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HYDROLYSIS OF AMYLOPECTIN BY THE ALPHA-AMYLASE OF *B. subtilis**

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ABSTRACT

The mixture of oligosaccharides obtained after initial hydrolysis of waxy-maize amylopectin by the alpha-amylase from *Bacillus subtilis* was fractionated on a Sepharose CL 6B column. The molecular-weight-distribution curves indicated that intermediate products of defined molecular weights were formed as the reaction proceeded towards smaller dextrins. The results can be explained in terms of the cluster model for the fine structure of the amylopectin molecules, assuming that the intermediate products represent one or more unit clusters or cluster residues. Simultaneously with the formation of the intermediate products of molecular weights ≥30,000, small dextrins were also produced, most probably representing the residues from outer chains in the macromolecule. Thus, alpha-amylase does not hydrolyse amylopectin molecules in a random manner, but, in the initial stages, preferentially attacks the glucosidic bonds between the unit clusters, which in turn are of defined sizes.

INTRODUCTION

Alpha-amylolysis of amylopectin is only a partly understood process, because the fine structure of the substrate is still obscure. *Bacillus subtilis* alpha-amylase produces, in addition to small carbohydrates also arising from the hydrolysis of amylose, several small, branched dextrins as end-products[1–4]. Gel filtration has been used to study the initial stages of the actions of *B. subtilis* alpha-amylase on wheat starch[5] and of barley alpha-amylases on barley starches[6,7]. The latter reaction followed a course suggested to depend on a non-random hydrolysis of the amylopectin component[6].

Studies of the fine structure of amylopectin have made great advances since the introduction of debranching enzymes. The chain profile of amylopectins of various origins usually has a bimodal distribution with longer and shorter chains

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having average lengths of 40–60 and 11–25 D-glucosyl residues, respectively[8–13]. In amylopectin from barley[14] and some mutants of maize[13], the shorter chains are divided further into two distinct groups with approximate chain lengths of 10–14 and 16–20. A cluster model for the structure of the macromolecule, introduced by French[15] in 1972, has been supported by several investigators[11,16–21]. A non-random alpha-amylolysis of amylopectin has also been suggested, to correlate with the cluster model[6].

The initial stages of the hydrolysis of amylopectin from waxy maize by *B. subtilis* alpha-amylase has now been investigated by gel filtration on Sepharose CL 6B, in order to determine whether the mode of action of the enzyme can be correlated with a systematic structure of the substrate.

EXPERIMENTAL

Waxy-maize starch granules (amylopectin, Sigma) were deproteinised by stirring overnight with chloroform–1-butanol (5:1), and defatted by extraction overnight with hot aqueous 85% methanol in a Soxhlet apparatus. The granules were then dried in ether.

The activity of alpha-amylase [(1→4)-α-D-glucan glucanohydrolase, EC 3.2.1.1, *Bacillus subtilis*; Koch–Light] was measured in 0.05M sodium acetate buffer (pH 5.5) at 22°. Soluble starch (Merck) at a concentration of 5 mg/mL was used as the substrate. The reducing power was determined with the Nelson reagent[22], using D-glucose as the standard. One unit was defined as one μmol of reducing groups formed per min and mL.

Hydrolysis of amylopectin. — A suspension of waxy-maize starch granules (200 mg) in deionised water (20 mL) was gelatinised by boiling for 1 h with continuous stirring, cooled to room temperature, and treated with alpha-amylase (20 mL; 3.3 μg/mL) in 0.05M acetate buffer (pH 5.5). The final enzyme concentration was 0.002 U/mg of amylopectin. At intervals, aliquots (2 mL) were treated with 5M KOH (50 μL) to stop the reaction.

Gel chromatography. — A column (2.5 × 90 cm) of Sepharose CL 6B (Pharmacia) was calibrated[23] with hydroxyethyl-starch samples and dextrans T10, T40, and T70 (Pharmacia) of known molecular weights ($\overline{M}_w$). Elution was performed with 0.5M KOH (1 mL/min).

Approximately 24 h before gel chromatography, 5M KOH (450 μL) was added to the sample aliquot and the whole sample (2.5 mL) was then applied to the column. Fractions (2 mL) were collected and analysed for carbohydrates, using the anthrone–sulphuric acid reagent[24] and the absorbance measured at 625 nm.

RESULTS

The gel-filtration chromatograms of the hydrolysis products obtained at different stages in the degradation of waxy-maize starch by *B. subtilis* alpha-

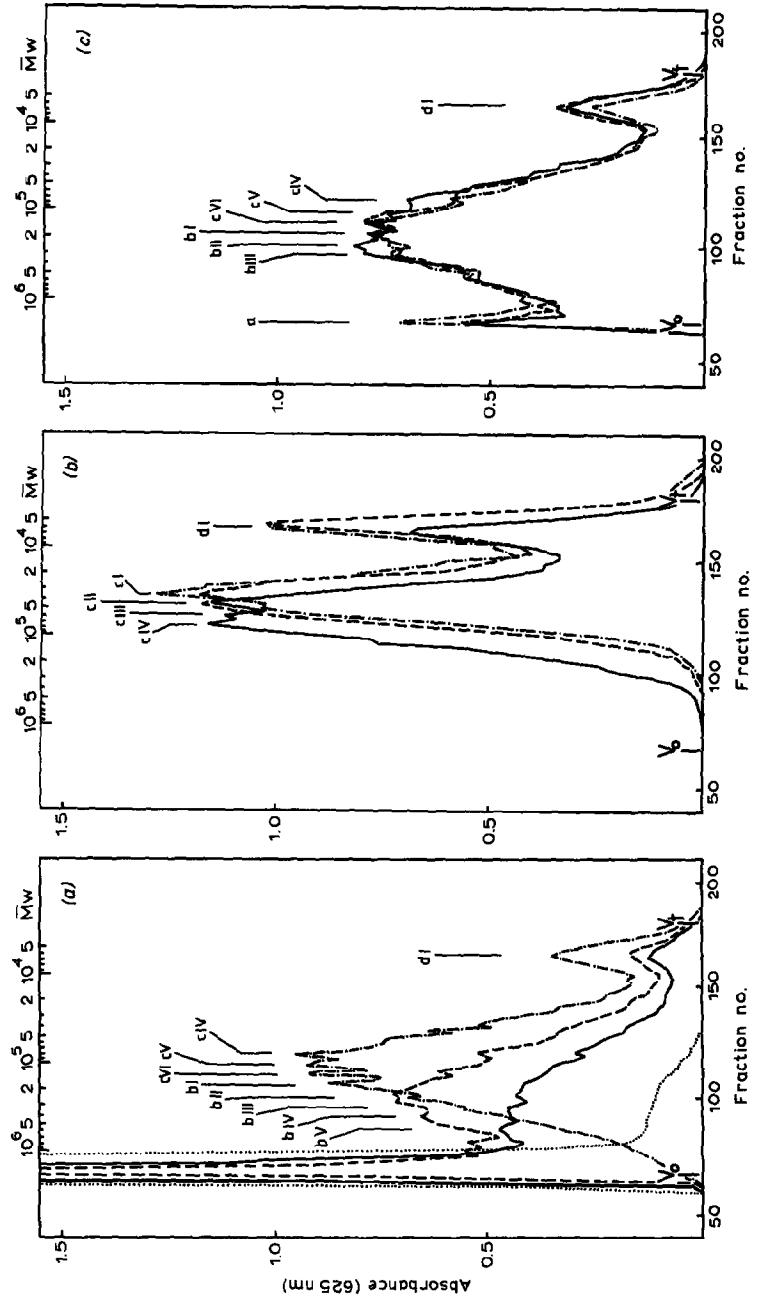

Fig. 1. Sepharose CL 6B fractionation, showing the changes in molecular weight of waxy-maize amylopectin during hydrolysis with *B. subtilis* alpha-amylase: (*a*) amylopectin before hydrolysis (·····) and the hydrolysate after 10 (——), 20 (— —), and 40 min (—·—); (*b*) amylopectin hydrolysate after 75 (——), 105 (— —), and 150 min (—·—); (*c*) amylopectin hydrolysate after 30 min of hydrolysis from three separate experiments, showing the reproducibility of the gel chromatograms; dI–bV indicate characteristic intermediate products.

amylase are shown in Fig. 1. Before the addition of the enzyme, the gelatinised amylopectin solution contains large macromolecules that are completely excluded from the gel. These molecules (fraction a) are hydrolysed rapidly to intermediate products. The molecular-weight-distribution curves show several clear peaks, indicating that the macrodextrins formed are of defined molecular weights. During the initial stages of the reaction, macrodextrins (bV–bI, Fig. 1a) of $\overline{M}_w$ >150,000 preponderated. As the reaction proceeded, new products (peaks cVI–cI) of smaller molecular weight appeared and the intermediate group b disappeared. At the same time, the number of peaks was reduced to two or three dominating peaks (cI, cII, and cIII; Fig. 1b).

Simultaneously with fractions b and c, small dextrins were continuously formed. These were eluted close to the total volume of the gel (peak dI). In the molecular weight range 14,000–30,000 (fraction dII), no clearly defined products were formed.

As an example of the reproducibility of the chromatograms, the intermediate products after 30 min of alpha-amylolysis from three separate experiments are shown in Fig. 1c. The shapes of the curves are similar, although all peaks are not present in all curves; *e.g.*, fraction bII is not seen in one run, but is clearly seen in the others. Further, the peaks were not always clearly separated due to the very small differences in elution volumes. The exact position of a peak was ±1 fraction. To obtain a correct picture of the intermediate products, it was therefore necessary to analyse several samples from closely selected time-intervals.

The recovery from gel chromatography was 101 ±7%. During prolonged use,

TABLE I

CHARACTERISTIC FRACTIONS OBTAINED AFTER GEL FILTRATION OF HYDROLYSATES FROM WAXY-MAIZE AMYLOPECTIN AFTER TREATMENT WITH ALPHA-AMYLASE

Fraction	$\overline{M}_w$ *range*
dI	<14,000
dII	14,000–30,000
cI	30,000–40,000
cII	40,000–50,000
cIII	50,000–65,000
cIV	65,000–80,000
cV	80,000–110,000
cVI	110,000–150,000
bI	150,000–200,000
bII	200,000–275,000
bIII	275,000–350,000
bIV	350,000–450,000
bV	450,000–700,000
a	>700,000

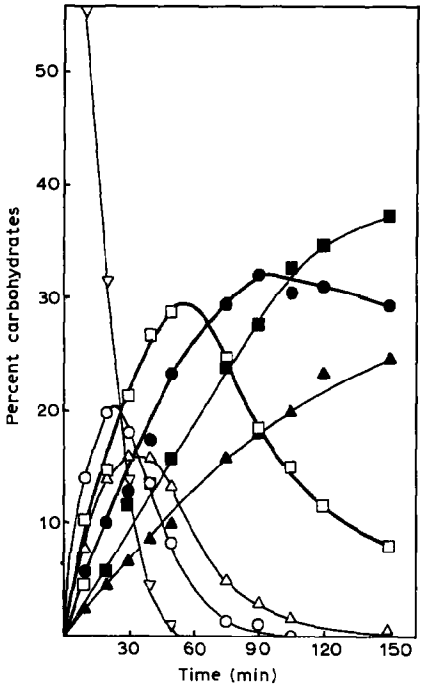

Fig. 2. Percent distribution of molecular weight as a function of time for alpha-amylolysis of waxy-maize amylopectin. The molecular weight ranges for fractions a (▽), bV–III (○), bII–I (△), cVI–IV (□), cIII–I (●), dII (▲), and dI (■) are given in Table I.

the background slowly increased due to the alkaline elution medium, which slowly released carbohydrates from the gel material, causing recoveries of >100%. The column therefore has to be repacked with fresh gel after 3–4 months of continuous use.

Table I contains a summary of the fractions obtained in the initial stages of the alpha-amylolysis. The molecular weight ranges given for the fractions are based on the results from three separate series of experiments. The changes in the composition of the hydrolysate as a function of time are illustrated by Fig. 2. It is clear that the hydrolysis is a consecutive reaction in which the intermediate fractions initially increase, pass through a maximum, and then decrease. However, the rate of decrease of the fractions cIII–I is much lower than that of the other intermediates. Fractions dI and dII increased continuously.

DISCUSSION

Alpha-amylases have a random pattern of action on amylose in the initial stages of the hydrolysis[25–28]. Small dextrins are not hydrolysed randomly because the reaction proceeds more slowly near the ends of a chain of D-glucosyl residues.

This is believed to be due to the protein configuration near the active site of the enzyme[25,26].

As distinct intermediate products are formed from amylopectin, it is apparent that the action of the enzyme on amylopectin is a non-random process. This is to be expected, since the average chain-length for most amylopectins is only 20–26 D-glucosyl residues (*e.g.*, refs. 12 and 14). The macromolecule therefore contains many non-reducing chain ends, which probably restrict the action of the enzyme. In addition, certain (1→4)-D-glucosidic linkages close to a branch point are resistant to hydrolysis[1]. If alpha-amylase attack is basically random, a non-random hydrolysis suggests that a highly ordered structure of amylopectin molecules forces the enzyme initially to attack some well-defined glucosidic bonds in preference to others.

Fig. 1 shows that, in the later stages of the hydrolysis, the number of intermediate products is reduced to two or three. Further hydrolysis of these products proceeded at a rate much lower than that of the others produced during the hydrolysis (Fig. 2). The cluster model of amylopectin structure[15] agrees with this result if it is assumed that the intermediate products that are more resistant to hydrolysis represent unit clusters or cluster residues in which the branch points are

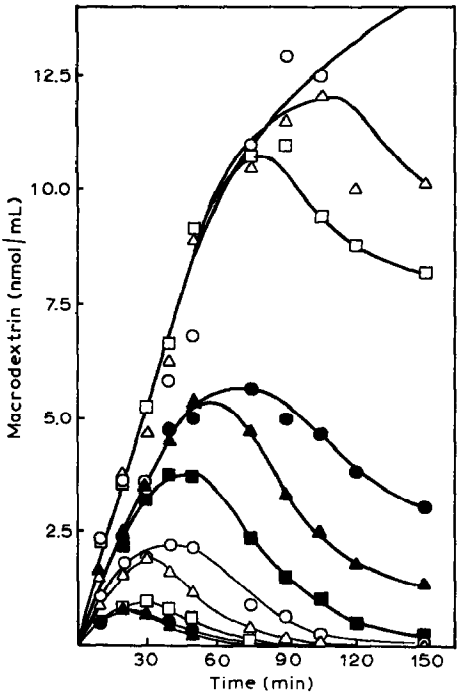

Fig. 3. Molar concentration of intermediate products obtained by alpha-amylolysis of waxy-maize amylopectin as a function of time. Peaks from fraction b are drawn with normal lines and those from fraction c with bold-face lines. Peak numbers are symbolised as follows: I (○), II (△), III (□), IV (●), V (▲), and VI (■).

so close to each other that the action of the enzyme is restricted. The small dextrins obtained in peak dI would then represent residues from partly hydrolysed outer chains in the macromolecule. The larger, non-resistant intermediates could represent two or more unit clusters connected to each other.

Using the indicated peak $\overline{M}_w$ values (Fig. 1), it is possible to make a rough calculation of the molar concentration of each fraction as a function of time. This is shown in Fig. 3. It is apparent that, in the early stages of the reaction, the intermediates formed can be differentiated into more or less well-defined groups. The molecular species within group b are clearly distinguishable from those in group c. Within group c, the fractions cI, cII, and cIII are formed in approximately equimolar amounts and constitute a group distinguishable from fractions cIV, cV, and cVI.

It is possible that the intermediate products cI–cIII represent three unit clusters of different sizes. Bender *et al.*[20] came to the same conclusion using cyclodextrin glycosyltransferase in combination with beta-amylase, pullulanase, and isoamylase. How these three units are interconnected is of course an open question. However, using the peak $\overline{M}_w$ values, it is possible theoretically to combine the fractions in different ways to obtain the larger intermediate fractions. One possible way is shown in Table II. The molecular weights calculated in this way agree fairly well with the peak molecular weights for the fractions after gel filtration.

TABLE II

POSSIBLE SYSTEMATIC CONNECTION BETWEEN INTERMEDIATE PRODUCTS OBTAINED DURING THE ALPHA-AMYLOLYSIS OF AMYLOPECTIN

Sub-fractions and peak $\overline{M}_w$[a]		*Fraction obtained*	
		Calc. $\overline{M}_w$[b]	*Peak $\overline{M}_w$ obtained*[a]
cI	cII	cIV	
36,000	44,000	80,000	76,000
cII	2 × cI	cV	
44,000	72,000	116,000	100,000
cIV	cIV	cVI	
76,000	76,000	152,000	140,000
cV	cII	cVI	
100,000	44,000	144,000	140,000
cVI	cIII	bI	
140,000	58,000	198,000	180,000
cVI	2 × cIII	bII	
140,000	116,000	256,000	240,000
bI	cVI	bIII	
180,000	140,000	320,000	305,000
bI	bII	bIV	
180,000	240,000	420,000	390,000
bI	bIV	bV	
180,000	390,000	570,000	550,000

[a]Obtained in gel filtration as indicated in Fig. 1. [b]Calculated as the sum of the $\overline{M}_w$ of the sub-fractions.

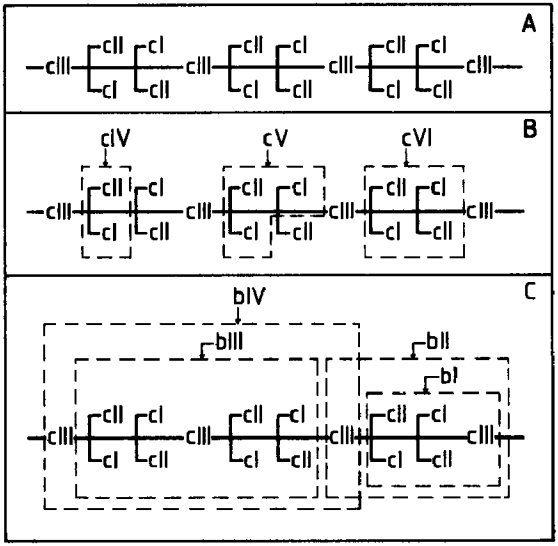

Fig. 4. Proposed systematic cluster structure for waxy-maize amylopectin. The intermediate products cI, cII, and cIII are clusters of different sizes that form the basic structure of the macromolecule (A). Two, three, or four connected clusters will form the intermediate products cIV–cVI (B). Fraction bI comprises a larger structural unit to which more clusters can be added, giving the intermediate products bII–bV, the latter being the whole structure drawn (C).

A schematic model for the structure of amylopectin, based on the theoretical calculations in Table II, is shown in Fig. 4. It is clear that a simple basic structure composed of the three unit clusters cI–cIII easily explains all the different intermediate macrodextrins obtained in the experiment. As indicated in Fig. 4, fraction bI ($\overline{M}_w$ 180,000) forms a larger structural unit. The possible existence of such unit "super-clusters" was suggested by Bertoft and Henriksnäs[6].

It is recognised that the suggested structure of waxy-maize amylopectin in Fig. 4 is just one of several possibilities. The purpose of the present study was to show how alpha-amylase, together with exo-amylases and debranching enzymes, could be a useful enzyme in structural studies of amylopectin.

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