The Resistance of Glycoproteins to Proteolytic Inactivation

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The carbohydrate constituents of globular glycoproteins are probably located at the surface and may thus be expected to act as local shields and enhance the protection of the polypeptide towards degradation by proteolytic enzymes. Supporting evidence for this hypothesis has been obtained by different groups,¹⁻³ and the results were obtained from experiments using glycoproteins found in nature.

A novel approach to such studies is provided by the possibility of attaching, covalently, soluble carbohydrates to polypeptides by the cyanogen bromide method.⁴ The evidence obtained so far indicates that a considerable increase in stability towards inactivation by heat and by proteolytic enzymes may be imparted to proteins in this way.^{5,6}

In the present communication we describe extensions to our previous experiments⁶ and have examined the proteolysis of different fractions of a lysozyme-dextran T10 preparation as well as the inactivation by autolysis of chymotrypsin, trypsin and their dextran conjugates.

Experiments. The enzymes used were egg white lysozyme, EC 3.2.1.17 (Nutritional Biochemicals Corporation, U.S.A.), alpha-chymotrypsin, EC. 3.4.21.1 (Koch-Light Laboratories, England) and trypsin, EC. 3.2.21.4 (Sigma Chemical Co., U.S.A.). Dextran-T10 (Pharmacia, Sweden) with an average molecular weight of 10 000 daltons was used as the carbohydrate attached to the enzymes. Partial characterisation of the preparations is included in Table 1.

The conjugates were prepared from cyanogen bromide activated T10⁴ (400 mg) which was attached to free amino- and carboxyl groups⁷ in

the proteins (100 mg). Subsequent to the coupling procedure, the lysozyme-T10 preparation was subjected to gel exclusion chromatography on Sephadex G-100 and the three fractions, A, B, and C were obtained from the early, middle, and late parts of the elution diagram, respectively.

Lysozyme was assayed using the lysis of bacterial cell walls⁸ and the proteases were assayed by the caseinolytic method.⁹ Relative specific enzyme activities were obtained by comparison with the original enzymes and the calculations were based on the protein content of the conjugates. Percentage inactivation was determined by comparing the assay readings to standard curves of "amount of enzyme" versus "assay reading".

The carbohydrate content of the lysozyme-T10 fractions was determined by gas chromatography of the trimethyl silyl derivatives of the methyl glucosides obtained by methanolysis¹⁰ but the compositions of the protease-conjugates were taken directly from the amounts used in the coupling procedure as no fractionation was carried out.

All proteolytic degradations were carried out in sealed tubes at 37 °C in the presence of traces of toluene in order to prevent bacterial growth. Lysozyme and the lysozyme-conjugates were incubated in 25 mM citrate-phosphate buffer (pH 6.2) containing 5 mM calcium chloride in order to stabilise chymotrypsin. The concentrations of lysozyme and chymotrypsin in these mixtures (Nos. 1-4) were 0.05 mg/ml and 0.2 mg/ml, respectively.

The autolytic inactivation of the protease conjugates and the free proteases were performed in 100 mM phosphate buffer (pH 7.5). The chymotrypsin concentrations in incubation mixtures 5 and 6 were 0.5 mg/ml and 0.7 mg/ml respectively, and the trypsin concentrations in mixtures 7 and 8 were 1.0 mg/ml and 1.7 mg/ml, respectively.

For control purposes the free enzymes were incubated in the presence of concentrations of dextran T10 equivalent to the amount of T10 present in the corresponding conjugate-incubations. This was done in case the dextran might have a modifying influence on the proteolytic enzymes.

Results and discussions. The results obtained by Marshall and Rabinowitz⁵ and by us⁶ (Table 2) show that the covalent attachment of carbohydrate to lysozyme, trypsin, and chymotrypsin render these proteins more resistant to proteolytic degradation. Our results show that the lysozyme fraction with the highest content of carbohydrate was most resistant to inactivation by chymotrypsin. Assays of the proteolytic activity in the different incubation mixtures with lysozyme and its conjugates indicate that the results can not be due to variations in the activity of chymotrypsin.

The protective influence of carbohydrate constituents attached by the cyanogen bromide

Table 1. Protein-dextran T10 preparations.

Preparation	Relative specific enzyme activity (%)	Carbohydrate content (% w/w)
Lysozyme-T10-A	16	40
Lysozyme-T10-B	39	29
Lysozyme-T10-C	24	17
Chymotrypsin-T10	55	80
Trypsin-T10	58	80

Table 2. Proteolytic inactivation of protein-dextran conjugates and free proteins.

Incubation mixture No.	Composition	Enzyme assay	Remaining enzyme activity (%)					
			Incubation time (days)					
			0	1	2	3	4	5
1	Lysozyme + T10	Lysozyme	100	13	0	0	0	0
2	Lysozyme-T10-A + chymotrypsin	Lysozyme	100	52	45	42	40	40
3	Lysozyme-T10-B + chymotrypsin	Lysozyme	100	40	32	28	27	25
4	Lysozyme-T10-C + chymotrypsin	Lysozyme	100	35	27	22	20	17
5	Chymotrypsin + T10	Chymotrypsin	100	52	41	29	17	
6	Chymotrypsin-T10	Chymotrypsin	100	72	60	50	40	
			Incubation time (h)					
			0	1	2	5	24	
7	Trypsin + T10	Trypsin	100	24	16	2	0	
8	Trypsin-T10	Trypsin	100	50	40	24	20	

method is consistent with the results obtained with "natural" glycoproteins.¹⁻³ Thus it appears that the phenomenon may be a general one. To what extent the type of carbohydrate and the size of the polysaccharide groups affect the protective influence is not yet known, but experiments to clarify this point should be relatively simple in view of the convenient method available for preparation of glycosylated proteins.

The reason for the enhanced resistance to proteolysis is not definitely known although it seems plausible that steric hindrance is, at least partly, responsible for the effect. However, if carbohydrate constituents cause a reduction in the tendency for the polypeptide chain to oscillate between different conformational states, this might also affect the susceptibility to proteases.¹¹ An effect of this type might possibly be related to the increased resistance to thermal inactivation.⁵

Stabilisation of enzymes by attachment of carbohydrate might have applications for industrial processes or for enzymes used commercially as for example in detergents. Enzymes linked to soluble carbohydrate could subsequently be attached to an insoluble support and linkage between the carbohydrate part and the solid support might offer advantages.¹²

Increased half-life of therapeutic macromolecules by conjugation with polysaccharides is a possibility and it has been shown that the coupling of heparin to ficoll gave a complex with very good anticoagulant activity and the duration *in vivo* (rabbits) was significantly longer than that of free heparin.¹³

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