ENZYMATIC CLEAVAGE OF GLYCOPEPTIDES

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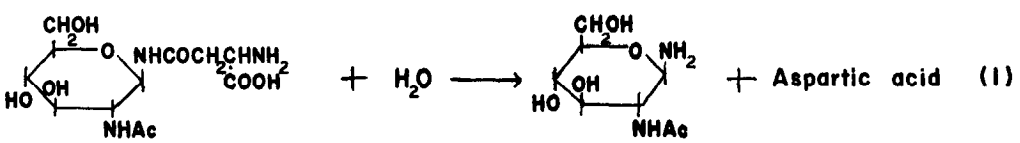
In many glycoproteins carbohydrate is linked to protein through the aspartylglycosylamine linkage (Yamashina et al., 1965, inter alia). We had been seeking an enzyme or an enzyme system working in the cleavage of this linkage using the synthetic β-aspartylglycosylamine (1-L-β-aspartamido-2-acetamido-1,2-dideoxy-β-D-glucose) and found that an enzyme from mammalian sera is capable of breaking down this linkage. Using a partially purified enzyme the reaction appears to proceed in two steps, (1) and (2), of which step (1) is catalyzed by the enzyme.



1-Amino-N-acetylglucosamine + H_2O ⟶ N-Acetylglucosamine + NH_3 (2)

There have been three reports on enzymatic cleavage of the aspartylglycosylamine linkage (Murakami and Eylar, 1965; Kaverzneva, 1965; Roston et al., 1965), but in none of them is the mode of enzymatic reaction definitely stated.

Materials and Methods: β-Aspartylglycosylamine was synthesized as described previously (Yamashina et al., 1965). 1-Amino-N-acetylglucosamine (1-β-amino-2-acetamido-1,2-dideoxy-D-glucose) was synthesized from 1-β-azido-2-acetamido-1,2-

dideoxy-D-glucose by catalytic reduction. The latter compound was derived from D-glucosamine-HCl via 1-α-Br-3,4,6-triacetylglucosamine-HBr, 1-β-azido-3,4,6-triacetylglucosamine and 1-β-azido-2,3,4,6-tetraacetylglucosamine. Experimental details for these syntheses will be published elsewhere.

In enzyme assays, ammonia was determined according to Lubochinsky and Zalta (1954), and N-acetylamino sugar by the Morgan-Elson reaction (Levvy and McAllan, 1959). Alkaline silver nitrate was used for detection of N-acetylamino sugars on paper (Trevelyan et al., 1950).

Results and Discussion: The structural similarity of β-aspartylglycosylamine to

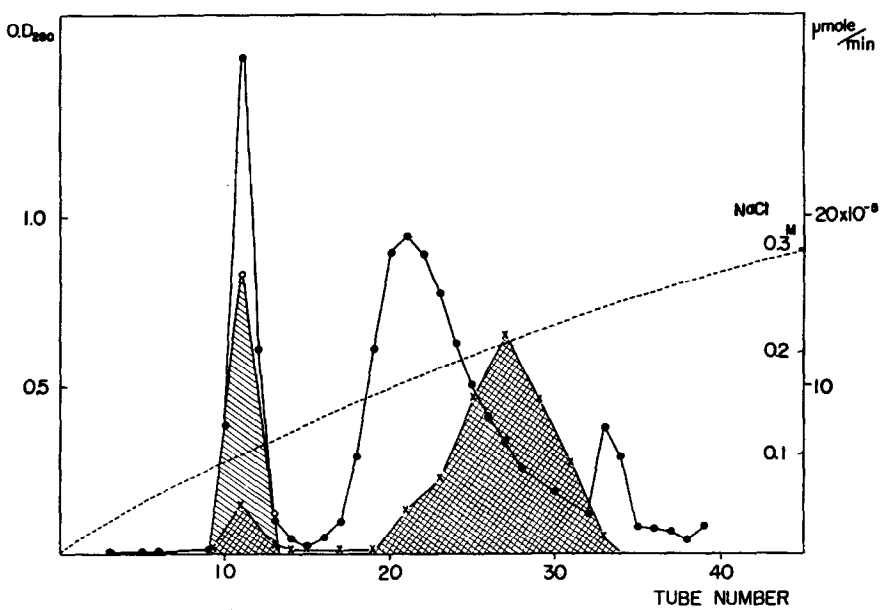


Fig. 1. Chromatography of the asparaginase fraction from guinea pig serum on DEAE-cellulose. Material for chromatography was the combined protein peak C and asparaginase peak obtained by chromatography according to Tower et al. (1963). The material (104 mg) was chromatographed on a 2.0 X 20 cm column which had been equilibrated with 0.02 M phosphate buffer, pH 7.35. Elution was by a gradient of NaCl in the same buffer, and each 4.4 ml fraction was collected with a flow rate of 4 ml per hour. Enzymatic reactions were carried out at pH 7.0 in 0.05 M phosphate buffer with substrate concentration 5 μmole/ml, and the ammonia liberation per min was measured. \\\\\\\ and XXXXXX indicate total activities of asparaginase and glycopeptide-cleaving enzyme of each fraction respectively. Asparaginase activity was traced with one thousandth scale of that indicated in the figure.

asparagine prompted us to test if asparaginase might hydrolyze the glycopeptide. Actually, guinea pig serum which is known to possess high asparaginase activity was capable of releasing ammonia and N-acetylamino sugar from the glycopeptide. Upon purification of the guinea pig asparaginase on DEAE-cellulose, however, about 90 % of the total activity in the serum could be attributed to another protein fraction than asparaginase (Fig. 1). The asparaginase fraction showed only marginal activity, the rate of the glycopeptide cleavage being less than one thousandth of that of asparagine hydrolysis.

The capacity to cleave the glycopeptide seems to be rather widely distributed among mammalian sera. Of three species tested, sera from guinea pig and pig had comparable activity, whereas bovine serum was much less active (about one fourth) than the other two.

Pig serum was fractionated by $(NH_4)_2SO_4$. Most of the activity was precipitable with $(NH_4)_2SO_4$ of 55 % saturation from the supernatant of the precipitation with $(NH_4)_2SO_4$ of 40 % saturation. The active fraction was then chromatographed on a DEAE-cellulose column and a CM-cellulose column successively. Thus, about 30 mg of the material were obtained from 100 ml serum with about 100 fold purification with reference to serum. At any stage of the purification, the enzymatic liberation of ammonia and N-acetylamino sugar ran parallel at pH 5.5 in 0.05 M phosphate buffer.

The enzyme preparation was readily soluble in water and stable over a pH range of 4 - 10 for at least 22 hours at 37°C. Heating at 100°C for 5 min destroyed the activity.

With synthetic β-aspartylglycosylamine, the Michaelis constant was about 10^{-3}M. Incubation of this substrate (5 μmole/ml) with the enzyme (substrate : enzyme = 5 : 1 by weight) in 0.05 M phosphate buffer, pH 7.0 at 37°C for 110 hours resulted in complete hydrolysis yielding equimolar amounts of ammonia, aspartic acid and N-acetylglucosamine.

The pH dependence of the activity is shown in Fig. 2. A remarkable finding was that at alkaline pH ammonia liberation dropped precipitously in con-

trast to a rather slow decline of amino sugar liberation. It was later found that the plateau of the curve for amino sugar liberation at pH 7 - 8.5 was due to formation of 1-amino-N-acetylglucosamine instead of N-acetylglucosamine in this pH range, the clour yield of the former amino sugar in the Morgan-Elson reaction being about 10 % higher than that of the latter.

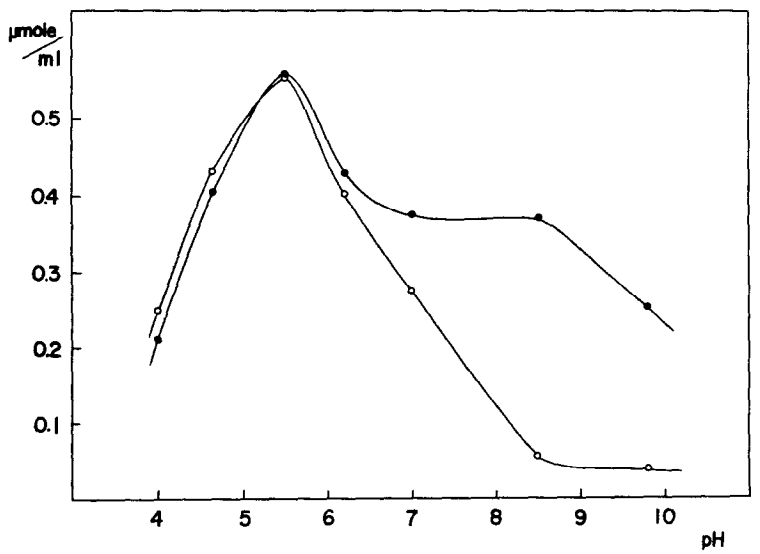


Fig. 2. The pH dependence of liberation of ammonia and N-acetylamino sugar in the enzymatic cleavage of the synthetic β-aspartylglycosylamine. —●—●— , N-acetylamino sugar; —○—○— , ammonia. Enzymatic reaction was carried out with substrate concentration 5 μmole/ml in the following buffers. 0.05 M Acetate buffer for pH's 4.0 and 4.7, 0.05 M phosphate buffer for pH's 5.5, 6.2 and 7.0, 0.05 M barbital buffer for pH 8.5, and 0.05 M carbonate buffer for pH 9.8.

The delay in ammonia formation at alkaline pH was considered to reflect the reaction sequence (1) and (2), as mentioned above. In order to prove this, 1-amino-N-acetylglucosamine was synthesized and its properties were investigated.

The pH dependence of the stability of the synthetic 1-amino-N-acetylglucosamine was as expected. When hydrolysis of 1-amino-N-acetylglucosamine (5 μmole/ml) to ammonia and N-acetylglucosamine was followed at various pH values for 20 hours at 37°C, the following values for hydrolysis % were recorded, i.e. 10 at pH 8.5, 75 at pH 7, and 95 at pH 5.5. At pH lower than 5, 100 % hydrolysis was

achieved in one hour. The hydrolysis was not accelerated by the enzyme preparation at any pH. Thus, the reaction (2) was concluded to be nonenzymatic.

Further support for the reaction sequence could be obtained by showing that the reaction mixture at pH 8.5 after 20 hours' incubation contained 1-amino-N-acetylglucosamine which was indistinguishable on paper chromatography and on paper electrophoresis from the synthetic amino sugar. For chromatography, ethyl-acetate-1-propanol-H_2O-aq.NH_3 of s.g. 0.90 (1 : 7 : 2 : 0.1) was used as a solvent (R_f = 0.41) and electrophoresis was carried out in 0.05 M barbital buffer, pH 8.5, at 3°C for 30 min at 50 volts/cm (migration towards cathode = 4.5 cm). Judging from the area and colour intensity of the spots on paper, this amino sugar was the major product of the enzymatic reaction at pH 8.5 whereas N-acetylglucosamine was in much smaller amount.

A glycopeptide from ovalbumin prepared according to Yamashina and Makino (1962) was as good a substrate as synthetic β-aspartylglycosylamine. The pH dependence of the reaction determined by ammonia estimation was also similar although there was no colour by the Morgan-Elson reaction in the enzymatic reaction at any pH, which could be due to substitution at both C-3 and C-4 of the terminal glucosamine of the oligosaccharide (Makino and Yamashina, 1966). Thus, after the reaction at pH 8.5, 1-amino-oligosaccharide could be isolated from the reaction mixture by gel filtration on a Sephadex G-15 column. This oligosaccharide liberated ammonia readily upon acidification.

All these facts are in accord with the proposed reaction sequence (1) and (2). Since the enzyme participates only in reaction (1), its action may be classified as a peptide or amide hydrolase.

Specificity of this enzyme was further investigated using several glycopeptides. Asp-Thr and Thr-Asp to which N-acetylglucosamine is linked to aspartic acid with the same type of linkage as in the β-aspartylglycosylamine (experimental details of synthetic procedures of these glycopeptides will be published elsewhere) were not attacked by the enzyme. Ovalbumin and α_1-acid glycoprotein from human plasma were not attacked, to judge from the appearance

of trichloracetic acid soluble carbohydrate or of reducing power. However, this enzyme might possess specificity toward peptide structures to which glucosamine is linked, or might display transferring action which apparently could not be detected in our assay system.

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