxysuccinimide ester to the amino groups of the lectins: 0.1 μ mol digoxigenin-carboxymethyl-*N*-hydroxysuccinimide es-

¹ Abbreviations used: anti-DIG-AP, polyclonal sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt; CHO, Chinese hamster ovary; CpY, carboxypeptidase Y; DIG, digoxigenin; EPO, erythropoietin; NBT, nitro blue tetrazolium chloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tPA, tissue plasminogen activator; ELISA, enzyme-linked immunosorbent assay.

ter in 50 μ l ethanol is added to 0.025 μ mol lectin/ml in 50 mM potassium phosphate buffer, pH 8.0. The mixture is stirred for 3 h at room temperature and then dialyzed against 50 mM Tris-HCl, 0.15 M sodium chloride, pH 7.5. The concentration was adjusted to 1 mg lectin/ml and the amount of digoxigenin incorporated determined with an ELISA system using anti-digoxigenin antibody. The incorporation was judged as one to three digoxigenin molecules incorporated per lectin molecule.

Others. Polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (anti-DIG AP), nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (BCIP), blocking reagent (Catalog No. 1096 176, casein, special quality), molecular weight markers for SDS-PAGE, and all enzymes used were also from Boehringer Mannheim. For blotting nitrocellulose BA85 from Schleicher & Schüll (Dassel) was used.

METHODS

Deglycosylation

A 10- μ g sample glycoprotein dissolved in 10 μ l H₂O is denatured by boiling for 2 min in the presence of 1% SDS. Buffer (90 μ l) for endoglycosidase F (endo- β -N-acetylglucosaminidase F): 50 mM sodium acetate, 25 mM sodium chloride, 0.2 mM sodium azide, pH 5.5 or buffer for peptide N-glycosidase F [peptide N⁴-(N-acetyl- β -glucosaminyl)asparagine amidase; Boehringer Mannheim name: N-glycosidase F]: 20 mM sodium phosphate, 5 mM EDTA, 0.2 mM sodium azide, pH 7.2 and 0.5% Nonidet P-40 (v/v) (Sigma) are added, and the mixture is again boiled for 2 min. The mixture is cooled to 37°C, the glycosidases (0.1 unit endoglycosidase F or 0.4 unit peptide N-glycosidase F) are added, and the reaction mixture is incubated for 18 h at 37°C. Neuraminidase (0.05 U) and O-glycosidase (2.5 mU) were included in the peptide N-glycosidase F incubations as indicated. 10- μ l (-1 μ g protein) were usually loaded into one lane of an SDS gel.

SDS-PAGE and Blotting

12 or 15% polyacrylamide gels were used according to the procedure of Laemmli (8). Gels were run in a Mini-Protean II apparatus from Bio-Rad (Munich) or a Minigel Twin from Biometra (Göttingen). A 1- μ g sample of glycoprotein in 10 μ l H₂O or 10 μ l from the deglycosylation assay were mixed with 10 μ l SDS sample buffer (0.05 M Tris-HCl, pH 6.8; 4% SDS (w/v); 20% glycerol (v/v); 10% 2-mercaptoethanol (v/v)), boiled for 2 min, and loaded into a gel slot. Proteins were transferred electrophoretically in a Mini-Transblot cell from Bio-Rad at 200 V constant for 1 h onto nitrocellulose as described by Burnette (9).

The protein transfer efficiency under these conditions was approximately 50%. For dot blots, glycoproteins were directly spotted onto dry nitrocellulose and dried at room temperature.

Protein Staining

Proteins in gels were silver stained after prestaining with Coomassie blue (Serva Blue G, Serva, Heidelberg) according to Heukeshoven and Dernick (10). Proteins on nitrocellulose were stained by a 5-min incubation at room temperature with 0.2% Ponceau S in 3% TCA, followed by destaining in H₂O until the protein bands were visible. The Ponceau S staining of the protein bands disappears during subsequent incubation of the membrane in the blocking solution.

Lectin Incubation and Immunodetection

The membrane is first treated for 30 min (or 15 h in the case of the dot blot samples with low amounts of glycoproteins) with 20 ml 0.5% blocking reagent (w/v) in TBS (50 mM Tris-HCl, 150 mM sodium chloride, pH 7.5).

After being washed two times with TBS and once with TBS, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.5 (50 ml each), the membrane is incubated for 1 h at room temperature with the lectin-DIG conjugate (AAA, DSA, GNA, and SNA, 1 μ g/ml; MAA, 5 μ g/ml; PNA, 10 μ g/ml) in 10 ml TBS, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.5. After the membrane was washed three times with 50 ml TBS, anti-DIG AP (750 U/ml) is added in a 1:1000 dilution in 10 ml TBS and incubated for 1 h at room temperature. The membrane is again washed three times with 50 ml TBS and the alkaline phosphatase reaction is carried out by incubating the membrane without shaking in 10 ml of the following solution (freshly prepared): 37.5 μ l BCIP (50 mg/ml, in dimethylformamide) and 50 μ l NBT (75 mg/ml, in 70% dimethylformamide) in 10 ml 0.1 M Tris-HCl, pH 9.5, 0.05 M MgCl₂.

The reaction is normally complete within a few minutes. The membrane is rinsed with H₂O to stop the reaction and allowed to dry.

RESULTS

Sensitivity and Specificity of the Detection

The sensitivity, with which various glycoproteins were detected by the different lectin-digoxigenin conjugates, is demonstrated in Fig. 1. The seven glycoproteins CpY, human serum transferrin, EPO, tPA, fetuin, asialofetuin, and lactotransferrin were spotted onto nitrocellulose in decreasing concentrations, and probed with the respective lectins listed in the middle of Fig. 1. The structural features of the glycoproteins responsible for the binding of the lectins are illustrated on the right

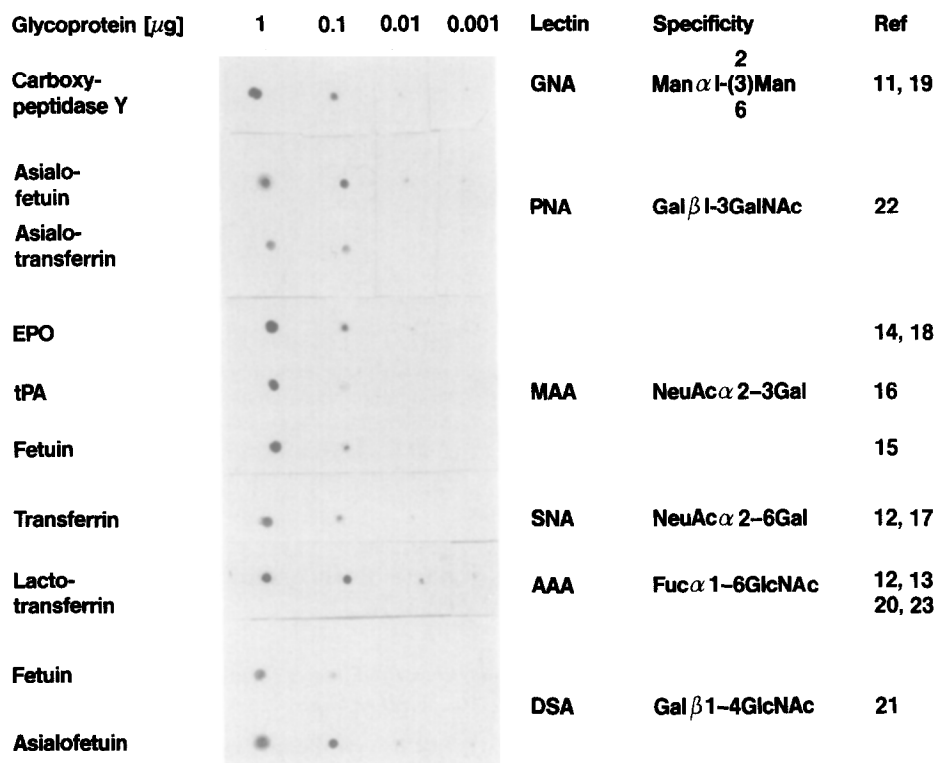


FIG. 1. 1 μ l of each glycoprotein dilution in H₂O were spotted onto nitrocellulose, resulting in the amounts of glycoproteins bound as indicated in the figure. The digoxigenin-labeled lectins were used at their optimal concentrations as described under Methods. Color development was 15 min for all incubations. The carbohydrate structures which are responsible for the recognition by the individual lectins are included on the right, together with the corresponding references where the respective specificities have been reported.

of Fig. 1. The references (11–23) indicated in the figure refer to the carbohydrate structure of the glycoprotein and the sugar specificity of the corresponding lectin. Each lectin–DIG conjugate was used in the optimal concentration which had been determined beforehand and is listed under Methods. The bound lectin–DIG conjugates were visualized by binding anti-DIG conjugated with alkaline phosphatase, and using the BCIP/NBT system for detection of AP activity.

The sensitivity with which the different glycoproteins are detected by the lectin–DIG conjugates is not the same. In several cases (e.g., GNA and SNA in Fig. 1), 1 ng of glycoprotein could be seen after 15 min of incubation in the AP reaction solution. With other lectins (e.g., MAA) the amounts of glycoproteins required for a positive reaction within the same incubation time were somewhat higher. The sensitivity of detection depends mainly from the lectin–DIG conjugate, and to a certain extent also from the individual glycoprotein. This can be seen from the results with MAA in Fig. 1, where different glycoproteins were probed with the same lectin. In all cases roughly similar amounts of the glycoproteins (EPO, tPA, and fetuin) were detected, although slight differences in the sensitivity of detection were still apparent. As with the DIG-based glycan detection system (6), a generally very low background was also

observed here with all lectin conjugates. This obviously contributes to the high sensitivity observed. Usually a 30-min incubation in the blocking solution is sufficient to allow the detection of glycoproteins down to about 10 ng. For the detection of lower amounts of glycoproteins, the incubation time in the blocking solution has to be increased, even to overnight incubations. This allows a prolonged incubation time in the NBT/BCIP solution by retaining a low background staining.

The colored precipitate formed upon the AP reaction with BCIP/NBT is stable and suitable for a direct documentation of the results. An example of the remarkable specificities of lectins, which can be employed to distinguish between subtle structural differences of glycoconjugates, is presented in Fig. 2. It illustrates the reactions of the lectins SNA (17) and MAA (18) with three glycoproteins, which have sialic acid bound to galactose either in α 2–3 or in α 2–6 or in both linkages. Transferrin with sialic acid-linked α 2–6 to galactose is recognized by SNA only. On the other hand erythropoietin, which contains sialic acid in α 2–3 linkage, is only recognized by MAA. Consequently, bovine fetuin, which contains sialic acid in both linkages, gives a positive signal with both lectins. Glycoproteins with no sialic acid (e.g., asialofetuin or CpY) do not react at all with these two lectins (data not shown).

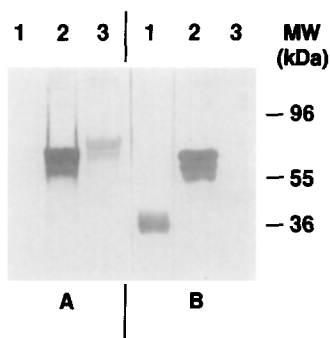


FIG. 2. 2 μ g of EPO (lane 1), fetuin (lane 2), and serotransferrin (lane 3) were run on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose. Blot A was probed with DIG-SNA and blot B with DIG-MAA.

Another remarkable example of lectin specificity is illustrated in Fig. 3. Complex type oligosaccharide chains of glycoproteins are often modified by the addition of fucose-linked α 1-6 to the innermost *N*-acetylglucosamine unit ("core-fucose"). The carbohydrate structure of milk transferrin, e.g., differs in this respect from that of human serum transferrin. The lectin from *Aleuria aurantia* (AAA) (20) clearly recognizes lactotransferrin, but not serum transferrin, as is demonstrated in Fig. 3. Erythropoietin, produced in chinese hamster ovary cells, also reacts with AAA, thus indicating the presence of core fucose. From studies with complex type oligosaccharides it is known that a fucose bound α 1-6 to the proximal *N*-acetylglucosamine is indispensable for a strong binding of AAA. Oligosaccharides, containing fucose only α 1-3 linked to *N*-acetylglucosamine in the outer chain, as it occurs in human α ₁-acid glycoprotein for instance, react only very weakly with AAA (20). However an outer chain α 1-3-linked fucose in addition to a fucose-linked α 1-6 to the proximal core *N*-acetylglucosamine enhances the binding (23). This remarkable specificity makes the *Aleuria* lectin

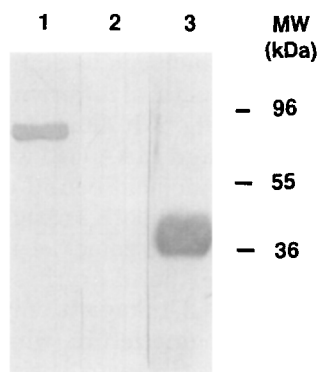


FIG. 3. 2 μ g of lactotransferrin (lane 1), serotransferrin (lane 2), and EPO (lane 3) were applied to SDS-PAGE, blotted onto nitrocellulose, and incubated with DIG-AAA.

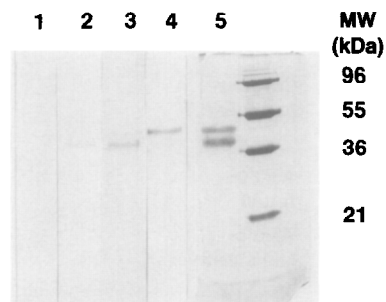


FIG. 4. 1 μ g of tPA, cleaved by plasmin into the A- and B-chains and carboxymethylated, were subjected to SDS-PAGE (15% gel) and transferred to nitrocellulose. Nitrocellulose strips were incubated with the DIG-lectins as described under Methods. Lane 1, SNA; lane 2, MAA; lane 3, DSA; lane 4, GNA; lane 5, silver stain (Lane 5 and molecular weight markers, silver stained in the gel.)

particularly useful for identifying fucose, bound α 1-6 to GlcNAc.

Glycan Chain Analysis of Glycoproteins Produced in CHO Cells

The combination of highly specific lectins, a sensitive detection system, and specific endoglycosidases can be used to obtain a considerable amount of information on the glycan chain structure of glycoproteins, as is demonstrated in the next two examples. tPA, cleaved in its two chains (A and B) by plasmin, was carboxymethylated, blotted after SDS-PAGE on to nitrocellulose and probed with the different lectin-DIG conjugates listed in Fig. 4. The protein band with the higher molecular weight (see lane 5) corresponds to the A-chain (275 amino acids; two potential glycosylation sites). Two forms of the A chain have been described, differing in either the presence (type I) or the absence (type II) of a glycan chain at asparagine-184 (15,24,25). The B-chain (253 amino acids) also contains two glycosylation sites, but only one is known to be used (15). The identity of the two bands on the blots was proven by probing with two specific antibodies against the A- and B-chain (data not shown). The lectin-binding studies illustrated in Fig. 4 allow the following conclusions about the glycan chains of this tPA expressed in chinese hamster ovary cells:

(i) The *Galanthus* lectin (GNA), specific for terminal bound mannoses (α 1-2, 1-3, or 1-6 to mannose), reacts with the A chain. This indicates the presence of a high mannose or hybrid type oligosaccharide structure (lane 4), because unlike concanavalin A, GNA does not react with biantennary complex type chains (26). The presence of a high mannose or hybrid structure was also confirmed by susceptibility toward endoglycosidase H (data not shown).

Besides the mannose structure, a complex type oligosaccharide, as it is found on various tPA forms (24,25),

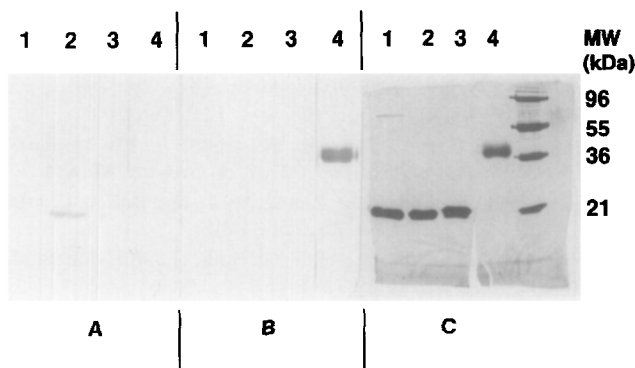


FIG. 5. EPO was deglycosylated as described under Methods. 1 μ g samples were loaded onto SDS gels, silver stained (C) or blotted onto nitrocellulose (A and B). Blot (A) was incubated with DIG-PNA, blot (B) with DIG-MAA. Lane 1, EPO, deglycosylated with *N*-glycosidase F, neuraminidase, and *O*-glycosidase; lane 2, EPO, deglycosylated with *N*-glycosidase F and neuraminidase; lane 3, EPO, deglycosylated with *N*-glycosidase F; lane 4, glycosylated form; MW, molecular weight markers: 96, 55, 36, and 21 kDa.

is not detectable in the A-chain, since it does neither react with the *Maackia* lectin, specific for α 2-3-linked sialic acid nor with the *Datura* lectin, specific for Gal-GlcNAc termini of complex oligosaccharides (lanes 2 and 3). This indicates that the A chain only contains a glycan chain of the high mannose or hybrid type.

(ii) The B-chain contains a complex type chain with sialic acid-linked only α 2-3 to galactose (positive reaction with MAA; no reaction with SNA; lanes 1 and 2). The presence of fucose could be demonstrated by a positive reaction with AAA (data not shown).

The combined use of lectins and glycosidases proved to be even more instructive in the case of erythropoietin glycan analysis, as is illustrated in Fig. 5. The EPO was also produced in CHO cells, and is known to contain three potential N-glycosylation and one O-glycosylation site (14). The following conclusions about the glycan structures could be drawn (not all of the experimental results are presented in the figure).

(i) The N-linked oligosaccharide chains are all of the complex type, most of them with more than two antennae (GNA negative; mainly resistant to endoglycosidase F). Sialic acid is only linked α 2-3 to galactose (MAA positive, SNA negative), and fucose α 1-6 linked is present (AAA positive; compare Fig. 3).

(ii) After removal of all N-linked oligosaccharides a sharp protein band is obtained in the SDS-PAGE (see lane 3 in Fig. 5C). That this band still contains carbohydrate has been shown by a positive reaction with a general glycan stain (6).

(iii) Combined neuraminidase and peptide-*N*-glycosidase F treatment gives rise to an even further reduction of the molecular weight (lane 2 in Fig. 5C). Due to overexposure of the silver staining the mobility shifts between bands 3, 2, and 1 appear small in this particular

experiment. They have been verified to be present in several similar experiments performed in our laboratory. The resulting form is recognized by the peanut lectin (lane 2 in Fig. 5A), yet not anymore after endo- α -*N*-acetylgalactosaminidase treatment (lane 1 in Fig. 5A). This proves the presence of an O-linked Gal β 1-3GalNAc unit, which is substituted by one or two sialic acids.

The lectin MAA does not recognize the sialic acid(s) on this O-glycan chain, nor does SNA (data not shown). This is in agreement with data in the literature in which MAA reacts only weakly with sialic acids bound to Gal-GalNAc of O-linked chains, even if the sialic acid is bound α 2-3 (18).

(iv) That the form obtained after incubation with peptide-*N*-glycosidase F, neuraminidase, and *O*-glycosidase (lane 1 in Fig. 5C) is completely devoid of carbohydrate, could be demonstrated by a negative reaction when applying a general carbohydrate stain (6).

DISCUSSION

It has been demonstrated that combining the use of lectins with the resolution power of electrophoresis and the DIG/anti-DIG immunological detection system proves to be useful for the characterization of carbohydrate chains of glycoproteins. Some advantages of this type of analysis are as following:

It requires only small amounts of glycoproteins, which can be present in protein mixtures and have not to be purified extensively. The experiments are performed quite easily and fast, and do not need expensive or sophisticated equipment. The well-known specificities of the lectins employed allow precise structural conclusions. Moreover by combination of this approach with the more general DIG-based glycan detection techniques (6) as well as with the various endo- and exoglycosidases of known specificities available, a considerable amount of structural information can be obtained. The application of these techniques for obtaining structural information should therefore prove to be valuable before employing other methods, which need highly purified material or which are much less available (like NMR or FAB-MS).

This method should be especially valuable for the in process monitoring of the glycan composition of biotechnologically produced glycoproteins.

The techniques applied here for the analysis of glycoproteins can likewise be applied, with certain modifications, for other glycosylated compounds. Thus the detection of HPLC separated glycopeptides, spotted on nitrocellulose, by using the DIG/anti-DIG AP general glycan detection system has been described (27). The use of DIG-lectins should now allow a more thorough structural characterization of the glycopeptides, thus allowing conclusions on the glycan microheterogeneity

of individual glycosylation sites. Similarly in addition to the sensitive detection of TLC separated glycolipids using the DIG/anti-DIG AP system, which has been described very recently (28), the DIG-lectins should also prove valuable here for obtaining more structural information. They also might be useful for the characterization of free oligosaccharides which are immobilized to a solid support.

A very important area for the application of lectins is their use in cytochemical studies (29–31). It has been demonstrated that digoxigenylated lectins are very useful for electron and light microscopic studies, e.g., being superior to the biotin/streptavidin system because of complete absence of endogenous binding sites (32). This advantage applies also to the studies of biological materials by electrophoresis and blotting procedures, albeit to a lesser extent. The generally very low background produced by this system compared, e.g., to the use of streptavidin conjugates, or lectins conjugated directly to peroxidase, together with the availability of further anti-DIG conjugates besides AP (e.g., POD; fluorescence; gold), and the availability of a large number of lectins with different specificities make this system very useful for a wide variety of applications.

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