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EXOENZYMIC ACTIVITY OF *alpha*-AMYLASE IMMOBILIZED ON A PHENOL–FORMALDEHYDE RESIN

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ABSTRACT

Amylose and amylopectin from two starch sources were partially degraded by *alpha*-amylase immobilized on a phenol–formaldehyde resin. The degradation products were fractionated by gel-permeation chromatography and high-pressure, liquid chromatography Two distinct fractions were obtained from tapioca amylose. One is a fragment having a molecular weight exceeding 200,000, and the other consists of oligosaccharides of low molecular weight with a degree of polymerization of 1–8. In contrast, treatment of tapioca amylose with soluble *alpha*-amylase produces a single fraction, nearly all of which has a molecular weight of $<35{,}000$, with only traces of small oligosaccharides detectable by high-pressure, liquid chromatography. Even wider differences were observed in degradation products from tapioca amylopectin. Similar activity-patterns were obtained with immobilized and soluble enzymes, using corn amylose and corn amylopectin as substrates Immobilization of *alpha*-amylase on the resin apparently restricts the activity of the enzyme to the ends of the starch molecules, making it appear to be limited to exoenzymic activity.

INTRODUCTION

Immobilization of *alpha*-amylase on an inert, protein-adsorbent resin changes the enzyme-activity pattern for starch substrates from the natural, endoenzymic[1,2] to an exoenzymic mode In the free, soluble state, *alpha*-amylase has easy access to the inner recesses of large starch molecules, where initial activity is favored[1]. However, immobilization of this enzyme seemingly limits its initial activity to the outermost molecular segments of the polysaccharide This change in behavior has been attributed to steric hindrance[3] to interaction between active sites of the immobilized enzyme and the amylaceous substrates We have now characterized the molecular-weight distributions in the degradation products that result from the action of soluble and immobilized *alpha*-amylase, respectively, on the two molecular species of which most starches are composed, namely, amylose (linear) and amylopectin (branched)

*Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S Department of Agriculture over other firms or similar products not mentioned

EXPERIMENTAL

Enzyme — Crystalline *alpha*-amylase (EC 3.2.1.1) from *Bacillus subtilis* was purchased from Sigma Chemical Co

Enzyme–resin — The *alpha*-amylase–resin complex was prepared by a modification of the method described by Olson and Stanley[4] Duolite S-761, a phenol–formaldehyde resin, was supplied by Diamond Shamrock Chemical Co To 0 1M acetate buffer (pH 5, 400 ml) containing crystalline *alpha*-amylase (910 mg) was added drained resin (200 g) Adsorption of the enzyme took place within 3 h at room temperature The resin was drained, and 1 25% glutaraldehyde solution (400 ml) was added and allowed to react during 90 min at room temperature Most of the excess of glutaraldehyde was decanted from the resin, which was then washed successively with acetate buffer (pH 5 and 6) and 0 1% solution of soluble starch (pH 6)

Substrates — Amylose and amylopectin, obtained by fractionation of tapioca starch in general accord with the method of Schoch[5], served as substrates without further purification of the initial precipitates The respective intrinsic viscosities of the amylose and amylopectin in 90% dimethyl sulfoxide at 25° were 2 56 and 1 42

A corn amylose, precipitated three times as the butyl alcohol complex, and a commercial, waxy-maize amylopectin (Amioca, National Starch and Chemical Co) served in two experiments that required uncontaminated amylose and amylopectin The intrinsic viscosities in 90% dimethyl sulfoxide at 25° were corn amylose, 0 94, and waxy-maize amylopectin, 1 44

Aqueous solutions of these substrates, of concentration 0 08% (w/v), were prepared by heating suspensions for 20 min at 95° with stirring An exception, however, was the corn amylose, which had retrograded A solution of amylose (0 4 g) in *M* potassium hydroxide (10 ml) at room temperature was made neutral with hydrochloric acid, and diluted to 0 08% for treatment with the enzyme

Enzyme digests — Soluble *alpha*-amylase (5 μg) in water (50 μl) was added to 100-ml portions of the substrates at 40° The mixtures were maintained at 40° in a shaking water-bath After 10 min, the mixtures were boiled for 2 min, and cooled before being analyzed for reducing end-groups and iodine-complex color

Immobilized-enzyme digests were prepared as described for soluble *alpha*-amylase, except that 5 g of the enzyme–resin complex was used and the digests were filtered after 20, or 40, min to remove the immobilized-enzyme complex

Assay methods — Reducing end-groups were determined on a Technicon Autoanalyzer by procedure A of Robyt *et al.*[6] with maltose standards

The iodine-stainability of the starch fractions was determined essentially by the method of McCready and Hassid[7] To samples of the enzyme digests was added 0 2% iodine in 2 0% potassium iodide solution (100 μl), and the volume was made to 10 ml with water. The optical absorbance at 590 nm was read on a Gilford 300-N spectrophotometer

Gel-permeation chromatography (*g p c*) — Molecular-size distributions were determined, with a Waters Anaprep gel-permeation chromatograph, on two columns

(122 cm × 9 5 mm i d) containing Waters Hydrogels IV and VI, these respectively produce molecular-weight separations for dextran of mol wt 500 to 10,000 and 1,000 to 2,000,000 The columns were calibrated with D-glucose and with Pharmacia dextrans T10 and T110 The operating conditions were solvent, water, sample concentration, 0 08%, sample size, 2 ml, flow-rate, 1 ml min^{-1}, oven temperature, 41° The samples were filtered through porous glass (pore size, 0 4–1 4 μm), and then injected into the columns The percentages of degradation products distributed above and below an arbitrarily chosen, molecular-weight value of 35,000 (effluent volume of 65 ml) were estimated by direct weighing of paper tracings of the curve areas recorded

High-pressure, liquid chromatography (*h p l c*) — Oligosaccharides of low molecular weight were separated on μ Bondapak Carbohydrate and μ Bondapak NH_2 columns (30 cm × 4 mm i d), in tandem, in a Waters Associates ALC-100 chromatography unit Samples containing carbohydrate (0 5 mg) in water (25 μl) were chromatographed with 7 3 acetonitrile–water at a constant flow-rate of 1 ml min^{-1} Malto-oligosaccharide standards ranging from D-glucose to maltononaose served as markers for calibrating the elution positions

RESULTS AND DISCUSSION

Reducing values and iodine-complex color of digests of the tapioca-starch fractions are summarized in Table I Also given in Table I are the relative proportions of degradation products having molecular weights above or below 35,000 The g p c curves of the substrates described in Table I are reproduced in Figs 1 and 2

The most striking difference between the action of soluble and immobilized *alpha*-amylase is the molecular-weight distribution of the degradation products This difference is dramatically illustrated in Fig. 1 by the g p c curves of tapioca-amylose digests respectively produced by the soluble (curve A) and immobilized (curve B) enzymes Although the digests have approximately the same reducing values and iodine color, the yield of products having a molecular weight of <35,000 is 97% for the soluble enzyme, but only 23% for the immobilized enzyme Furthermore, the degree of polymerization (d p) of such products from the immobilized enzyme, estimated from the reducing value of the whole digests, is only one-quarter (8 5/34 6) of that of the products from the soluble enzyme Analysis by h p l c of the oligosaccharides of low molecular weight (see Fig 3) isolated from the immobilized-enzyme digests of tapioca amylose revealed only eight peaks, the largest being that for malto-octaose The average d p value (namely 4) of the observed malto-oligosaccharides is in reasonable agreement with those calculated from the reducing ends (7 and 8 5), which include degradation products having a molecular weight of up to 35,000 The double peak in the region of high molecular weight in curve C (Fig 1) may have resulted from the presence of an amylopectin contaminant, because only one peak was observed in this region during the course of hydrolysis of purified, corn amylose by immobilized enzymes

TABLE I

PROPERTIES OF DIGESTS OF TAPIOCA-STARCH FRACTIONS WITH FREE AND BOUND ENZYME

Substrates	*Digest time (min)*	*Iodine blue color (%[a])*	*Reducing value (%[b])*	*Molecular-weight distribution[c] (%)*	
				Below 35,000 (d p)[d]	*Above 35,000*
Soluble enzyme					
Amylose	10	77 5	2 8	97 (34 6)	3
Amylopectin	10	51 2	4 9	96	4
Immobilized enzyme					
Amylose	20	79 5	2 7	23 (8 5)	77
	40	30 3	10.7	75 (7 0)	25
Amylopectin	40	89 0	2 0	9	91

[a]Percent of value obtained in unhydrolyzed substrates [b]Percent of theoretical for anhydrous D-glucose [c]Determined from relative areas of gel-permeation chromatographic curves [d]Degree of polymerization (d p) calculated on the assumption that all of the reducing power is present in this fraction

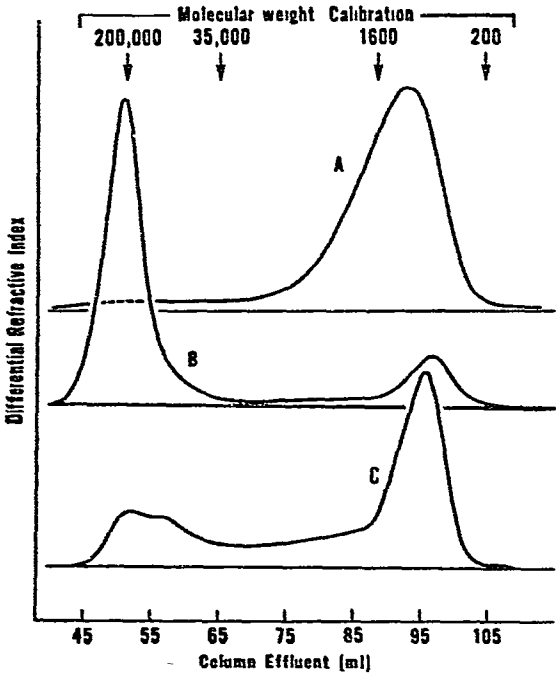

Fig. 1 Molecular-weight distributions of tapioca amylose in digests from soluble and immobilized *alpha*-amylases by g p c [A is a 10-min digest with soluble enzyme, B and C are 20- and 40-min digests, respectively, with immobilized enzyme]

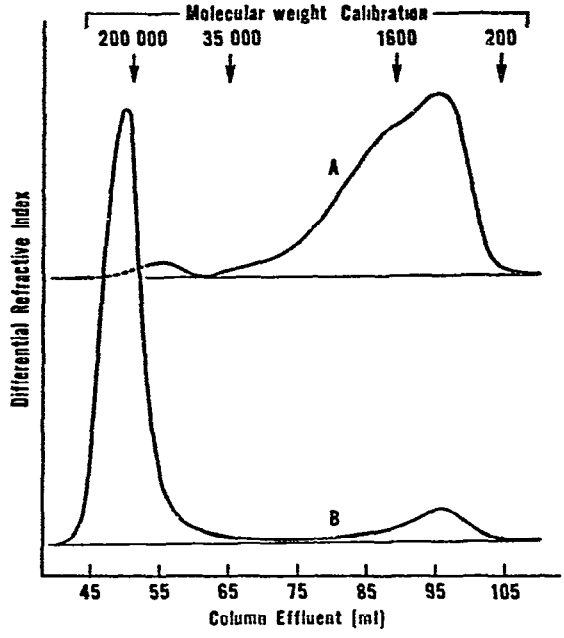

Fig 2 Molecular-weight distributions of tapioca amylopectin in digests from soluble and immobilized *alpha*-amylases by g p c [A is a 10-min digest with soluble enzyme, B is a 40-min digest with immobilized enzyme]

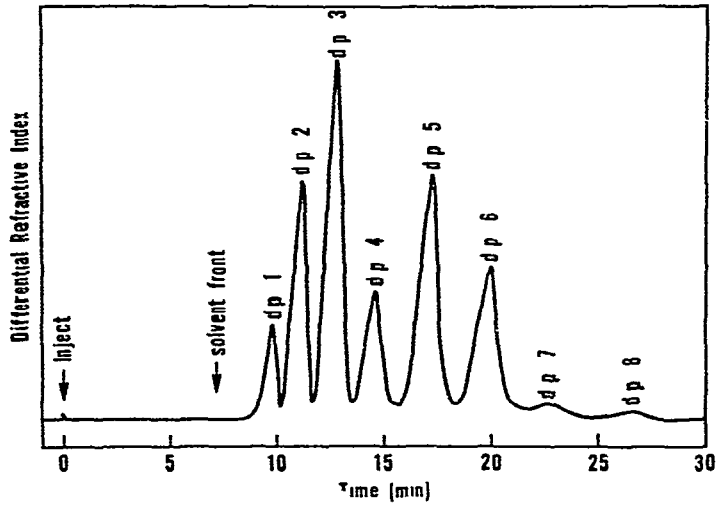

Fig 3 High-pressure, liquid chromatography of a digest (50 min) of tapioca amylose with immobilized *alpha*-amylase, showing the distribution of malto-oligosaccharides

Another significant difference between the activity of the soluble and the immobilized enzyme is the rate of hydrolysis of amylose and amylopectin within each system For example, the reducing value (see Table I) of the 40-min immobilized enzyme digest of amylose is 5 3 (10 7/2 0) times that of the comparable amylopectin digest. However, when waxy-maize starch, assumed to be essentially pure amylopectin, was treated with the immobilized enzyme for 60 min, little or no enzymic degradation occurred Probably little or no tapioca amylopectin was degraded by the immobilized enzyme during the 40-min digest, as the low reducing-power (2 0) could have resulted from degradation of an amylose impurity Comparison of the hydrolysis rates of amylose and amylopectin revealed that, with soluble enzyme, amylopectin is hydrolyzed somewhat faster than amylose Also, the soluble-enzyme digests of amylose and of amylopectin contain almost identical proportions of materials of high and low molecular weight, and practically no small oligosaccharides were detectable with the h p l c system used These patterns stand in marked contrast to the large differences in distributions obtained with the immobilized enzyme

CONCLUSIONS

The results of this preliminary study indicate that immobilized *alpha*-amylase primarily displays exoenzymic activity, which may be due to steric hindrance between the immobilized enzyme and the substrates of high molecular weight derived from starch Cleavage of only the readily accessible, peripheral D-glucosidic linkages would produce small oligosaccharides plus macromolecular residues The steric effect is particularly pronounced with amylopectin, which, in addition to having high molecular weight, is highly branched

The results suggest that controlled hydrolysis of starches, particularly cereal starches, with immobilized amylases could produce modified starches and oligosaccharides that can readily be separated from one another The modified starch residues, which would be chiefly amylopectin of high molecular weight, could have possible value as nongelling, thickening agents

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