CLEAVAGE OF THE CARBOHYDRATE-PEPTIDE BOND IN THE POLYSACCHARIDE-ASPARAGINE FRAGMENT OF OVALBUMIN BY ENZYMES FROM AN EXTRACT OF ARION SUBFUSCUS

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After the establishment of the chemical nature of the bond between the carbohydrate and the peptide fraction of ovalbumin [1, 2, 3], a search was begun for enzymes splitting this bond. This search was all the more well-founded since the type of bond found proved to be widely distributed in the animal kingdom (pleuromucoid [4], the acidic α_1-glycoprotein of the blood [5], gonadotropin [6]) and is apparently found in plant proteins (hemagglutinin of soybeans [7]).

Table 1

Carbohydrate Content of the Neutral Fractions of the Enzyme Hydrolyzate

Substance	Hydrocarbons			
	after 6 days		after 10 days	
	mg	% of the amount in the sample	mg	% of the amount in the sample
Substrate + enzyme (experiment)	3.94	39	7.3	68
Substrate (control 1)	0	—	0.15	—
Enzyme (control 2)	0	—	0.33	—

Recently enzymes have been found which cleave this bond not in the native proteins but in low-molecular-weight glycopeptides. Montgomery et al., [8] have cleaved the polysaccharide-aspartic acid from ovallsumin by means of emulsin (pH 4.1), and Murakami et al., [9] have found that the synthetic simplest compound containing this bond, 1-β-aspartamido-2-acetamido-D-glucosamine, is cleaved under the action of an extract of sheep epididymides (pH 6.5).

Table 2

Molar Ratio of Aspartic Acid and Glucosamine in the Ammoniacal Eluates, mole*

Eluate	Aspartic acid (after 6 days)	Glucosamine (after 6 days)	Ratio	Aspartic acid (after 10 days)	Glucosamine (after 10 days)	Ratio
Experiment	2	1.10	2:1.10	2	2.10	2:2.10
Control - 1	1	2.60	2:2.97	1	2.77	2:4.82
Control - 2	1	0.37		1	2.05	

*Polysaccharide–aspartic acid: calculated aspartic acid: glucosamine = 1:3.

We have found enzymes which cleave this bond in molluscs. The polysaccharide-aspartic acid isolated from ovalbumin is cleaved under the action of a partially purified enzyme preparation from an extract of the slug Arion subfuscus at pH 6.8 with the formation of free aspartic acid.

Experimental

We obtained the enzyme extract from three slugs after grinding them in a homogenizer and digesting them in the cold for 1 hr in 9 ml of a 0.05 M acetate buffer of pH 6.8. After centrifuging, the solution was treated with 1.4 volume of saturated ammonium sulfate solution, and after 1 hr the precipitate that had formed was separated off, dissolved in 0.1 M acetate buffer, and left to dialyze against water with traces of sodium acetate. Then the solution was concentrated in the cold to a volume of 3 ml.

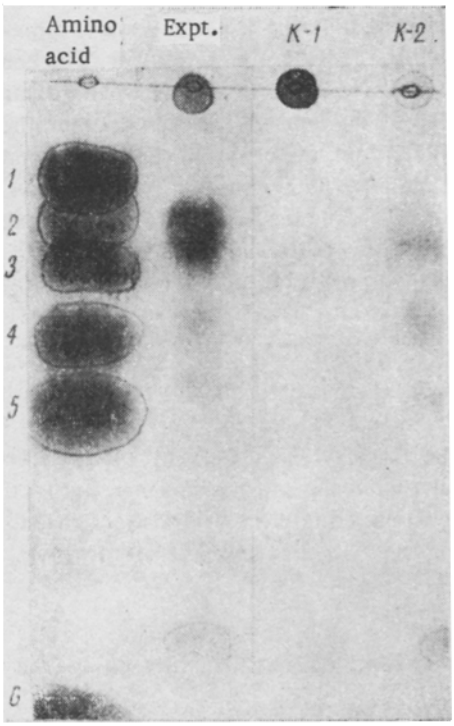

Fig. 1. Chromatogram of the ammonical eluates after the action of enzymes on the polysaccharide-aspartic acid in the butan-1-ol-acetic acid-water (4:1:5) system. K-1, substrate without enzyme; K-2, enzyme without substrate; 1) lysine; 2) aspartic acid; 3) glucosamine; 4) threonine; 5) alanine; 6) isoleucine.

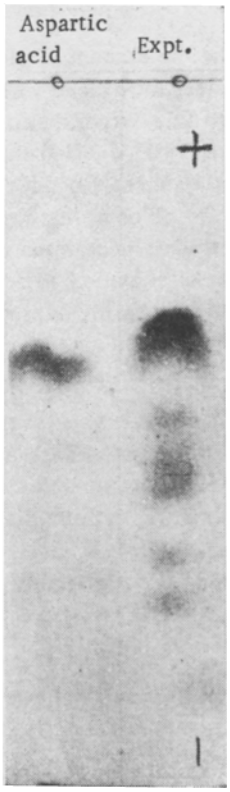

Fig. 2. Electrophoregram of the ammoniacal eluate after the action of enzymes on the polysaccharide-aspartic acid (acetic acid, pH 2.6, 2 hr at 1000 V).

The experiments on the action of the enzyme separation on polysaccharide-aspartic acid were carried out with two controls. The experimental solution contained 20 mg of substrate in 1 ml of 0.1 M acetate buffer and 1 ml of enzyme preparation; the 1st control (K-1) contained only 20 mg of substrate in 2 ml of buffer, and the 2nd control (K-2) contained 1 ml of enzyme and 1 ml of buffer. The solutions, covered with toluene, were kept in a thermostat at 37° C. After 6 and 10 days, 0.8-ml samples were taken from them for analysis. The experimental samples and the control samples were transferred to similar columns of "Dowex-50 × 4" (H^+) with dimensions of 0.9 × 7.0 cm. The neutral components were eluted from the column with 25 ml of water and the components containing basic groups were displaced with 4 N NH_4OH and were dried under vacuum.

The neutral fractions of the experimental solutions were found to contain carbohydrates (Table 1), while there was practically none in the controls. The amount of carbohydrates, determined by means of the colorimetric reaction with anthrone-sulfuric acid was 39% of the initial amount of mannose in the polysaccharide-aspartic acid after 39 days and 68% after 10 days. However, this still did not make it possible to answer the question of cleavage at the carbohydrate-asparagine bond, since the carbohydrate part could also have been cleaved at glycosidic bonds under the action of a group of enzymes.

The analysis of the ammoniacal eluates from the column enabled us to answer this question. On paper chromatograms and electrophoregrams the experimental solutions gave a distinct and intense spot of aspartic acid together

with a number of other ninhydrin-positive spots (Figs. 1 and 2).

For quantitative analysis, the eluates were subjected to hydrolysis (2 N HCl, 2 hr, 105° C, in sealed capillaries) and the content of glucosamine and amino acids was determined by means of electrophoresis-chromatography and in an amino acid analyzer. In addition to traces of other amino acids, the peaks of glucosamine and aspartic acid were clearly distinguished on the curve. Table 2 gives the ratios of glucosamine and aspartic acid obtained.

The control K-1, containing only the polysaccharide-aspartic acid gave ratios of 1:2.60 and 1:2.77 fairly close to the theoretical (1:3), which shows the considerable stability of this compound under the experimental conditions.

To determine the amount of glucosamine split off in the experimental solution it was necessary to make a correction for the impurities introduced with the enzyme, taking into account the fact that the total of the two control solutions corresponded to the experimental solution. Consequently, in order to equalize the conditions, the figures of the ratio for the experimental solution were doubled. The apparent increase in the amount of glucosamine in the experiment after 10 hr is apparently connected with an enhanced liberation of it from the enzyme preparation. Calculation showed a loss of 1.87 glucosamine residues per molecule of aspartic acid after 6 days and 2.72 after 10 days. This means that of the three glucosamine residues in the polysaccharide part of the molecule 1.13 remained attached to the aspartic acid after the enzyme had acted for 6 days and only 0.28 after ten days, which is equivalent to the complete cleavage of the polysaccharide-aspartic acid. If it is borne in mind that the material in 20 mg of substrate was treated with an enzyme preparation obtained from one slug with a weight of 3 g, it may be assumed that the specific glucosaminidase contained in it possesses a considerable activity.

In addition to the cleavage of the carbohydrate-peptide bond under the action of the enzyme preparation on the substance investigated, partial decomposition of the polysaccharide itself took place. This follows from the fact that the chromatograms of the ammoniacal eluate of the 6-day hydrolyzate had several spots giving positive reactions with ninhydrin and silver nitrate. They disappeared after 10 days' hydrolysis. Evidently, because of cleavage at the bond with the aspartic acid, these products of the carbohydrate decomposition passed into the neutral fraction. No free acetylglusamine was found in the latter.

Summary

A partially purified preparation from an extract of Arion subfuscus contains enzymes which cleave the amide bond in the polysaccharide-asparagine fragment of ovalbumin.

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