ENZYMATIC DEGRADATION OF THE COMPONENTS OF ASPARAGINYL-CARBOHYDRATE FROM CHICKEN OVALBUMIN

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Summary

Five components of asparaginyl-carbohydrate, prepared from crystalline chicken ovalbumin by digestion with pronase, have been isolated and purified. The homology of their structures has been demonstrated by enzymatic hydrolysis, using α-D-mannosidase and N-acetyl-β-D-glucosaminidase.

Three structures have been proposed for the carbohydrate group in chicken ovalbumin (1,2,3). In each case the classical methods of carbohydrate chemistry were applied to a carbohydrate glycopeptide (1) or the asparaginyl carbohydrate (AC*) obtained by proteolytic digestion of the glycoprotein. Subsequently, the AC was separated into five components (4,5,6) each with a different composition, so that the previous structures represented an average of the structural features of the molecules in this mixture. The first indications of the individual structures are described in the present paper and it will be noted that the original observations on the enzymatic digestion of AC were in error (1,7,8).

*AC is used as the abbreviation for the mixture of asparaginyl carbohydrates from chicken ovalbumin with each of the five components represented AC-A, AC-B, AC-C, AC-D and AC-E.

Experimental

Proteolysis of Ovalbumin. Ovalbumin (recrystallized four times) was digested four times with pronase as described previously (9). The mixture was gel filtered on Sephadex G25 and the fractions containing carbohydrate were combined and freeze-dried.

Fractionation of AC. The chromatographic procedure of Cunningham et al. (4) was followed in general, but with several modifications. Dowex-50-X2 (Bio-Rad AW 50-X2, 200-400 mesh) was regenerated with 2 N NaOH, washed well with sodium acetate starting buffer, pH 2.6 and 10^{-3} M in Na^+, and poured into a column (2.0 x 150 cm), which was equilibrated with the same buffer for 2 days at a pumping speed of 60 ml per hr. The mixture of glycopeptides, containing 250 to 300 mg equivalents of D-mannose, was applied to the column in 5 ml of starting buffer and the chromatography was continued at the same pumping speed. Aliquots (0.25 ml) of the fractions (15 ml) were analyzed for carbohydrate and the five peaks, corresponding to those described (4,5) were each combined, freeze-dried and rechromatographed until free from other peaks. Each peak was then purified further by zone electrophoresis on cellulose in 0.1 M pyridine acetate buffer, pH 6.4, as described earlier for ovalbumin glycopeptide mixtures (10), and finally desalted on Sephadex G-25. The pure fractions were analyzed for D-mannose, N-acetyl-D-glucosamine, and amino acids, only aspartic acid being detected. The composition of each fraction is given in Table 1.

Action of α-D-mannosidase on AC-fractions. α-D-Mannosidase was prepared by the method of Snaith and Levvy (11) and demonstrated to be free from any N-acetyl-β-D-glucosaminidase, using p-nitrophenyl β-D-glucosaminidine as substrate. To each AC-fraction (containing about 260 µg equivalents of D-mannose) in 0.05 M sodium acetate, pH 5.0, 0.01 M in NaCl and 0.1 mM in $ZnSO_4$, at 25° was added 4 units of α-D-mannosidase (12) and the final volume was adjusted to 500 µl with buffer. Incubation was continued in a saturated atmosphere of toluene to prevent bacterial contamination. A control solution

Table 1

Enzymatic Hydrolysis of Asparaginyl Carbohydrate Fractions from Chicken Ovalbumin

AC-Fraction	Mole % of AC	Composition[a]		Sugar Residues Hydrolyzed by	
		Man	GNAc	α-D-Mannosidase	N-Acetyl-β-D glucosaminidase
A	5.0	6.01	5.00	6.1	4.0
B	12.5	5.10	5.08	5.1	3.9
C	35.8	5.92	4.05	6.2	2.9
D	27.0	5.94	2.18	6.0	1.0
E	19.7	5.01	1.96	5.1	1.1

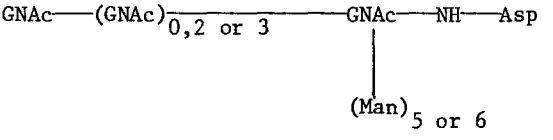
[a]Values are given with reference to one mole of aspartic acid.

was prepared identically. Periodically, aliquots (5 µl), of the reaction solutions were removed, heated for 3 min in a boiling water bath and evaporated to dryness. The D-mannose released was determined by the method of Park and Johnson (13). After 96 hr a second aliquot of α-D-mannosidase was added to insure that the reaction was as complete as possible. The amount of D-mannose released from each AC-fraction is summarized in Table 1.

Action of N-acetyl-β-D-glucosaminidase on AC-fractions. N-Acetyl-β-D glucosaminidase (1 unit), kindly supplied by Dr. Y. T. Li (14), was added to each AC-fraction (containing about 260 µg equivalents of D-mannose) dissolved in 0.05 M citrate buffer, pH 5.0 (500 µl). The reaction was followed at 25° under an atmosphere of toluene by periodically withdrawing aliquots (25 µl), which were heated in a boiling water bath for 3 min, evaporated to dryness, and the free N-acetyl-D-glucosamine determined in the residue (15). When the rate of hydrolysis slowed down more enzyme (0.5 unit) was added until the release of hexosamine stopped. This involved from two to four additions of enzyme and a total reaction time of up to 400 hr. A control was followed concomitantly. The results are summarized in Table 1.

Results and Discussion

α-D-Mannosidase hydrolyzes all the D-mannose in each of the AC fractions without any release of N-acetyl-D-glucosamine. N-Acetyl-β-D-glucosaminidase hydrolyzes all but one of the glucosamine residues in each of the AC-fractions without any release of D-mannose. These results are consistent with the general structure of the carbohydrates in the ovalbumin AC-fractions shown in I.



I

The structures represented in I are compatible with many of the observations made earlier on AC with the outstanding exception of the reported release of an average of one residue of glucosamine instead of the average of two residues noted in the present study. The earlier studies gave no indications of the rate of hydrolysis of the N-acetyl-D-glucosamine or whether the reaction was complete. Fraction AC-C, which represents about 36% of AC, released 0.62, 1.52 and 2.38 moles of N-acetyl-D-glucosamine after hydrolysis for 96, 240 and 340 hr, respectively, a fresh aliquot of enzyme being added after each time interval noted, with a final total formation of 2.94 moles of hexosamine after 384 hr. This relatively slow release of the hexosamine residues is not due to the steric inhibition of the D-mannose-oligosaccharide sidechain because AC-D and AC-E are each hydrolyzed to give one residue of N-acetyl-D-glucosamine after 150 hr of hydrolysis under identical conditions. The possibility exists that the glucosamine units may not be linked to each other in an identical fashion so that the rates of glycosidic cleavage may differ.

With the increasing availability of specific carbohydrases, their application to structural studies will be even more general than at present.

The present investigation points to the need for caution in the interpretation of these results in terms of the primary sequence of the monosaccharide residues.

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