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Preparation of Deglycosylated Egg White Avidin

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ABSTRACT

A simple procedure for the preparation of deglycosylated avidin is described. Commercially obtained avidin was treated with a mixed microbial culture. The cells were capable of growing on the oligosaccharide residues, but generally ignored the polypeptide portion of the egg white glycoprotein. The resultant deglycosylated avidin retained its biotin-binding characteristics. The major bacterial strain (strain BECH080), responsible for the deglycosylation, was isolated. On the basis of elementary biochemical tests, fatty acid, and phenotypic analyses, the isolate was identified as a strain of *Flavobacterium meningosepticum*. The primary enzymatic activity that caused the removal of the oligosaccharide residues of avidin appeared to be similar to endoglycosidase F.

Index Entries: Avidin; avidin-biotin system; deglycosylation; glycoprotein; *Flavobacterium meningosepticum*.

INTRODUCTION

Avidin is a glycoprotein found in the egg whites and various tissues of birds, reptiles, and amphibians. Interest in avidin has continued throughout the years because of its remarkably strong binding to the vitamin biotin (*1*) and consequent use in biotechnological processes (*2,3*).

Avidin is a tetramer (62,700 Dalton), composed of identical monomers, each of which bears a single biotin-binding site and a single oligosaccharide chain. The latter includes mainly mannose and *N*-acetyl glucosamine

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residues, and accounts for roughly 10% of the molecular weight of the protein (*4*). Only one of the asparagine residues, Asn-17, is glycosylated per avidin monomer. The carbohydrate moieties are heterogeneous and are thought to comprise three structurally different types, including oligomannosidic, bisected, and nonbisected hybrid components (*5*).

The presence of the sugars in avidin is known to cause spurious interactions that interfere with its application in avidin-biotin systems (*6*). For this reason, streptavidin, the naturally nonglycosylated bacterial cognate of avidin, has found broad application in its stead—despite the excessive price of the bacterial protein. We were therefore interested in producing a deglycosylated form of the glycoprotein that would help restore its preponderant usage in avidin-biotin technology.

In an earlier publication (*7*), we noted that a commercial preparation of avidin consisted of two SDS-polyacrylamide gel electrophoresis (SDS-PAGE)-resolved polypeptide bands (M_r 18,000 and 15,500, respectively), both of which exhibited biotin-binding activity. Further analysis revealed that the sole difference between the two forms of avidin monomer was the lack of carbohydrate in the low-mol-wt band. Based on this finding, we were able to isolate homotypic deglycosylated tetramers of avidin on a Concanavalin A (Con A) affinity column. Interestingly, determination of the three-dimensional structure of this "nonglycosylated" form of avidin revealed a single *N*-acetyl glucosamine residue still associated with the protein (*8*).

However, preparation of the deglycosylated tetramer from commercial samples of avidin resulted in very low yields (<20%). Moreover, the extent of glycosylation of the starting material was highly variable; some samples were almost totally glycosylated, and we could not expect to isolate any deglycosylated tetramer from such material. We were therefore interested in controlling the deglycosylation process, in order to improve the yields.

In the present work, we describe the isolation of a mixed microbial culture that grows on avidin as the sole source of carbon and energy. In doing so, the polypeptide portion of the glycoprotein remains intact, whereas the oligosaccharide moiety is degraded completely. The process is amenable to scale-up procedures, and we have succeeded in converting kilogram quantities of the glycoprotein to the deglycosylated product.

MATERIALS AND METHODS

Materials

Partially deglycosylated preparations of egg white avidin were obtained from Belovo Chemicals (Bastogne, Belgium). The fully glycosylated avidin was provided by STC Inc. (Winnipeg, Manitoba, Canada). Con A-agarose was the product of Makor Chemicals, Jerusalem, Israel. The mixed

microbial culture used for deglycosylation was provided by Belovo Chemicals, and strain BECH080 was isolated in Bastogne as described in the Results and Discussion.

Typical Deglycosylation Procedure

A solution (between 50 μL and 200 L) of avidin (between 2 and 5 mg/mL) was prepared in phosphate-buffered saline, pH 7.4, with added 1% (v/v) Vogel's minimal medium (*9*), from which all carbon- and nitrogen-containing constituents were omitted. To this solution, a 5% (v/v) inoculum of the desired (mixed or pure) culture was added. For volumes larger than 1 L, starter and seed cultures were used. The cultures were maintained at room temperature under stationary conditions, and the status (i.e., oligosaccharide content) of avidin monomers was examined by SDS-PAGE. The cells were removed by centrifugation.

Phenotypic Analysis

An API 20NE strip, containing conventional and assimilation tests, was inoculated with the bacterial isolate (strain BECH080) and cultured at 28°C for 48 h. The results were interpreted according to the instructions of the manufacturer (API System S.A., La Balme les Grottes, Montalieu Vercieu, France). The resultant code (2676305) was compared with the API profile recognition program (Version 3.1). Additional tests were carried out using conventional plating techniques.

Fatty Acid Composition of Strain BECH080

The fatty acid composition of the bacterial cell membranes was determined by gas chromatography. The extraction procedure and the analysis were performed in accordance to the recommendations of the MIDI identification system (Microbial Identification System, Inc., Delaware). The resulting profile was compared with the MIDI commercial data base.

Miscellaneous Methods

SDS-PAGE was carried out under denaturing conditions (boiled samples) on either 10 or 15% gels as described previously (*7*). The presence of oligosaccharide residues on monomers of avidin was determined by the mobility of the denatured samples of SDS-PAGE and by staining with Con A. The biotin-binding capacity of avidin samples was determined in solution by the HABA method (*10*). Affinity chromatography on Con A-agarose columns was carried out as described earlier (*7*). Distilled water was used as effluent; 25 m*M* Tris-HCl buffer (pH 7.4), containing 150 m*M* NaCl, 1 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$, was employed to elute the deglycosylated avidin tetramers, and a 5% (v/v) solution of methyl α-D-mannoside (αMM) in the same buffer was used to elute the residual glycosylated material.

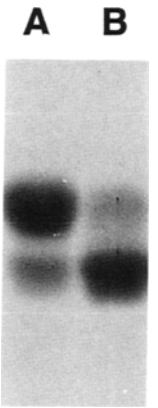

Fig. 1. Deglycosylation of avidin by microbial growth. A solution containing glycosylated avidin (Belovo Chemicals, lot #0586) was inoculated with the mixed microbial culture, and the mixture was kept at 28°C. The bacterial cells were centrifuged, the supernatant fluids were subjected to SDS-PAGE, and the resultant gel was stained using Coomassie brilliant blue. Lane A, avidin solution before inoculation of culture; lane B, inoculated solution after a 7-d growth period. The upper band represents the glycosylated avidin monomer, which corresponds to a molecular mass of about 15,700 Dalton; the lower band indicates the deglycosylated monomer, calculated at 14,500 Dalton.

RESULTS AND DISCUSSION

Enrichment of the Deglycosylation Culture

The original source of the microbial culture used in this work was a solution of avidin from Belovo Chemicals that had been stored at 4°C for several months. It had been observed that the avidin derived from this sample was partially deglycosylated, and we postulated that this was a function of microbial cells that grew mainly on the oligosaccharides of avidin. Indeed, addition of an aliquot of this "contaminated" solution of avidin to samples of purified avidin resulted in a deglycosylation of the purified glycoprotein (Fig. 1). No deglycosylation was observed in the presence of inhibitors of microbial growth or by passage of the culture through a Millipore filter prior to use.

Typically, a 20-fold dilution of the mixed microbial culture was added to purified avidin, and periods of 3–4 wk were required to effect substantial deglycosylation of commercial preparations of avidin. Not surprisingly, the procedure was much more rapid for partially deglycosylated avidin (i.e., from Belovo Chemicals) than for the fully glycosylated protein (i.e., from STC Inc.), indicating that other microbial glycosidases may have facilitated the rate of deglycosylation. Nevertheless, even highly glycosylated samples of avidin could eventually be deglycosylated

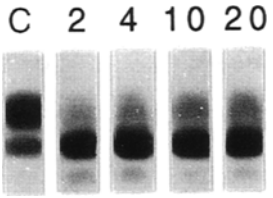

Fig. 2. Deglycosylation as a function of avidin concentration. Samples of different concentrations of glycosylated avidin (lot #0586) were inoculated with the mixed microbial culture, and the mixture was maintained at 28°C. After 28 d, the suspensions were centrifuged, brought to a concentration of 1 mg/mL protein, and the supernatant fluids were subjected to SDS-PAGE. The number above the lane denotes the concentration of avidin (2, 4, 10, or 20 mg/mL) in the initial sample. C, control sample of avidin—not inoculated.

to apparent completion, using the mixed microbial culture. In all samples, the biotin-binding activity and susceptibility to denaturants were essentially unaffected (unpublished results).

For scaling up, we examined the efficiency of microbial degradation of higher concentrations of avidin. It was found that the optimal concentration of substrate was about 4 mg/mL (Fig. 2). At higher concentrations, the rate was increased, but the yield was lower, reflecting the lower solubility level of the deglycosylated product. Nevertheless, effective deglycosylation of avidin concentrations as high as 20 mg/mL could easily be achieved.

The rate of deglycosylation could be hastened in two ways: (1) the culture could be enriched by successive weekly passages into new solutions of avidin, and (2) minimal medium could be added to improve the culture conditions. Following the enrichment procedure, nearly full deglycosylation could be achieved after a 2-wk growth period on avidin. However, if minimal medium was added to the culture, the polypeptide chain of avidin was completely hydrolyzed within 3 d, indicating that the culture was now enriched for protease-producing strains. If lower amounts of salts and nutrients were added (e.g., 100-fold dilution of minimal medium), a more rapid rate of deglycosylation was observed, and the polypeptide chain of avidin remained intact. Addition of $CaCl_2$ and $MgCl_2$ (0.1 mg/L each, final concentration) produced a similar effect.

Isolation and Identification of the Major Deglycosylating Bacterial Strain

It was interesting to determine whether the complement of microbial strains present in the mixed culture was required for efficient deglycosylation of avidin or whether a single microbe was responsible. The enriched culture was therefore streaked on nutrient agar, and single colonies were selected and propagated. Following growth on nutrient broth, the cells

Table 1
Some Properties of the Isolated Bacterium (Strain BECH080)

α-Glucosidase	+	Casein	+
β-Galactosidase	+	Gelatin	+
Indole	+	Citrate	+
Catalase	+	Glucose	+
Urease	–	Mannose	+
Amylase	–	Mannitol	+
Lipase	–	*N*-Acetyl glucosamine	+
Lysine decarboxylase	–	Maltose	+
Arginine dihydrolase	–	Arabinose	–
Reduction of nitrates	–	Gluconate	–
H_2S production	–	Caprate	–
Gas production	–	Adipate	–
Acidification of glucose	–	Malate	–
		Phenyl acetate	–

were washed by centrifugation with phosphate-buffered saline, pH 7.4, and resuspended in solutions of avidin. After a 2-wk growth period, the samples were examined for the extent of deglycosylation of avidin. In one of the samples, avidin was extensively degraded.

The isolate (strain BECH080) was examined for purity on tryptone-soya agar, on nutrient agar, and on plate-count agar. The culture showed homogeneous growth on all three media. In the presence of horse blood, the strain was strongly hemolytic. Optimal growth was achieved at 33°C; moderate levels of growth were observed at 26 and 37°C.

Strain BECH080 was observed to be gram-negative, nonspore-forming rods. The rods were small (2–8 μm × 0.5–0.7 μm), irregular, slightly S-shaped, and nonmotile. The bacterium produced a yellow pigment after long periods of growth, but failed to grow on MacConkey's agar. It degraded glucose under anaerobic conditions, but failed to degrade glucose, lactose, or sucrose aerobically. The strain grew on casein and gelatin, reflecting strong proteolysis. It grew on brilliant green (12.5 mg/L) and also grew on proteins in its presence. The isolate also grew on citrate.

Other properties of the bacterial strain are presented in Table 1. The fatty acid composition of the cell membranes was consistent with that of *Flavobaterium meningosepticum* giving a score of 0.788.

Purification of Deglycosylated Avidin on Con A-Sepharose

Despite the efficient degradation of avidin oligosaccharides by *F. meningosepticum* strain BECH080, it was clear that the mixed culture was more efficient in removal of the sugars. In this regard, other indigenous strains may contribute other types of glycosidases (e.g., mannosidases) that

facilitate the action of the enzyme(s) from strain BECH080. It was also observed that the extent of deglycosylation was generally more complete using the mixed culture. Even so, a residual band of glycosylated avidin monomers, accounting for about 5% of the total protein, usually remained. This could reflect either the heterogeneity of the avidin oligosaccharides, which may include recalcitrant forms, or the action of other glycosidases, which could lead to "dead-end" intermediates. In any case, it is frequently desirable to remove avidin tetramers, which may retain residual carbohydrate on one or more of their monomers.

Con A affinity chromatography has previously been used to separate deglycosylated tetramers from glycosylated forms of avidin (*7*). This was originally achieved on a partially deglycosylated preparation, which exhibited a near-equivalent mixture of glycosylated and deglycosylated monomers. However, the final yields of the deglycosylated product were only about 15% of the total protein applied (Fig. 3A). The superficial reason for obtaining such low yields in this case is inherent in the tetrameric structure of avidin. Thus, individual molecules (tetramers) of avidin would have 0, 1, 2, 3, and 4 oligosaccharide chains remaining. Only those totally lacking available carbohydrate would pass through the Con A column; molecules of avidin with even one glycosylated monomer would still be retained on the column.

Deglycosylation of avidin yielded a product that appeared to be nearly void of glycosylated monomers (Fig. 3B, insert). The sample was purified further by passage through the affinity column. About 70% was isolated as a homogeneous preparation of the deglycosylated tetramer. This value is consistent with a 5% subpopulation of avidin tetramers, which include a single glycosylated monomer.

On the basis of biochemical and chemical analyses (*7*), it had been assumed that the deglycosylated avidin tetramers were entirely devoid of carbohydrate. Consequently, we thought that a bacterial enzyme equivalent to Peptide:*N*-glycosidase (EC 3.2.2.18) was responsible for the observed deglycosylation. However, recent X-ray crystallographic studies (*8,11*) have shown that a residual *N*-acetyl glucosamine residue remained on the glycoprotein, thus implicating a type of enzyme similar to endoglycosidase F (i.e., endo-β-*N*-acetylglucosaminidase F, EC 3.2.1.96).

The availability of glycosylated and deglycosylated avidin will enable further study of the importance of the sugar residue in the structure and function of the protein. For example, we can now investigate the importance of the oligosaccharide moiety in folding and stabilization of the avidin monomer and the respective interactions among their amino acid residues. Since the avidin-biotin system has now become a tool in different in vivo studies (e.g., tumor localization and imaging [*12*] as well as drug delivery [*13,14*]), the importance of the sugars for these functions should be evaluated.

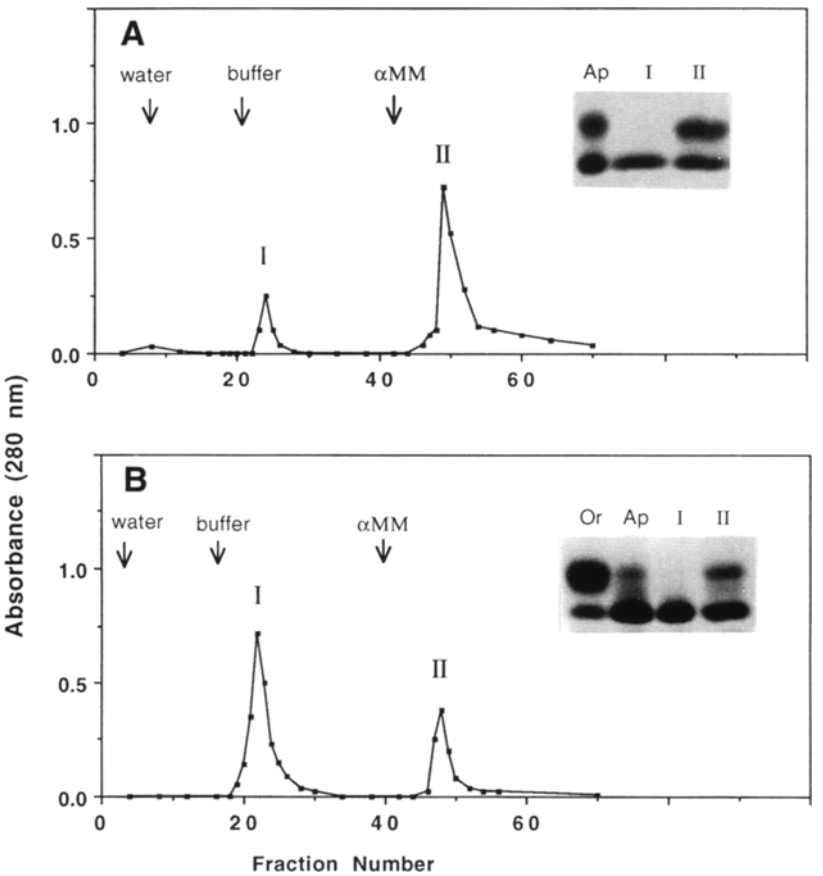

Fig. 3. Affinity chromatographic separation of deglycosylated and glycosylated avidin tetramers on Con A-Sepharose. (**A**) A sample (2 mg protein in 2 mL of distilled water) of an untreated, commercially obtained preparation of avidin (lot #1283) that exhibited equivalent amounts of glycosylated and deglycosylated monomeric forms was applied to a 2-mL Con A-Sepharose column. As designated (arrows), buffer and competing saccharide (αMM) were added. Insert: The SDS-PAGE profile of the applied sample (Ap) and the peaks eluted with buffer or competing sugar (I and II, respectively). (**B**) An equivalent sample of deglycosylated avidin, produced by interaction of a heavily glycosylated preparation with the mixed microbial culture, was analyzed on the same column. Insert: The SDS-PAGE pattern of: Or, the original protein (prior to microbe-induced removal of oligosaccharide chains); Ap, the applied deglycosylated sample; I and II, the corresponding peaks eluted with buffer and competing sugar.

The two main types of biotin-binding protein—avidin and streptavidin—are remarkably similar in their structure (*11,15,16*). Although streptavidin is nonglycosylated, we have recently shown that the protein adheres to cell surfaces via an intrinsic RYD sequence that is analogous to the RGD cell-binding motif (*17,18*). The availability of various glycosy-

lated and deglycosylated forms of avidin will allow us to examine the involvement of the carbohydrate chain in different processes, such as cell adhesion, targeting to different tissues, and renal clearance (*19*).

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REFERENCES

1. Green, N. M. (1975), *Adv. Protein Chem.* **29,** 85–133.
2. Bayer, E. A. and Wilchek, M. (1980), *Methods Biochem. Anal.* **26,** 1–45.
3. Wilchek, M. and Bayer, E. A., eds. (1990), in *Methods of Enzymology,* vol. 184, Academic, San Diego, p. 746.
4. DeLange, R. J. and Huang, T. S. (1971), *J. Biol. Chem.* **246,** 698–709.
5. Bruch, R. C. and White, H. B. (1982), *Biochemistry* **21,** 5334–5341.
6. Bayer, E. A. and Wilchek, M., in *Egg Uses and Processing Technologies,* Sim, J. S. and Nakai, S., eds., CAB International, Wallingford, UK, pp. 169–187.
7. Hiller, Y., Gershoni, J. M., Bayer, E. A., and Wilchek, M. (1987), *Biochem. J.* **248,** 167–171.
8. Bayer, E. A., Livnah, O., Sussman, J. L., and Wilchek, M. (1993), *Glycoconj. J.* **10,** 276–277.
9. Vogel, H. J. (1956), *Microbiol. Gen. Bull.* **13,** 42.
10. Green, N. M. (1970), *Methods Enzymol.* **18A,** 418–424.
11. Livnah, O., Bayer, E. A., Wilchek, M., and Sussman, J. L. (1993), *Proc. Natl. Acad. Sci. USA* **90,** 5076–5080.
12. Mangili, F., Stella, M., Sassi, I., Ferrara, F., Paganelli, G., De Nardi, P., and Cantaboni, A. (1993), *Eur. J. Histochem.* **37,** 53–57.
13. Schechter, B., Arnon, R., and Wilchek, M. (1992), *Cancer Res.* **52,** 4448–4452.
14. Bickel, U., Yoshikawa, T., Landaw, E. M., Faull, K. F., and Pardridge, W. M. (1993), *Proc. Natl. Acad. Sci. USA* **90,** 2618–2622.
15. Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J., and Salemme, F. R. (1989), *Science* **243,** 85–88.
16. Hendrickson, W. A., Pähler, A., Smith, J. L., Satow, Y., Merritt, E. A., and Phizackerley, R. P. (1989), *Proc. Natl. Acad. Sci. USA* **86,** 2190–2194.
17. Alon, R., Bayer, E. A., and Wilchek, M. (1990), *Biochem. Biophys. Res. Commun.* **170,** 1236–1241.
18. Alon, R., Bayer, E. A., and Wilchek, M. (1992), *Eur. J. Cell Biol.* **58,** 271–279.
19. Lis, H. and Sharon, N. (1993), *Eur. J. Biochem.* **218,** 1–27.