Carbohydrate Research, 208 (1990) 306–311
Elsevier Science Publishers B.V., Amsterdam

Note

The effects of deglycosylation on the properties of native and biotinylated bovine testicular hyaluronidase

Diane Lace, Anthony H. Olavesen, and Peter Gacesa*
Department of Biochemistry, University of Wales College of Cardiff, P.O. Box 903, Cardiff CF1 1ST (Great Britain)
(Received March 14th, 1990; accepted for publication, April 30th, 1990)

Mammalian hyaluronidases (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) are endoglycosidases that can hydrolyse the β-(1→4)-glycosidic bonds in hyaluronic acid and the chondroitin sulphates. Testicular hyaluronidase has been purified from a variety of sources[1] and the properties of the enzymes are well documented[2].

Intravenously administered hyaluronidase is of potential use for the treatment of myocardial infarction[3] and other circulatory disorders[4], and there have been pharmacological studies of preparations of hyaluronidase labelled with ^{125}I (ref. 5) or biotin[6]. A major problem is the relatively rapid removal of the enzyme from the blood stream[5], which may be mediated by the high-mannose oligosaccharide subunits of the enzyme[5]. In order to study this uptake phenomenon, the deglycosylation of bovine testicular hyaluronidase has been investigated. It is important that deglycosylation should not alter the properties of hyaluronidase that are essential for monitoring its uptake during studies of metabolism.

We now report on the deglycosylation of preparations of bovine testicular hyaluronidase that have been used clinically[7], on a biotin-labelled derivative, and on the effects of deglycosylation on certain of the properties of the enzyme preparations.

Preliminary studies were done to ascertain the kinetics of deglycosylation of native and biotinylated hyaluronidases by endoglycosidase F, an enzyme that catalyses the hydrolysis of the chitobiose unit of certain mannose-containing N-linked oligosaccharide units[8]. Samples of hyaluronidase were removed from the reaction mixture at various times up to 5 h and analysed by SDS-PAGE. Staining of the gels with Coomassie Blue revealed one major and several minor bands. The presence of multiple-molecular-weight forms in highly purified preparations of hyaluronidase has been confirmed by electrophoresis and immunoblotting with monoclonal anti-hyaluronidase antibody[9,10].

The mobility of each of the molecular weight species increased as the deglycosyla-

* Author for correspondence.

0008-6215/90/$ 03.50

tion progressed up to but not beyond 2.5 h. Western blotting of the gels and analysis of the enzyme with Con A–peroxidase, anti-hyaluronidase antibody, streptavidin–peroxidase (in the case of the biotinylated enzyme), and the Schiff periodate reagent revealed identical patterns of mobility. Efficient transfer of the protein to the membrane was confirmed by staining with Amido Black. It was estimated that the major band of hyaluronidase decreased in molecular weight from 62 000 to 57 000, a reduction that is comparable to the estimated size (~4800) of the three major oligosaccharide units present in hyaluronidase[11].

Deglycosylation did not affect the activity of the enzyme, and similar results were obtained with biotinylated hyaluronidase except that this preparation could bind streptavidin.

Prior to deglycosylation, hyaluronidase activity, protein, and total carbohydrate were co-eluted from a column of Sephadex G-100. After incubation with endoglycosidase F for 5 h (Fig. 1), 66% of the total carbohydrate had been removed from the hyaluronidase and was eluted as a separate peak of low-molecular-weight material. This percentage is comparable to that found on deglycosylation of coat proteins of the Sendai virus[12].

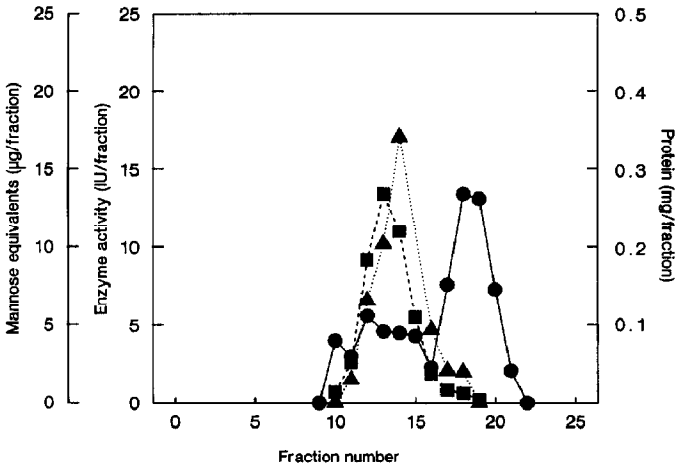

Fig. 1. Gel-permeation chromatography of hyaluronidase following deglycosylation with endoglycosidase F. The sample (0.16 mL) was applied to a column (0.9 x 30.0 cm) of Sephadex G-100 superfine and eluted at 6 $mL.h^{-1}$ with 0.1M NaCl. Fractions (0.93 mL) were assayed in duplicate for enzyme activity (■), protein (▲), and carbohydrate content (●).

The carbohydrate composition of hyaluronidase, before and after treatment with endoglycosidase F, was assessed by g.l.c. of the trimethylsilylated products of methanolysis. D-Mannose and 2-acetamido-2-deoxy-D-glucose were the only sugars detected in significant quantities; the molar ratio was 1.9:1.0 for hyaluronidase and 0.6:1.0 for the deglycosylated preparation, which indicated selective removal of mannose-containing moieties.

The affinity of Con A, quantified using the Con A–peroxidase conjugate, for native and biotinylated hyaluronidase decreased progressively as deglycosylation progressed but was still significant when the reaction with endoglycosidase F was complete (Fig. 2). Thus, either Con A can still bind to the remaining single 2-acetamido-2-deoxy-D-glucose residues or to residual oligosaccharide units. Only certain high-mannose type oligosaccharides are hydrolysed[12] by endoglycosidase F.

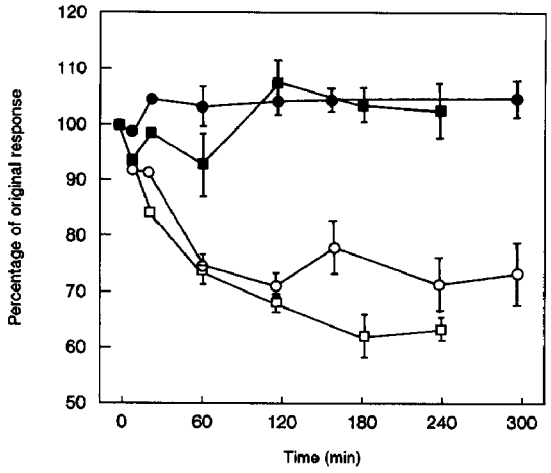

Fig. 2. The effect of deglycosylation on the ability of hyaluronidase to bind Concanavalin A. Native (o, ●) and biotinylated (□, ■) hyaluronidase were each incubated with endoglycosidase F. The deglycosylated (o, □) and parent hyaluronidases (●, ■) were tested for their ability to bind Con A–peroxidase conjugate. The results (determined in triplicate) are expressed as mean values ± standard deviation.

Deglycosylation did not alter the antigenicity of hyaluronidase (Fig. 3a), but the antigenicity of the biotinylated enzyme increased to a maximum of 140% (Fig. 3b). This result indicated that the presence of both the oligosaccharide units and the biotin residues masks the epitope for the anti-hyaluronidase antibody.

Treatment of the biotinylated hyaluronidase with endoglycosidase F resulted in an increased avidity for peroxidase-conjugated streptavidin up to 170% (Fig. 4); it is possible that the oligosaccharide units sterically hinder the binding of the streptavidin conjugate to the biotin residues.

Thus, both native and biotinylated hyaluronidase may be deglycosylated without loss of enzyme activity or immunological properties. The deglycosylated biotinylated enzyme showed enhanced binding of antibody and streptavidin, which may increase the sensitivity of its detection studies of metabolism.

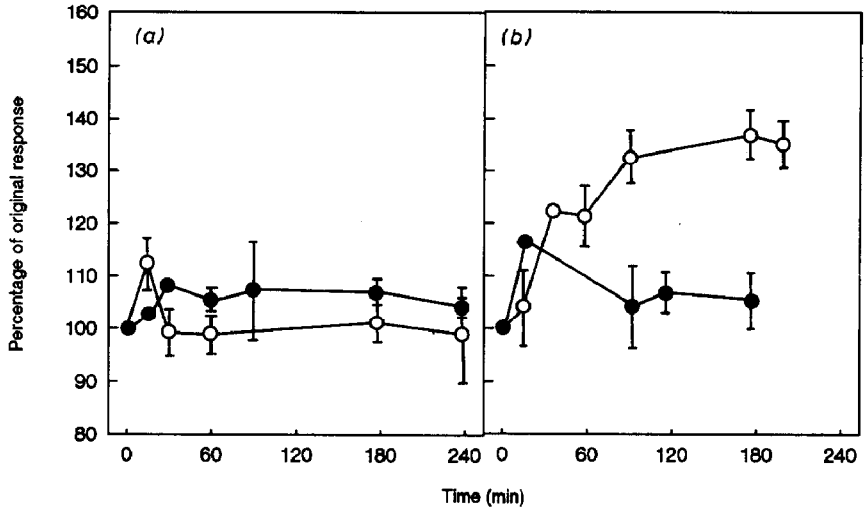

Fig. 3. The effect of deglycosylation on the binding of anti-hyaluronidase antibody. (*a*) Native and (*b*) biotinylated hyaluronidases were each incubated with endoglycosidase F. The deglycosylated (o) and parent hyaluronidases (●) were tested for their ability to bind rabbit anti-hyaluronidase. The results (determined in triplicate) are expressed as mean values ± standard deviation. The values for the biotinylated enzyme (180 min) are significantly different ($p < 0.001$), whereas those for the native enzyme (240 min) are not as determined by Student's t-test.

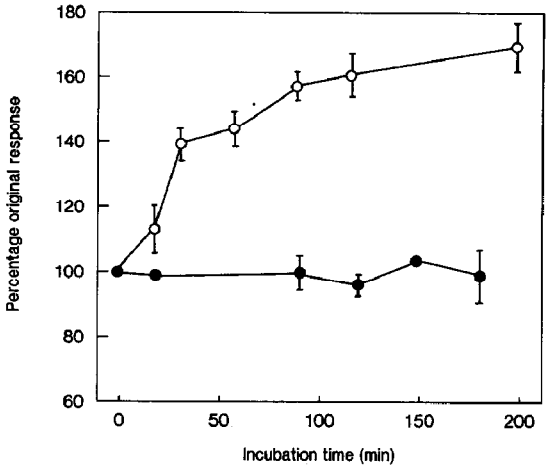

Fig. 4. The effect of deglycosylation on the binding of streptavidin to biotinylated hyaluronidase. Biotinylated hyaluronidase was incubated with endoglycosidase F, and the deglycosylated (o) and non-deglycosylated hyaluronidases (●) were tested for their ability to bind streptavidin–peroxidase conjugate. The results (determined in triplicate) are expressed as mean values ± standard deviation. The control (180 min) and test (200 min) are significantly different ($p < 0.001$).

EXPERIMENTAL

Preparation and assay of hyaluronidase. — A highly purified preparation of bovine testicular hyaluronidase (> 40 000 IU/mg), prepared according to the method of Pope *et al.*[13], was a gift from Biorex Laboratories Ltd. Hyaluronidase activity was measured by quantitation[14] of the release of reducing equivalents of 2-acetamido-2-deoxy-D-glucose from a solution of potassium hyaluronate[15]. Enzyme activity is expressed in international units (IU) of hyaluronidase as defined by the World Health Organisation[16].

Hyaluronidase was biotinylated[17] using a 2000-fold molar excess of biotin–*N*-hydroxysuccinimide ester to give preparations that contained 35 ± 5 mol of biotin/mol of enzyme (mean ± S.D. of three experiments).

Deglycosylation of hyaluronidase preparations. — Typically, aqueous solutions (125 μg/mL) of native or biotinylated hyaluronidase were incubated at 37° with endoglycosidase F (20 U/mg; 0.28 U/mL; Boehringer) in 0.1M sodium acetate buffer (pH 5.1). Samples were removed at intervals and analysed by sodium dodecyl sulphate–poly(acrylamide) gel electrophoresis (SDS-PAGE) and Western blotting.

Electrophoresis and Western blotting. — Samples of the native enzyme and the mixture obtained on deglycosylation were each added to 0.5 vol. of reducing buffer solution[18] and heated for 5 min at 100°. Portions of each sample (5–10 μg of protein) were applied to SDS-poly(acrylamide) gels (3.75% stacking gel and 10% separating gel) and then subjected to electrophoresis[18], and the gels were stained with Coomassie Blue.

Alternatively, proteins were Western blotted on to nitrocellulose membranes by electrophoresis[19] and analysed using either the Amido Black protein stain[20], a polyclonal anti-hyaluronidase antibody, peroxidase-conjugated Con A, or a peroxidase-conjugated streptavidin. The detection of antigen, protein-associated carbohydrate, and biotin-labelled hyaluronidase was performed essentially as described by Towbin *et al.*[21]. Antigen was detected using a 1000-fold dilution of rabbit anti-hyaluronidase followed by a 500-fold dilution of peroxidase-conjugated goat anti-rabbit IgG. Carbohydrate was estimated using 0.5 μg/mL of Con A conjugate, whereas the biotin label was determined using a 1000-fold dilution of peroxidase-conjugated streptavidin.

Quantitative estimation of the carbohydrate content of the enzyme. — Samples of native and deglycosylated hyaluronidase were methanolysed and trimethylsilylated according to Sweeley *et al.*[22], with mannitol as the internal standard. The derivatives were analysed on 3% of SE-30 on Gas-Chrom Q (80–100 mesh) with an N_2 flow rate of 30 mL/min and the temperature programme of 1°/min from 135–250°.

Enzyme-linked solid-phase assay methods. — The wells (4–8 for each sample) of a microtitre plate were primed by incubation overnight at 4° with appropriate samples (50 μL per well) of heat-treated (100° for 5 min) hyaluronidase [diluted with phosphate-buffered saline (PBS, pH 7.2) to a concentration range of 0–0.5 μg/mL]. After three washes with PBS, any remaining protein-binding sites were blocked by incubation with a solution (10 mg/mL) of bovine serum albumin (100 μL per well) for 0.5–2 h at 25°.

The relative antigenicities of hyaluronidase preparations were determined using

rabbit polyclonal anti-hyaluronidase (Biorex Laboratories Ltd.) diluted 1000-fold in PBS containing 5% of foetal calf serum. After incubation with the antibody for 2 h at 37°, the plates were washed three times with PBS. Peroxidase-conjugated goat anti-rabbit IgG (500-fold dilution in PBS containing 0.05% of Nonidet P-40) was added to each well and allowed to incubate for 2 h at 37° followed by five washes with the detergent-containing PBS. The binding of antibody was quantitated by addition of a chromogenic substrate for peroxidase, which comprised 0.4 mg/mL of 1,2-benzenediamine in citrate–phosphate buffer (0.32M citric acid–0.34M Na_2HPO_4, pH 5.0). Hydrogen peroxide (20 μL of a 30% solution) was added to 50 mL of substrate solution immediately before use. The combined peroxide–chromogenic substrate solution was added (50 μL per well) and allowed to incubate at room temperature in the dark for up to 20 min prior to stopping the reaction by addition of 25 μL of 2.5M H_2SO_4. The absorbance at 492 nm was proportional to the amount of antibody bound by the enzyme.

The assay of Con A and of streptavidin binding was performed in an analogous manner to that described for the polyclonal antibody. A 1000-fold dilution of peroxidase-conjugated Con A or peroxidase-conjugated streptavidin in PBS was used instead of the first and second antibodies.

ACKNOWLEDGMENTS

The S.E.R.C. and Biorex Laboratories Ltd. are thanked for a CASE studentship (to D.L.).

REFERENCES

1 A. J. Bollett, W. M. Bonner, and J. L. Nance, *J. Biol. Chem.*, 238 (1963) 3522–3527.
2 K. Meyer, in P. D. Boyer (Ed.), *The Enzymes*, Vol. V, Academic Press, London, 1971, pp. 307–320.
3 P. R. Maroko, L. D. Hillis, J. E. Muller, L. Tavazzi, G. R. Heyndrickx, M. Ray, M. Chiariello, A. Distante, J. Askenaski, J. Salerno, J. Carpentier, N. I. Reshetnaya, P. Radvany, P. Libby, D. S. Raabe, E. I. Chazov, P. Bobba, and E. Braunwald, *N. Engl. J. Med.*, 296 (1977) 898–903.
4 J. B. Elder, A. T. Raftery, and V. Cope, *Lancet*, i (1980) 648–649.
5 J. S. Earnshaw, C. G. Curtis, G. M. Powell, K. S. Dodgson, A. H. Olavesen, and P. Gacesa, *Biochem. Pharmacol.*, 34 (1985) 2199–2203.
6 D. Lace, Ph.D. Thesis, University of Wales, 1988.
7 D. G. Julian, B. L. Pentecost, J. M. Simpson, R. H. Smith, P. J. Cadigan, and M. P. Petri, *Circulation*, 72 (suppl. III) (1985) 222.
8 F. Maley, R. B. Trimble, A. L. Tarentino, and T. H. Plummer, Jr., *Anal. Biochem.*, 180 (1989) 195–204.
9 R. A. P. Harrison, *Biochem. J.*, 252 (1988) 865–874.
10 R. A. P. Harrison, *Biochem. J.*, 252 (1988) 875–882.
11 J.-C. Michalski, A. H. Olavesen, P. J. Winterburn, D. J. Pope, and G. Strecker, *Biochem. Soc. Trans.*, 12 (1984) 655–656.
12 P. Hsieh, M. R. Rosner, and P. W. Robbins, *J. Biol. Chem.*, 258 (1982) 2548–2561.
13 D. J. Pope, C. Rhodes, and S. D. Gorham, Br. Pat. 1,425,918 (1976); *Chem. Abstr.*, 85 (1976) P1805h.
14 P. Gacesa, M. J. Savitsky, K. S. Dodgson, and A. H. Olavesen, *Anal. Biochem.*, 118 (1981) 76–84.
15 J. L. Reissig, J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.*, 217 (1955) 959–966.
16 J. H. Humphrey, *Bull. W.H.O.*, 16 (1957) 291–294.
17 D. Lace, K. S. Dodgson, P. Gacesa, and A. H. Olavesen, *Biochem. Soc. Trans.*, 15 (1987) 402–403.
18 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
19 W. N. Burnette, *Anal. Biochem.*, 112 (1981) 195–203.
20 W. Schaffner and C. Weismann, *Anal. Biochem.*, 56 (1973) 502–514.
21 H. Towbin, T. Staehelin, and G. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350–4354.
22 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.