ACTION OF PANCREATIC AMYLASE ON STARCH OLIGOSACCHARIDES CONTAINING SINGLE GLUCOSE SIDE CHAINS*

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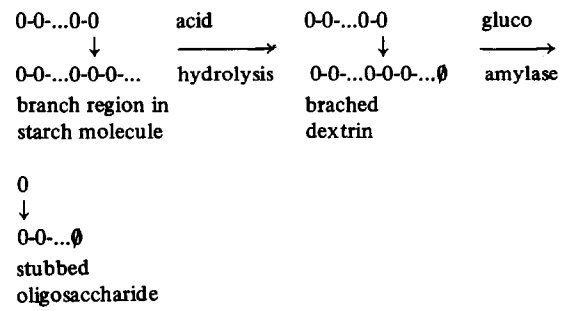
1. Introduction

The experiment described in this letter was undertaken to establish the specificity of porcine pancreatic amylase acting on "stubbed" [1] starch oligosaccharides.

The term "stubbed" oligosaccharides as used here refers to otherwise α-1,4-linked starch oligosaccharides, to which single glucose units (stubs) are linked by α-1, 6-bonds. Such stubs could be attached to any glucose unit in the main chain, but we are concerned primarily with oligosaccharides containing stubs attached to the non-reducing terminal glucose unit. Stubbed oligosaccharides are produced during enzymic degradation of starch [2–4]. The rationale behind our method of preparation of stubbed oligosaccharides is shown in Scheme 1. Under our conditions procine pancreatic amylase is without action on the stubbed tri- and tetrasaccharide. However the stubbed pentasaccharide and all higher saccharides are converted into the stubbed tetrasaccharide, together with glucose or maltose or both. Additionally, our data indicate the presence in waxy maize starch of chains holding branches separated by only a single glucose unit.

Scheme 1
Preparation of stubbed oligosaccharides.†



† Symbols and abbreviations: O, D-glucose unit;-, α-1,4 bond; ↓α-1,6-bond; ∅, reducing terminal D-glucose unit; ↑, point of amylase attack; ↓, point of pullulanase attack; a series of three dots (...) indicates an indefinite continuation of the starch chain.

2. Experimental

2.1. *Materials and methods*

Waxy maize starch was a commercial sample donated by National Starch and Chemical Co. It was defatted by extraction with 85% methanol according to the procedure of Schoch [5]. Glucoamylase from *Aspergillus niger* ("Diazyme 160") was a gift from Miles Laboratories Inc. Pullulanase was prepared from *Aerobacter aerogenes* essentially by the method of Wallenfels et al. [6]. Procine pancreatic amylase was a 2X crystallized preparation purchased from Worthington Biochemical Corp.

Two-dimensional chromatography was run by the method of French et al. [7]. Following chromatography in the first direction, enzyme treatment was effected by spraying the oligosaccharide zone with 10 ml

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of procine pancreatic amylase solution (1000 I.U.* of amylase in 0.011 M sodium chloride and 0.04 M pyridine, adjusted to pH 6.9 with acetic acid). The chromatogram was incubated in a moisture-satured chamber for 24 hr at 40°C and dried. Following chromatography in the second direction, the entire chromatogram was dried and dipped in an acetone suspension of Diazyme 160 that contained 1 g of crude enzyme preparation in 1 l of 80% aqueous acetone. Such glucoamylase treatment converts the weakly-reducing higher oligosaccharides into glucose, thereby greatly enchancing the extent of silver reduction.

Reducing values and total carbohydrate were determined by the Technicon Autoanalyzer methods of Robyt and French [9] and Robyt and Bemis [10].

2.2. *Preparation of stubbed oligosaccharides*

Defatted waxy maize starch, 5 g dry basis, was suspended in 1 l of 0.08 N HCl and heated over a 40 min period to boiling. It was then boiled under reflux for 70 min. After cooling, the solution was passed through an ion-exchange column (Amberlite 4-B; column dimensions 17 X 2.0 cm). The reducing value corresponded to a degree of hydrolysis of 0.27. A 100-ml portion of the solution was treated with Diazyme (25 mg/2.5 ml of 0.2 M pyridine-acetic acid buffer, pH 4.8) for 120 min at 40°C. The glucoamylase was inactivated by heating to boiling, and the digest was cooled to room temperature and treated with pullulanase (12.5 I.U.) for 48 hr at 40°C. The mixture was heated to boiling for 10 min, cooled to room temperature, and again treated with glucoamylase for 20 min. After inactivating the glucoamylase a 300 μl portion of the mixture was submitted to 2-dimensional paper chromatography. The results are presented in fig. 1.

In a control experiment, recrystallized potato amylose [9] was used in place of waxy maize starch. Stubbed oligosaccharides could not be detected; therefore the stubbed oligosaccharides from waxy maize starch are not artifacts of the preparation method.

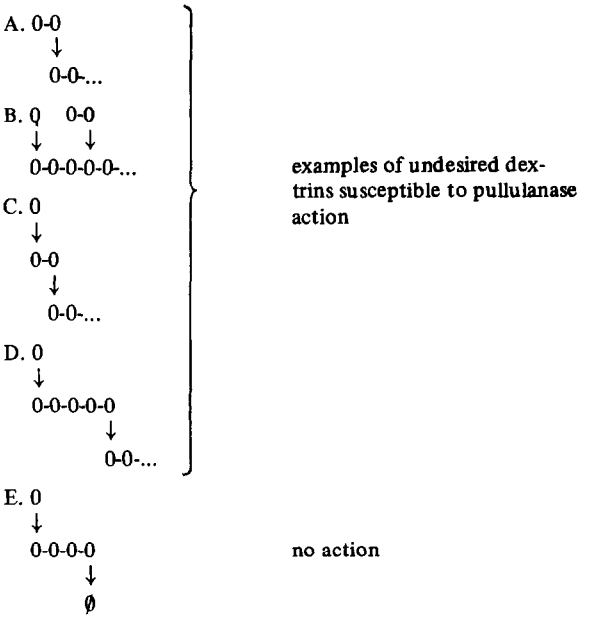
* One International Unit (I.U.) as defined according to the International Commission on enzymes is the amount of enzyme that will hydrolyze one micromole of glycosidic bonds per minute under optimal conditions [8]. For crystalline porcine pancreatic amylase, there are approximately 500 I.U. per mg of protein.

3. Discussion

Stubbed oligosaccharides have been produced in previous work on starch structure analysis or as substrates for various enzymes [2,4,11]. Stark [1] has recently reported the preparation of stubbed glucans by consecutive actions of acid, beta-amylase, and pullulanase [12]. Our method (Scheme 1) is somewhat similar in execution though different in concept. The initial reaction with acid was aimed at breaking down the complex starch to give a variety of relatively simple branched compounds (degree of hydrolysis 0.27). Glucoamylase was selected to remove the bulk of the linear oligosaccharides greater than maltose, and to remove glucose units from the non-reducing ends of the branched molecules, down to the branch points. Action of glucoamylase is prefential for α-1,4-links but it attacks maltose only slowly, in comparison with higher oligosaccharides, and it also cleaves α-1,6-links in stubbed oligosaccharides at a slow rate [13]. Conditions were selected by trial to give a reasonable yield and purity of the desired structural type. Following

Scheme 2

Pullulanase action on various dextrins produced from waxy maize starch by acid hydrolysis and glucoamylase action.



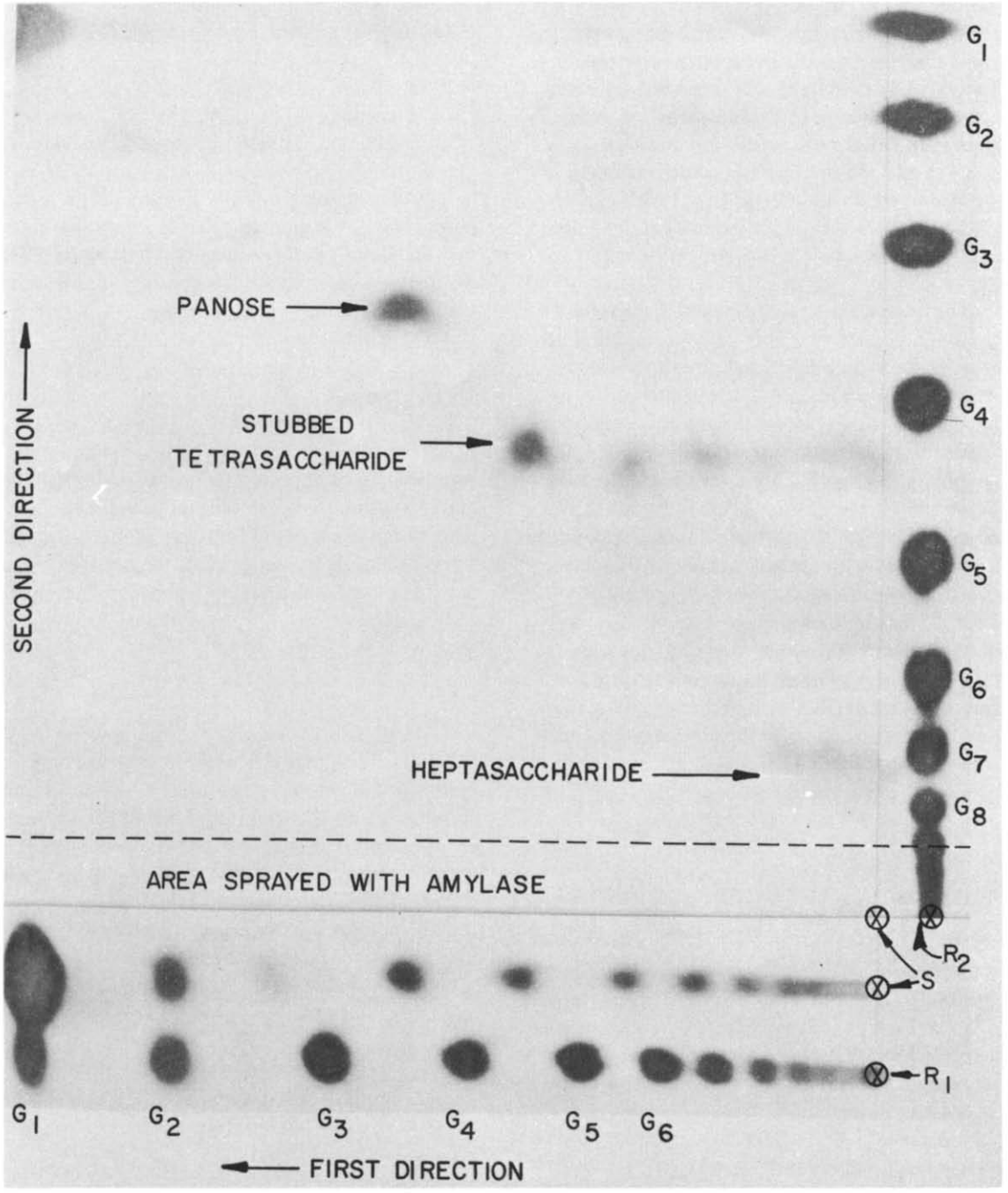
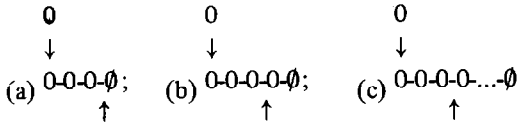


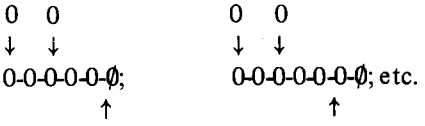
Fig. 1. Two-dimensional chromatogram showing action of procine pancreatic amylase on "stubbed" oligosaccharides; the lower portion of the figure shows the one-dimensional separation prior to amylase action. R_1: reference series of glucose (G_1), maltose (G_2), etc. for first direction. S: Sample; following chromatography in the first direction, the lower portion of the chromatogram was removed and the test sample area was sprayed with amylase, allowed to react, and dried; a second reference series was applied at R_2 and the chromatogram was developed in the second direction.

glucoamylase action, it may be expected that there will remain some oligosaccharides having undesired structural types (see A–D, Scheme 2). Pullulanase has no action on stub branches, and so it is effective in removing the unwanted compounds without acting on the stubbed oligosaccharides. Presumably only trivial amounts of undesired structure (such as E, Scheme 2) would remain. Pullulanase action liberates new non-reducing chains ends and linear oligosaccharides that may be removed by a second treatment with glucoamylase. For our purpose it was unnecessary to fractionate the reaction mixture before the chromatographic experiment.

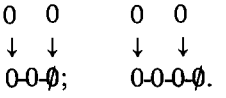
In other work we have found that 2-dimensional paper chromatography is a most effective method of surveying enzyme action on a mixture of substrates [7]. This method was therefore applied to action of porcine pancreatic amylase on the stubbed oligosaccharide mixture. Results are shown in fig. 1. Chromatography in the first direction revealed the presence of glucose and a series of oligosaccharides, which from the method of preparation would contain a 1→6-linked-α-glucose unit at the non-reducing end, i.e. isomatose, 6^2-α-glucosylmaltose (panose), 6^3-α-glucosylmaltotriose etc. Following treatment with α-amylase and chromatography in the second direction, one can identify the products of amylase action on the various stubbed components. The tri- and tetrasaccharides are resistant to amylase action. Stubbed pentasaccharide and higher components give stubbed tetrasaccharide according to the following reactions:



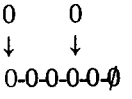
where the upward arrows indicate the point of cleavage. Linear oligosaccharides higher than maltose, produced in reaction (c) are simultaneously converted into glucose and maltose. No products are produced by the following reactions:



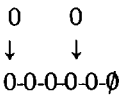
Traces of compounds moving as penta- and hexasaccharide remain unttacked by the amylase. Presumably these compounds are doubly-stubbed components of the original mixture:



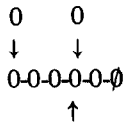
The chromatogram shows a weak indistinct band of products with chromatographic mobility slightly less than the doubly-stubbed heptasaccharide. At present this remains unidentified. We presume that it is not the doubly-stubbed octasaccharide:



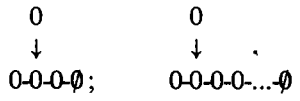
which would be expected to have a substantially lower mobility. Admittedly, resolution of 2-dimensional chromatograms is poor in this low-mobility region, and so we cannot rigorously exclude the possibility that the indistinct band is the doubly-stubbed octasaccharide:



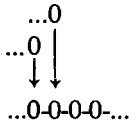
which would be expected to have a substantially lower mobility. Admittedly, resolution of 2-dimensional chromatograms is poor in this low-mobility region, and so we cannot rigorously exclude the possibility that the indistinct band is the doubly-stubbed octasaccharide. If it were, however, one would expect it to be produced in amounts more nearly comparable with the doubly-stubbed heptasaccharide. Therefore, we tentatively conclude that this octasaccharide is cleaved by amylase as follows:



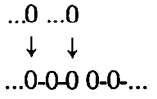
Absence of a stubbed pentasaccharide band indicates that the glucoamylase treatment has removed all structures of the following type:



Since this stubbed pentasaccharide ("fast B_5") [4] is totally resistant to pancreatic amylase under our conditions. Absence of a hexasaccharide band most likely means the branching structure:



does not occur in starch. Conceivably, however, such a compact branching arrangement might not be satisfactorily trimmed by the glucoamylase treatment and therefore might not survive pullulanase action. By contrast, the following branching pattern



which results in formation of doubly-stubbed heptasaccharide, represents a very significant structural arrangement in waxy maize starch.

References

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