The Mechanism of Carbohydrase Action

9. HYDROLYSIS OF SALEP MANNAN BY PREPARATIONS OF α -AMYLASE*

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Salep mannan is a water-soluble polymer of β -D-mannose, the sugar residues being joined by 1 $\rightarrow$ 4-linkages (Husemann, 1940). Extracts of *Bacillus subtilis* and *Aspergillus oryzae* were reported to hydrolyse this polysaccharide and the enzyme responsible was said to be α -amylase (Husemann & Lindemann, 1954). The susceptibility of the mannan to attack by α -amylase was ascribed to the similar *cis* relation between the C-2 hydroxyl group and the glycosidic oxygen bridge in the α -1 $\rightarrow$ 4-bonded glucose residues of starch and the β -1 $\rightarrow$ 4-linked mannose units in salep mannan. This explanation, if it were correct, would have important implications in elucidating the mechanism of α -amylase action. If, on the other hand, the mannan-splitting enzyme was not α -amylase, then purification and crystallization of the amylase should remove the ability to attack mannan. This paper shows that the two hydrolytic activities are separable.

EXPERIMENTAL

Enzymes. Crystalline salivary α -amylase was prepared from human saliva as by Fischer & Stein (1961a) and crystallized three times. Pig-pancreatic α -amylase, twice-crystallized, was obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. *B. subtilis* and *A. oryzae* α -amylases were prepared from crude enzyme powders supplied by the Takamine Laboratory Inc., Clifton, N.J., U.S.A. Solutions of crude enzyme were obtained by suspending the powder (1.8 g.) in 10 ml. of ice-cold 2 mM-sodium glycerophosphate-hydrochloric acid buffer, pH 5.7, centrifuging the suspension and using the clear supernatant solution. Once- and five-times-crystallized *B. subtilis* α -amylase was prepared as by Fischer & Stein (1961b) and once-crystallized *A. oryzae* α -amylase as by Fischer & Stein (1954). The four-times-crystallized *A. oryzae* enzyme was the gift of Dr E. A. Stein. α -Amylase activity is reported in

terms of the units described by Fischer & Stein (1961a) and the method of assay was the same except for measurement of reducing power, which was made with a copper reagent (Shaffer & Hartmann, 1921).

Salep mannan. The polysaccharide was kindly provided by Professor E. Husemann.

Enzymic digestions of salep mannan. The digests (20 ml.) contained enzyme (17 units/ml.), mannan (2.5 mg./ml.), 2 mM-sodium chloride and 4 mM-calcium acetate. The digests containing *B. subtilis* and *A. oryzae* α -amylases were buffered at pH 5.7 with 4 mM-glycerophosphate-hydrochloric acid and those containing the salivary and pancreatic enzymes were buffered at pH 6.5 with 125 mM-sodium citrate. The digests were layered with toluene and incubated at 30°. Reducing power was measured by adding 1 ml. portions of digest to the Shaffer & Hartmann (1921) copper reagent, calibrated against mannose. α -Amylase activity in the digests was measured at frequent intervals and in all cases the activity was at 75–100% of the original after 160–190 hr.

RESULTS

We confirmed that crude preparations of *B. subtilis* and *A. oryzae* α -amylases slowly attack salep mannan. The rates of hydrolysis were, however, unequal despite the use of equal amounts of amylase in terms of starch-splitting activity (Fig. 1a, b). In once-crystallized-enzyme preparations the relative activity against mannan was considerably diminished and after several recrystallizations it was very slight (Fig. 1a, b). Crystalline human-salivary and pig-pancreatic α -amylases also caused a very slight hydrolysis of mannan during several days of incubation (Fig. 1c). However, this action and that of the recrystallized fungal and bacterial amylases cannot have any significance in relation to theories of the specificity of α -amylase since the rates of hydrolysis of the mannan are of an entirely different order of magnitude from that of starch. The amount of α -amylase in each digest was sufficient to liberate reducing substances from starch equivalent to

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Fig. 1. Hydrolysis of salep mannan by α -amylase preparations. (a) *A. oryzae* α -amylase: (1) crude enzyme; (2) once-crystallized enzyme; (3) four-times-crystallized enzyme. (b) *B. subtilis* α -amylase: (1) crude enzyme; (2) once-crystallized enzyme; (3) five-times-crystallized enzyme. (c) Human-salivary (○) and pig-pancreatic (●) α -amylases, crystallized thrice and twice respectively. Conditions were as described in the Experimental section.

170 mg. of glucose in 3 min., under the conditions of the activity determination (Fischer & Stein, 1961a). The reducing substances formed from mannan by the recrystallized fungal and bacterial amylases were equivalent only to about 1.5 mg. of mannose after 100 hr., and to about 0.5 mg. for the salivary and pancreatic enzymes. It is clear that most, if not all, of the hydrolysis of mannan caused by the crude *A. oryzae* and *B. subtilis* amylases is due not to α -amylase but to a separate

mannanase which is gradually removed as the amylase is purified. The recrystallized amylases are without significant action on the mannan.

SUMMARY

1. A reported hydrolytic attack of the α -amylases of *Aspergillus oryzae* and *Bacillus subtilis* on salep mannan was investigated. Crude preparations of the enzymes hydrolyse the polysaccharide but mannanase activity is lost when the enzymes are purified and crystallized. Several-times-crystallized specimens are without appreciable action on the mannan.

2. Crystalline α -amylases of human saliva and pig pancreas similarly have no appreciable action on the polysaccharide.

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The Biosynthesis and Metabolism of Oestrogens in the Rat

1. CONVERSION OF [4-¹⁴C]TESTOSTERONE INTO [¹⁴C]OESTRADIOL-17 β BY THE RAT OVARY

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Despite the fact that the rat is used extensively in endocrinological and biochemical research, little work has been published on the nature of the steroid hormone secretion in this animal, compared with man and other species. Thus, although Ketzer & Witt & Mitzner (1961) reported the identification of oestrone, oestradiol-17 α and oestradiol-17 β in the urine of the rat, there appears to be little or no other information on the secretion of oestrogen in this species.

The difficulty of determining steroids, particu-

larly oestrogens, in the body fluids of small rodents has hampered the interpretation of the role of endogenous sex hormones in carcinogenesis in these animals. Studies by Cronkite, Shellabarger, Bond & Lippincott (1960), however, suggest that the neoplastic response of breast tissue in the rat may be very dependent on the secretion of oestrogen by the ovary after whole-body irradiation.

One way of circumventing the difficulty of determining the secretion of small amounts of steroids is to measure the rate of biosynthesis of