ACTION OF PANCREATIC ALPHA-AMYLASE AND SWEET POTATO BETA-AMYLASE ON 6^2- AND 6^3-α-GLUCOSYLMALTO-OLIGOSACCHARIDES*

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1. Introduction

In our studies on the branching patterns in starch and glycogen, it was important to define accurately the specificities of the various enzymes which act on starch dextrins. The ordinary amylases have no action whatsoever on the branch (α-1,6) links, and in fact, such branches act as impediments to attack of some of the adjacent linkages which would otherwise be susceptible to amylase attack. A major difficulty in trying to delimit the specificity of the various amylases has been the unavailability of substrates with defined structure. We have recently reported [1] preparation of homologous oligosaccharides with single α-1,6-linked glucose unit "stubs" at the non-reducing end of the oligosaccharide chain. We report here two similar series of starch oligosaccharides with the α-1,6-linked glucose stub located on the second or third glucose units from the reducing chain end. The action of porcine pancreatic alpha-amylase and sweet potato beta-amylase on these substrates has been examined by the 2-dimensional chromatographic method [2]. Our results are summarized in scheme I.

2. Experimental

2.1. *Enzymes*

Twice-crystallized porcine pancreatic α-amylase, prepared as by Caldwell et al. [3], and crystalline sweet potato β-amylase, prepared as by Nakayama and Amagase [4] were purchased from Worthington Biochemical Corp. *Bacillus macerans* transglycosylase (amylase) was prepared as by Tilden and Hudson [5]. Crude glucoamylase from *Aspergillus niger* ("Diazyme 160") was a gift from Miles Laboratories, Inc. Pullulanase was prepared as by Wallenfels et al. [6].

2.2. *Preparation of substrates*

Stubbed oligosaccharides containing the panose structure in the reducing end were prepared by the *B. macerans* amylase coupling reaction [7, 8]. Panose was prepared from hydrol by charcoal chromatography [9] and crystallized twice from 80% methanol. Crystalline panose (50 mg) and 50 mg of twice crystallized cyclohexaamylose [10] were incubated with 4 Tilden and Hudson units [5] of *B. macerans* amylase [5, 10] in a total volume of 3 ml at 40°C for 7 days. A small crystal of thymol was included to inhibit microbial growth. The reaction mixture was boiled to inactivate the enzyme, cooled and treated with 2 IU** of pullulanase [6] at pH 5 for 2 days. This treatment removed

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** One International Unit (IU) as defined according to the International Committee on Enzymes is the amount of enzyme that will hydrolyze one micromole of glycosidic bonds per minute under optimal conditions. For crystalline pancreatic amylase there are approximately 500 IU per mg of protein; for crystalline sweet potato beta-amylase there are approximately 1100 IU per mg of protein. Our preparations of pullulanase, assayed on pullulan, contain about 0.1–1 IU per mg of dry powder.

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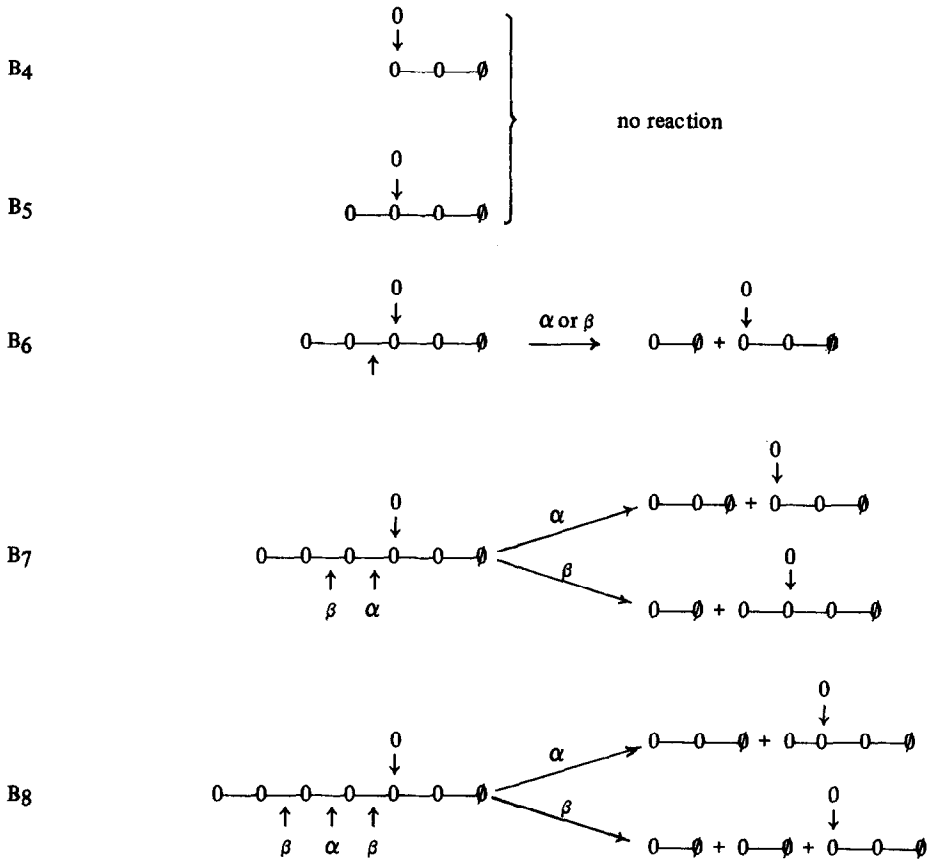
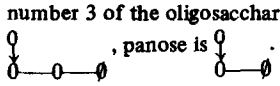


Scheme I. Position specificity and products of action of porcine pancreatic alpha-amylase (α) and sweet potato beta-amylase (β) on B4-coupled stubbed oligosaccharides*. Analogous results were obtained for substrate having the panose structure at the reducing end (panose-coupled oligosaccharides).

branches with two or more glucose units, leaving only linear and "stubbed" oligosaccharides [11]. It was observed that very extensive reaction with pullulanase was required to give complete debranching with the panose-coupled oligosaccharides. Completeness of the debranching was tested by adding a 10-fold excess of of pullulanase. This treatment gave no increase in reducing value. To prepare the B_4-coupled stubbed oligosaccharides, a 1% solution of waxy maize starch was hydrolyzed with porcine pancreatic amylase to the "second stage" [12]. At this point, the reducing value of the digest was 83% theoretical maltose. All linear fragments were converted to G_1, G_2 and a trace of G_3, and most of the branch points were in single-branched oligosaccharides (tetra- to heptasaccharide). The crude branched pentasaccharide fraction as obtained by charcoal chromatography [9, 13] was purified by paper chromatography (largescale descending chromatography at 40°C using Whatman 3MM paper irrigated with 65% propan-1-ol. For the coupling reaction, 50 mg of the pentasaccharide was treated as in the panose-coupling reaction. Debranching of the coupled product

* Symbols and abbreviations: 0, D-glucose unit; ——, α-1,4-linkage; ↓, α-1,6-linkage; ∅, reducing terminal D-glucose unit; ↑, point of amylase attack; G_1, G_2, G_3, etc. are glucose, maltose, maltotriose, etc.; B_4, B_5, etc., are "stubbed oligosaccharides", i.e., otherwise α-1,4-linked oligosaccharides containing a single α-1,6-linked glucose unit at glucose number 3 of the oligosaccharide chain. Specially, B_4 is  , panose is .

using pullulanase was considerably more facile than with the panose coupled product. We designate these compounds as "B_4-coupled oligosaccharides" because they all have the B_4 structure at the reducing end (cf. scheme I).

2.3. *Two-dimensional paper chromatography*

To survey the action of procine pancreatic alpha-amylase and sweet potato beta-amylase on the stubbed oligosaccharides, the 2-dimensional chromatographic method was used [2]. The chromatographic arrangement is illustrated in fig. 1 and fig. 2. The oligosaccharide mixture and suitable reference materials were subjected to five ascents, using the solvent system butanol: pyridine: water (6:4:4 parts by volume). After irrigating in the first direction and removing the reference channels, the area containing the resolved oligosaccharides was sprayed with enzyme solution. For pancreatic amylase, the solution contained 1000 IU of enzyme in 10 ml of 10 mM NaCl and 10 mM phosphate buffer, pH 6.8. The enzyme was allowed to react on the paper for 24 hr in a moist chamber at 40°C. For beta-amylase, 400 IU of enzyme were dissolved in 10 ml of 100 mM pyridine-acetic acid buffer, pH 4.8. This buffer is very desirable for applications involving paper chromatography, since it is completely volatile. After drying and applying a new reference spot, the chromatogram was again irrigated in the direction perpendicular to the first direction. The dried chromatogram was dipped rapidly in an enzyme suspension made by dissolving 1 g of Diazyme 160 in 200 ml of pyridine-acetic acid buf-

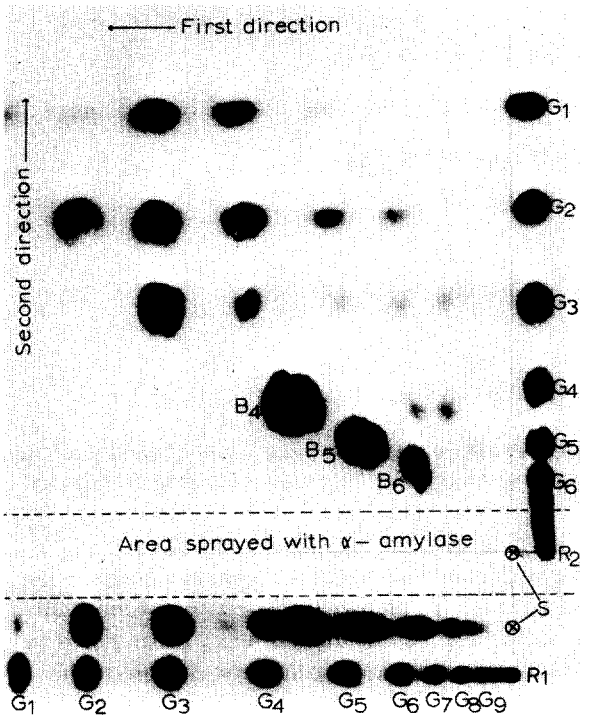


Fig. 1. Two dimensional chromatogram showing action of porcine pancreatic alpha-amylase on oligosaccharides containing a single α-1,6-linked glucose unit at position 3 (B_4-coupled oligosaccharides). R_1 and R_2 are reference series for the first and second direction of chromatography; S indicates the point of application of the stubbed oligosaccharide sample. After irrigation in the first direction, the lower part of the chromatogram, containing R_1 and one of the S channels, was cut off for reference. The remaining S channel was sprayed with alpha-amylase solution. After allowing adequate enzyme action on the paper, the chromatogram was dried, reference R_2 was applied, and the chromatogram was re-developed in the vertical direction.

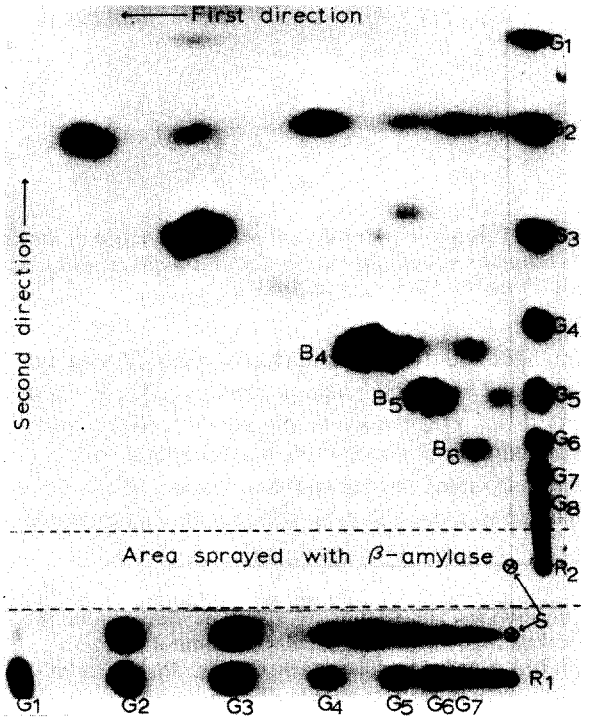


Fig. 2. Two-dimensional chromatogram showing action of sweet potato beta-amylase on oligosaccharides containing a single α-1,6-linked glucose unit at position 3. Symbols are as in fig. 1.

fer, then adding 800 ml of acetone. The wet chromatogram was hung in air for 1–2 min to evaporate the acetone, and allowed to incubate in a moist chamber for 30 min at 65°C. This Diazyme treatment was very effective in hydrolyzing the higher oligosaccharides to glucose, thereby greatly intensifying the silver dip reaction [14] for spot revelation. The extent of amylase action on the oligosaccharides was judged by the extent of hydrolysis of G_3, since G_3 is relatively resistant to both beta-amylase and pancreatic amylase.

3. Results and discussion

3.1. *Action of porcine pancreatic alpha-amylase*

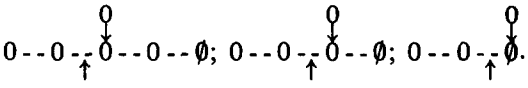
The results of action of this enzyme on the B_4-coupled stubbed oligosaccharides are illustrated in fig. 1. Enzyme action has been extensive judging from the degree of hydrolysis of G_3 to G_1 and G_2. All higher linear oligosaccharides have been completely degraded to G_1, G_2 and G_3. There was no detectable hydrolysis of B_4 or B_5. B_6, though rather resistant, has been partly hydrolyzed to G_2 plus B_4. B_7 and B_8 were completely hydrolyzed to give B_4 and B_5, respectively, and G_3. The original oligosaccharide sample did not contain visible amounts of the higher stubbed homologs.

The high degree of position specificity of pancreatic amylase acting on these oligosaccharides indicates that both the single glucose stub and the non-reducing end of the oligosaccharide chain are critical in positioning the substrate at the binding site of the amylase. Previously it has been shown that pancreatic amylase has a binding site for an amylose chain of five glucose units [15]. The subsites for the individual glucose units can be numbered 1–5, starting from the reducing end of a maltopentaose chain. This places the catalytic site between subsites 2 and 3. The results of the present study, with our previous findings [1], indicate that stubs or branches can be at positions 1 or 5 of the binding site, without blocking enzyme action. Stubs or branches at positions 3 or 4 prohibit enzyme action. A branch at position 2 severely restricts but does not absolutely block action.

3.2. *Action of sweet potato beta-amylase*

The rate of attack of beta-amylase on G_3 is only about 0.1% of that on starch. Therefore, the information of a siginificant amount of G_1 and G_2 from G_3 in fig. 2. is evidence that enzyme action has been fairly extensive.

Results of this study are in agreement with the earlier proposal [8] that a *single glucose stub branch* does not block beta-amylase action at the adjacent linkage on the non-reducing side of the branch point. Thus, the following three oligosaccharides are cleaved as indicated by the upward arrow:



This result is in contrast to the inability of beta-amylase to cleave such a linkage if the branch contains two or more glucose units [8]. It is rather remarkable that the ability of beta-amylase to attack this linkage depends on the structure (or possibility the bulk) of the branch. If the oligosaccharide contains an odd number of glucose units on the non-reducing side of the branch, this problem does not arise. Beta-amylase can remove G_2 units leaving a single glucose unit regardless of whether the branch is a single glucose unit stub or a chain of two or more glucose units.

It has been suggested that the substrate binding site for beta-amylase is specific for an amylose chain of six glucose units [16]. If the glucose units are numbered 1–6, beginning from the reducing end, our results indicate that branches may be on positions 1, 2 or 3 without preventing enzyme action. At position 4, a branch prevents enzyme action, but a single glucose stub only retards it. No reaction occurs if a stub or branch is at positions 5 or 6. If the substrate site binds some number other than six glucose units, the same arguments would hold. but it would be necessary to renumber the individual positions.

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