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Note

On the ability of pullulanase to stimulate the enzymic digestion of raw starch

SEINOSUKE UEDA* AND J. JOHN MARSHALL†
Laboratories for Biochemical Research, Howard Hughes Medical Institute, and Departments of Biochemistry and Medicine, University of Miami School of Medicine, Miami, Florida (U.S.A.)
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Enzymic digestion of raw-starch granules takes place much more slowly than does the breakdown of soluble or gelatinized starch[1]. Many efforts have been made to define the factors that affect starch granule enzymolysis, but as yet it has not proved possible to demonstrate efficient granule-breakdown *in vitro* by any amylase. Consideration has been given to the possibility that the process requires the synergistic action of two or more enzymes. Indeed, the suggestion was made many years ago that a hypothetical "raw-starch factor" is involved in the process[2], and this possibility has not yet been eliminated, although the nature of the so-called "raw-starch factor" has not been identified. It has generally been assumed that such a factor would be enzymic in nature, and increasing attention has recently been paid to the possibility that two or more amylolytic enzymes acting together may function synergistically in raw-starch digestion. In view of the observation that debranching enzymes may assist amylolysis of barley-starch granules[3], we have further investigated this possibility. Our results show that pullulanase has little effect on the α-amylolysis of waxy-maize, starch granules, but stimulates β-amylase action greatly.

EXPERIMENTAL

Materials. — *Bacillus polymyxa* β-amylase was obtained by chromatography of a culture filtrate of the organism on DEAE-cellulose and treatment with starch granules to remove contaminating α-amylase. Sweet-potato β-amylase was prepared by the method of Nakayama and Amagase[4] and chromatographed on DEAE-Sephadex to remove maltase[5]. Barley-malt α-amylase was a crude preparation from Sigma Chemical Co. (St. Louis, MO 63178), pullulanase (crystalline) from

*Permanent address: Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
†To whom enquiries and reprint requests should be addressed, at: Laboratories for Biochemical Research, Howard Hughes Medical Institute, P.O. Box 016039, Miami, FL 33101, U.S.A.

Hayashibara Biochemical Co. (Okayama, Japan), and soluble starch from J. T. Baker Chemical Co. (Phillipsburgh, NJ 08865). Waxy-maize starch was prepared by the method of Schoch[6]. All chemicals were of the highest quality available.

Measurement of carbohydrate. — Reducing sugars were determined with an alkaline copper reagent[7], calibrated against maltose. Total carbohydrate measurements were made with the phenol–sulfuric acid method[8].

Determination of raw-starch digestion. — To determine raw-starch digestion, reaction mixtures (5.0 mL) containing raw starch (100 mg), an appropriate buffer, and enzyme solution were incubated at 37° with occasional shaking. Samples were removed at suitable time-intervals, centrifuged, and analyzed for reducing sugars as maltose and for total, soluble carbohydrate produced.

RESULTS AND DISCUSSION

The effect of pullulanase on raw-starch digestion by malt α-amylase was tested by use of reaction mixtures containing malt α-amylase (0.03 U/mL) and 200mM acetate buffer (pH 6.0), with and without pullulanase (0.8 U/mL). Samples were removed for maltose determinations and total carbohydrate measurements at intervals of up to 65 h. Measurement of reducing sugars indicated a substantial stimulation of the conversion into maltose in the presence of pullulanase. Thus, α-amylase alone caused a conversion of 8.8% into maltose after 65 h, whereas, in the presence of pullulanase, a conversion of 13.3% was achieved. Pullulanase itself had negligible action. However, the stimulating effect of pullulanase is apparent rather than real, because measurement of total carbohydrate produced showed this to represent a conversion of 13.8% in the case of α-amylase alone, and of 15.0% when α-amylase and pullulanase were used together.

On the other hand, when pullulanase and β-amylase acted together, a genuine synergistic effect was apparent. Reaction mixtures contained 200mM acetate buffer (pH 6.0) and β-amylase (0.23 U/mL in the case of *Bacillus polymyxa* β-amylase, and 1.5 U/mL in the case of the sweet-potato enzyme), with or without pullulanase

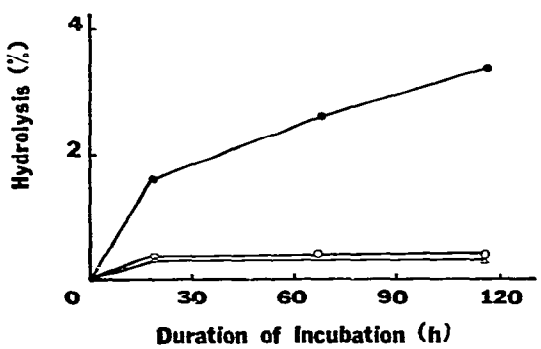

Fig. 1. Time course of raw, waxy-corn starch digestion: (●), sweet-potato β-amylase + pullulanase; (○), sweet-potato β-amylase alone; and (△), pullulanase alone.

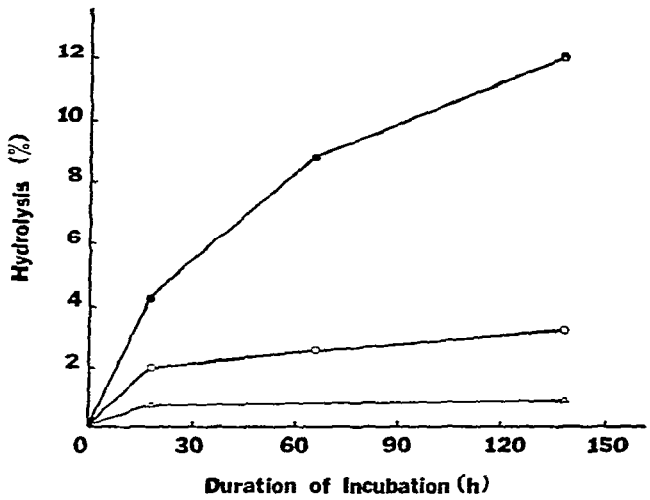

Fig. 2. Time course of raw, waxy-corn starch digestion: (●), *Bacillus polymyxa* β-amylase + pullulanase; (○), *Bacillus polymyxa* β-amylase alone; and (△), pullulanase alone.

(2.1 U/mL). Samples were removed for measurement of reducing sugars and total soluble carbohydrate produced at intervals of up to 140 h. The results are shown in Figs. 1 and 2.

In interpreting the results of studies on raw-starch digestion by use of combinations of enzymes, it is important not to reach misleading conclusions caused simply by one of the enzymes modifying the primary products produced by the other. Thus, while, on the basis of maltose measurements, action of α-amylase is apparently stimulated by pullulanase, the total amount of carbohydrate solubilized is the same in the presence and absence of the debranching enzyme. Thus, pullulanase probably serves only to hydrolyze the α-limit dextrins produced by α-amylase with a concomitant increase in the apparent conversion into maltose.

Plant β-amylase has long been known to have little action on raw-starch granules, but its action is stimulated over six-fold by the presence of pullulanase (Fig. 1). The enzyme from *Bacillus polymyxa*, which has recently been shown to be of the β-amylase type[9], acts on raw-starch granules at a considerably higher rate than does the sweet-potato enzyme. Possibly this difference between the two types of β-amylase can be related to the difference in their abilities to adsorb to starch granules[10,11], and it provides further support for the idea that adsorption of an enzyme to starch granules favors granule enzymolysis[12,13]. The action of the bacterial β-amylase on starch granules is stimulated 4-fold by pullulanase (Fig. 2).

It may be concluded that β-amylase and pullulanase act synergistically in raw-starch granule digestion, although the mechanism of the process remains to be determined. The joint action of bacterial β-amylase and pullulanase on raw, waxy-corn starch results in rates of breakdown similar to those reported earlier where glucoamylase I and pullulanase were used[14]. Further studies on the joint action of

the bacterial enzyme and pullulanase on starch granules would be justified on the basis that optimization of the conditions may form the basis of a process for maltose production from ungelatized starch.

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