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SHORT COMMUNICATION

Identification of major glycoconjugates from *Mycobacterium bovis* culture filtrate by biotin-hydrazide labeling

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To identify *Mycobacterium bovis* glycoproteins, carbohydrates present in a delipidized *M. bovis* culture filtrate protein extract were biotin-hydrazide labeled. 11 carbohydrate-containing protein with a moleculra weight of 15-, 19-, 25-, 32-, 35-, 39-, 42-, 48-, 52-, 58-, and 62-kDa were detected. The 52- and 32-kDa protein were deglycosylated by endoglycosidase-F.

Keywords: **M. bovis, glycoconjugate, glycoprotein, biotin-hydrazide, n-linked oligosaccharides**

Introduction

Identification and characterization of oligosaccharide-containing *Mycobacterium tuberculosis* [1] and *M. bovis* [2] antigens are of particular importance [3, 4]. So far, isolation of mycobacterial glycoconjugates has been achieved mainly through *Canavalia ensiformis* and *Lens culinaris* affinity chromatography [5, 6], but the main problem is the restriction of lectins for specific saccharide sequences [7]. In this study, we report the efficacy of biotin-hydrazide labeling of carbohydrates present in a delipidized *M. bovis* culture filtrate protein extract. Eleven glycoproteins were obtained; six of them represented 86% of all the glycoconjugate present in the filtrate. A 32- and a 52-kDa antigen were deglycosylated by endoglycosidase-F.

Material and Methods

Culture filtrate protein extract

M. bovis strain AN-5 bacilli were grown in protein-free Proskawer-Beck-Youmans medium for ten weeks at 37°C. Bacterial mass was separated by sequential filtration, using Whatman #3 paper filter, followed by 0.45 μm membrane filtration (Millipore Corp., Milford, MA). Proteins secreted to the culture medium were precipitated with solid ammonium sulfate at a final saturation of 80%, centrifuged for 90 minutes at 23,700 *g*, resuspended in 5 ml of 0.015 M PBS, pH 7.4, containing 20 μg/ml phenyl-methyl-sulfonyl fluoride (Sigma Chem., St. Louis, MO) and dialyzed against PBS [8]. The lipids remaining were removed by sequential extraction with chloroform:methanol:water (3:3:1, v/v), followed by chromatography on a PBS equilibrated 10×1.2 cm Biogel P-2 (Bio-Rad, Richmond, CA) column. Fractions with a positive spectrophotometric reading at 280 nm were pooled, concentrated in an Amicon ultrafiltration cell; protein concentration was determined by the Bradford method [9] using BSA as standard, and stored at −20°C until use.

Carbohydrate biotinylation

In a 100 mM sodium acetate buffer, pH 5.5, 700 μl (1.62 mg) of *M. bovis* culture filtrate protein extract were made to react with 300 μl of a 30 mM sodium m-periodate (Bio-Rad, Richmond, CA) stock solution, at 4°C for 1 h in the dark; excess sodium periodate was eliminated by dialysis. Oxidized glycoproteins were incubated with 5 mM Biotin-Hydrazide (Pierce, Rockford, IL) in 1 mM sodium acetate buffer for 1 h at room temperature. The reaction was stopped with 500 μl of 0.1 M Tris-HCl, pH 7.5, and excess biotin was eliminated by extensive dialysis against PBS. Aliquots of 30 μl were electrophoretically resolved in 15% SDS-polyacrilamide gels, under nonreducing conditions [10], and transferred to nitrocellulose (0.45 μm; Schleicher

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& Schuell, Keene, NH) with a Trans-Blot Cell (Bio-Rad, Richmond, CA), as described by Towbin *et al*. [11]. After washing the blots, they were incubated with peroxidase-labeled streptavidin (Sigma Chem., St. Louis, MO) for 1 h at room temperature, washed again and developed with 3,3 diamino-benzidine (DAB) for 5 min. Biotinylated molecular weight markers (Sigma Chem., St. Louis, MO) were always included.

Avidin affinity-chromatography

Biotin-labeled glycoproteins were isolated by affinity chromatography on a 2×1 cm agarose-coupled, monomeric avidin (Sigma Chem., St. Louis, MO) column activated according to the manufacturer's instructions [12]. Bound glycoproteins were eluted with 0.1 M glycine-HCl, pH 2; fractions were collected and immediately neutralized to pH 7.0 with 0.1 M Tris-HCl, pH 7.5. Protein, detected by ultraviolet spectrophotometry, was dialyzed against PBS, aliquoted and kept at −20°C until use. Aliquots of 25 µl were resolved in 15% SDS-PAGE, transferred to nitrocellulose, incubated with peroxidase-labeled streptavidin, and developed with DAB, as mentioned above.

Enzyme treatment

Affinity chromatography purified glycoprotein, 0.05 mg/ml, was extensively dialyzed against 0.015 M PBS, pH 8.6, incubated overnight at 37°C with 0.011 IU of Endoglycosidase-F (DuPont Co., Boston, MA); aliquots of 20 µl were resolved in SDS-PAGE and transferred to nitrocellulose. The blots, incubated with peroxidase-labeled streptavidin for 1 h at room temperature, were developed with DBA.

Densitometry

Affinity chromatography purified glycoproteins were transferred to nitrocellulose paper and endoglycosidase-treated, and nontreated controls were scanned in a model GS-670 densitometer (Bio-Rad, Richmond, VA) and analyzed with the image analysis software Molecular Analyst/PC version 1.1 (Bio-Rad, Richmond, VA).

Results

Crude *M. bovis* culture filtrate protein extract labeled with biotin-hydrazide showed a rather weak signal on the initial blot experiments, as only a small percentage of the total protein content contained carbohydrates. To enrich the glycoconjugate fraction, we poured the biotinylated material onto an affinity chromatography monomeric avidin column. The glycine-eluted material was then electrotransferred to nitrocellulose, incubated with peroxidase-labeled streptavidin, and visualized with DBA. Six major and five minor carbohydrate-containing bands were detected (Table 1 and Figure 1A). The molecular weight of the main carbohydrate-labeled proteins were 15-, 19-, 25-, 42-, 48-, and 52-kDa and represented 86% of all affinity chromatography purified proteins.

Table 1. Comparison of biotin-labeled M. bovis CFPE glycoconjugates before and after endoglycosidase-F treatment

Nonenzyme treated peak no. M.W. (%)	*Enzyme treated peak no. M.W. (%)*
1- 62(<1%)	1- 58(<1%)
2- 58(2%)	2- 54(1%)
3- 52(26%)	3- 48(14%)
4- 48(16%)	4- 45(3%)
5- 42(5%)	5- 42(2%)
6- 39(1%)	6- 40(3%)
7- 35(1%)	7- 38(<1%)
8- 32(1%)	8- 36(4%)
9- 25(16%)	9- 34(1%)
10- 19(18%)	10- 25(25%)
11- 15(5%)	11- 19(34%)
	12- 17(1%)
	13- 15(1%)

Peak number refers to the peaks depicted in Figures 1A and 1B. M.W. = molecular weight expressed in kDa. Values in parentheses represent the corresponding percentage of total biotin-labeled glycoconjugate determined by the densitometric analysis of the blot.

After the affinity chromatography purified proteins were digested with endoglycosidase-F, we detected the permanence of five of the six major carbohydrate-containing proteins, the 15-, 19-, 25-, 42-, and 48-kDa bands, and the disappearance of the biotin-labeled 52-kDa band. Eight new bands, representing 24% of the total area under the curve, appeared (Table 1 and Figure 1B).

It was interesting to observe that after endoglycosidase-F treatment the 19- and the 25-kDa proteins increased their percentages to 34% and 25%, respectively; the 48 kDa protein was hardly modified (from 16% down to 14%); and the 15- and 42-kDa protein bands diminished their percentages from 5% each, to 1% and 2%, respectively.

Discussion

Purification of mycobacterial glycoconjugated proteins has been traditionally performed with *Con-A*, but the specificity of the lectin [7] and the fact that the lack of lectin-binding does not necessarily indicate the absence of a particular carbohydrate residue restrict its use. Due to the biological importance of mycobacterial saccharide-containing antigens, we approached the isolation of these glycoconjugates in a different manner. An *M. bovis* culture filtrate protein extract, treated with m-periodate to oxidize all sugar moieties and generate aldehydes, was made to react with biotin-hydrazide. The resulting material was purified on an avidin

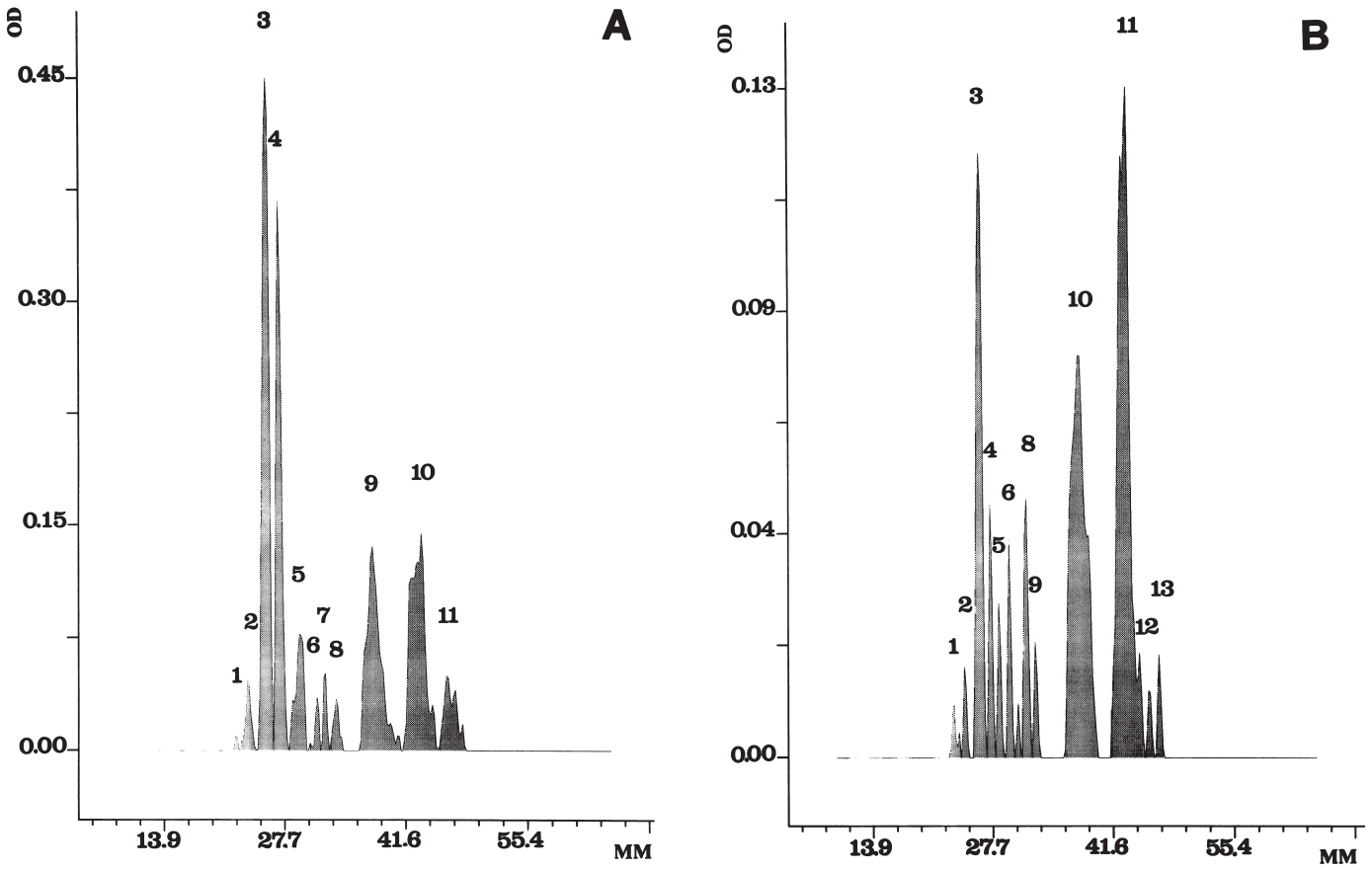

Figure 1. Densitometric analysis of biotin-labeled *M. bovis* culture filtrate protein extract. Graph A corresponds to Biotin-Hydrazide labeled glycoproteins purified by means of monomeric avidin affinity-chromatography; graph B corresponds to affinity-chromatography purified glycoproteins after endoglycosidase-F treatment. The densitometer was set to eliminate background signals from the graph. The corresponding molecular weight and relative percentage of each numbered peak is shown in Table 1.

column, resolved in SDS-PAGE, and blotted and analyzed on a densitometer. Aldehyde formation has been used to biotin-label antibodies, functional groups on membrane proteins, LDL receptor, and β-nerve growth factor [13–16]. To determine if the biotin-labeled mycobacterial glycoconjugates possessed *N*-linked saccharides, we performed experiments with endoglycosidase-F, an enzyme that acts specifically on the inner *N*-acetylglucosamine of biantennary oligosaccharides [17] and has no effect whatsoever on the protein mainframe nor on the O-glycosidically linked glycoconjugates.

Although 11 biotin-hydrazyde labeled proteins were detected, four of them, with an m.w. of 19-, 25-, 48-, and 52-kDa, represented 76% of all labeled material. The 19-kDa and the mannose-rich 25-kDa glycoconjugates, which have been already reported as *Con-A*–binding antigens [18], did not modify their molecular weight after endo-F treatment; on the contrary, their relative proportion increased considerably. Endoglycosidase-F treatment reduced the percentage of the 48 kDa glycoprotein from 16% of the total glycoconjugate content to 14%, and in a manner that resembles an antigen of 47 kDa degradable to 45 kDa described in *M. bovis* [2], a new band of 45 kDa, which represented 3% of the total glycoconjugate content, appeared. There is evidence of O-glycosylation sites in 19- and 45-kDa *M. tuberculosis* glycoproteins [19,20]. The 52 kDa antigen, which corresponds to approximately 3% of all the proteins (data not shown), represented 26% of all the glycoconjugate content and the biotin label disappeared completely after endoglycosidase-F treatment, thus suggesting the presence of *N*-linked biantennary oligosaccharides. The fact that we were able to detect biotin label in the 19-, 25-, and 48-kDa glycoproteins after endo-F treatment suggests that saccharides in these proteins are either O-linked or are attached in a different manner (i.e., glycation). There are reports of a ≅39 kDa antigen that also binds to *Con-A*, but recent information suggests it might be secondary to a nondescript carbohydrate contamination [21,22].

Of the minor glycoconjugates that we identified, there was one 32 kDa glycoprotein (corresponding to 1% of the total) that completely lost the biotin label after endogly-

cosidase-F treatment. An antigen of identical molecular weight has been isolated from *M. tuberculosis* by *Lens culinaris* lectin affinity chromatography [5]. Collateral experiments have shown that the addition of tunycamicin to the culture medium inhibits bacterial growth but does not interfere with cell viability (unpublished data).

Since many important questions about structure, biosynthesis, and function of these glycoconjugates are still unanswered, we believe that the method described here permits easy purification of sufficient amounts of well-characterized mycobacterial glycoconjugates.

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