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Selective susceptibility of *O*-(carboxymethyl)starch to amyloglucosidase activity

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INTRODUCTION

Enzymes hydrolysing starch, belonging to the class of polysaccharidases, are of considerable importance in industry[1]. These enzymes fall mainly into three groups, namely the endo-(1→4)-α-D-glucosidases or α-amylases (EC 3.2.1.1), the β-amylases (EC 3.2.1.2), and the amyloglucosidases (EC 3.2.1.33). Each group has specific uses in commerce. All may be assaysed, in general, by their activity in the liberation of reducing groups from starch[2,3]. The dextrinization of starch by α-amylases is also followed by the loss of iodine colour[4]. However, no specific assay has been available for estimating the amyloglucosidase activity of enzyme preparations. We report here the preparation of an *O*-(carboxymethyl) derivative of starch (CMS) that was found to have selective susceptibility to amyloglucosidase.

Modified substrates have had limited application in the assay of polysaccharidases. The preparation of *O*-(carboxymethyl)starch has been reported[5,6], but the derivatives were not tested for their susceptibility to different amylases. However, the *in vitro* digestibility by pancreatin of hydroxypropyl derivatives of starch was reported by Leegwater and Luten[7].

RESULTS AND DISCUSSION

A series of *O*-(carboxymethyl) derivatives of soluble starch was prepared by treating preheated (70–80°), gelatinized starch with different molar portions (excess) of monochloroacetic acid. The temperature of the exothermic reaction was maintained between 70 and 80° by external cooling. No carboxymethylation was achieved when the reaction was conducted initially at room temperature. The extent of carboxymethylation, as determined by the titratable acidity (m-equiv. of H^+/g) of the products increased with increase in the proportion of monochloroacetic acid in the reaction mixture up to 7 moles per mole of glucose residues in starch (Fig. 1). There was a sharp fall at molar excesses greater than 9. The maximum carboxymethylation, measured in terms of titratable acidity, was about 1.57 m-equiv. of H^+ per g of derivative. This value

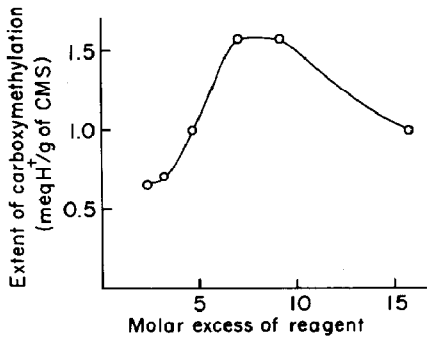

Fig. 1. Carboxymethylation of starch by monochloroacetic acid. CMS (100 mg) in 10 mL of deionized water was titrated with either 0.1M HCl or 0.1M NaOH in a Radiometer pH-stat.

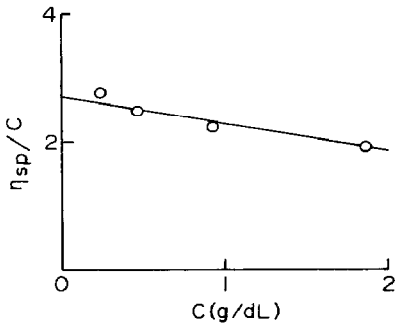

Fig. 2. Intrinsic viscosity of aqueous solutions of *O*-(carboxymethyl)startch.

corresponds to one carboxymethyl group per three to four glucose residues in starch, although monosubstitution may not be the only pattern of derivatisation. The intrinsic viscosity of the aqueous solution of the derivative was found to be 2.75 $dL.g^{-1}$ (Fig. 2) and the specific rotation, $[a]_{589}^{25}$, +156° (*c* 1.0 in water). In the carboxymethylation of starch the derivatisation of both primary and secondary alcoholic groups of the sugar residues is possible under the experimental conditions used, although the *O*-methylation of starch by sodium hydroxide and dimethyl sulfate was reported[8] to derivatise primary alcoholic groups. Determination of the consumption of periodate by carboxymethylated starch might indicate the extent of substitution of secondary alcoholic groups, since the derivatisation of primary alcoholic groups should not affect periodate consumption.

The consumption of periodate by starch and CMS was measured in buffers of pH 7.2 and 9.0. The increase in total periodate consumption with increasing carbohydrate concentration was observed to be linear only at starch concentrations greater than 1.5 $mg.mL^{-1}$. About 1.45 and 1.05 moles of periodate per mole of glucose equivalent were taken up by starch during oxidation at pH 7.2 and 9.0, respectively. Under similar conditions, the molar ratio of periodate consumed to glucose equivalents in CMS was found to be about 1.05 and 0.75 at pH 7.2 and pH 9.0, respectively. The lower (by 26.5 ± 3.5%) periodate consumption of CMS compared to that of starch indicated probable

TABLE I

Comparison of the specific activities of different amylases with starch and CMS as substrates[a]

Amylase	*Concentration (μg protein/mL)*		*Specific activity[b] (u/mg protein)*		*Specific activity ratio[b] (starch:CMS)*
	Starch	*CMS*	*Starch*	*CMS*	
α-Amylase (*A. oryzae*)	5.9	2362	0.22 ± 0.03	$5.72 \pm 0.11 \times 10^{-4}$	385 ± 50
α-Amylase (*B. subtilis*)	7.8×10^{-2}	313	84.2 ± 9	$4.08 \pm 0.09 \times 10^{-3}$	20 600 ± 180
Salivary amylase	2.1×10^{-2}	1.0	2340 ± 15	65.2 ± 5.9	36 ± 4
Pancreatic amylase	6.9×10^{-2}	3.4	765 ± 10	5.73 ± 0.53	134 ± 10
β-Amylase (sweet potato)	1.9	39	0.76 ± 0.08	0.023 ± 0.003	33 ± 1
Amyloglucosidase (*A. niger*)	3.1×10^{-3}	0.25	0.43 ± 0.06	0.16 ± 0.02	2.69 ± 0.1

[a] The enzymes were suitably diluted and assayed according to methods described in the experimental section.
[b] Values are averages from three sets of experiments.

substitution of some of the secondary alcoholic groups in the derivatised polysaccharide.

The susceptibility of CMS to a number of amylases from microbial, plant, and mamalian sources was compared with that of starch. The linearity of the assay system was initially determined for each substrate by using a series of dilutions of each enzyme preparation. Specific activities were calculated from the results of assays in which the amount of enzyme was in the proportionality range (Table I). It was of interest to observe that α-amylase from *B. subtilis* was nearly inactive on CMS, having a specific activity for the derivative only one 20 000th of that for starch. α-Amylases of fungal and mammalian sources were similarly found to have much lower specific activities on CMS, compared to starch. However, it is evident from Table I that the specific activity ratios for fungal and pancreatic amylases are much higher than that for salivary amylase. Thus, salivary amylase appears to be more active on CMS than pancreatin and *A. oryzae* enzyme. Pancreatin was also reported to be active on a hydroxypropyl derivative of starch, but its activity decreased exponentially with increase in the degree of substitution[7].

β-Amylase showed a relative activity on CMS, compared to starch, similar to that of salivary amylase. However, it was observed that amyloglucosidase was more or less equally active on the two substrates, its specific activity on starch being only 2.7 fold higher than that for CMS. In this connection, it may be mentioned that α-amylase obtained from *B. subtilis* was free of any glucoamylolytic activity, whereas the crude preparation of α-amylase from *A. oryzae* probably was contaminated with an amyloglucosidase[1]. So the higher activity of the *A. oryzae* enzyme on CMS, compared to that of *B. subtilis* enzyme, may be due to a contaminating amyloglucosidase in the fungal preparation. In order to ascertain something about changes in the binding affinity and catalytic activity of different amylases resulting from carboxymethylation of the substrate, the respective K_m and V_{max} values were determined from Lineweaver–Burk plots and compared with those for starch as substrate (Table II). It is apparent from the table

TABLE II

K_m And V_{max} values for the different amylases with respect to starch and CMS as substrates

Amylase	*Substrate*					
	Starch		*CMC*		*Ratios*	
	K_m *(mg/mL)*	V_{max} [a]	K'_m *(mg/mL)*	V'_{max} [a]	K'_m/K_m	V_{max}/V'_{max}
α-Amylase (*A. oryzae*)	6.25	0.42	28.7	3.86×10^{-3}	4.57	108
α-Amylase (*B. subtilis*)	5.40	178	50	4.4×10^{-2}	9.26	4040
Salivary amylase	4.06	4170	66.7	872	16.4	4.78
Pancreatic amylase	1.58	1002	100	145	63.3	6.89
β-Amylase (sweet potato)	1.7	1.0	15.4	9.14×10^{-2}	9.06	10.9
Amyloglucosidase (*A. niger*)	1.12	0.55	9.52	0.45	8.5	1.22

[a] V_{max} And V'_{max} are expressed as μmol of glucose liberated/min/mg of enzyme protein. Values were determined from Lineweaver–Burk plots of data for variable concentrations of starch (1–10 mg/mL) and CMS (5–20 mg/mL) as substrates.

that the changes in the K'_m/K_m values for each enzyme are within the range 4.6 to 16, except for pancreatic amylase, which exhibited a K'_m/K_m ratio of 63. Thus the binding affinity of these enzymes, other than pancreatic amylase, for derivatised starch was lowered to a limited extent. On the other hand the V_{max}/V'_{max} values of the α-amylases from *A. oryzae* and *B. subtilis* were much higher than those for the other enzymes, which varied within the limit 1.2 to 11. The activity of amyloglucosidase was not seriously altered when derivatised starch, which had an average carboxymethyl substitution of

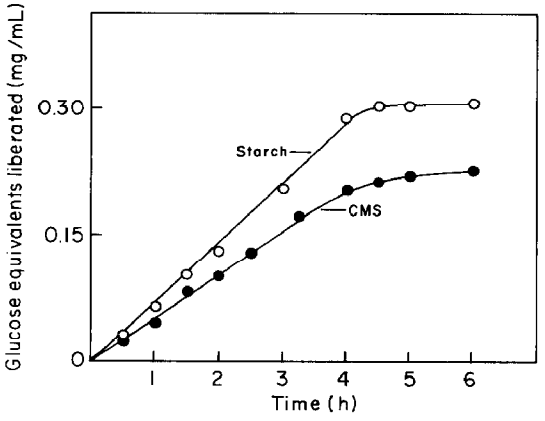

Fig. 3. Kinetics of the action of amyloglucosidase on starch and CMS. The incubation mixture containing starch (4.14 mg/mL) or CMS (35 mg/mL) and the enzyme (1.56×10^{-5} mg/mL) in 0.1M acetate buffer, pH 5.0, was incubated at 40° and reducing power was measured at as described in the text.

one per 3 or 4 residues, was used as substrate. In this context, it may be mentioned that pancreatic amylase has been reported to be active on hydroxypropylstarch containing more than 5 contiguous glucose residues free in a (1→4)-*α*-glucosidic chain[9].

The carboxymethylation of starch appeared to substantially affect either the catalytic activity or the binding affinity of all the amylases for their substrate, except for amyloglucosidase, whose catalytic activity remained nearly unchanged. It may be that amyloglucosidase can find numerous free terminal glucose residues in the derivative. However, the kinetics of reducing-group liberation from starch and CMS, under conditions of saturation by substrate, were more or less similar (Fig. 3) for both substrates, so it may be that amyloglucosidase can hydrolyse glycosidic linkages involving *O*-(carboxymethyl)glucose. All these studies conclusively show that CMS would be an excellent substrate for the specific assay of amyloglucosidase activity in any enzyme preparation. In addition, it is expected that the product may find use as a carbon source for the screening of amyloglucosidase-liberating microbial strains.

EXPERIMENTAL

Starting material. — Soluble starch from potato (Glaxo laboratories, Bombay, India) was used in the investigation.

O-*Carboxymethylation of starch.* — Starch (10 g) was gelatinised with water (10 mL) at 70–80° in a water bath. The mass was then removed from the bath and derivatised with different amounts (excess) of monochloroacetic acid. In practice, various volumes (7.5–50 mL) of 70% (w/v) NaOH were added in aliquots to the gelatinised starch. Each aliquot of alkali was followed by an equivalent amount of saturated aqueous monochloroacetic acid. The mass was thoroughly mixed and the temperature of the mixture was maintained at 70–80° by external cooling of the reaction vessel. Additions were completed within 30 min, and the reaction was allowed to proceed for a further 2 h at room temperature. The mass was then acidified with 6M HCl and CMS was precipitated with 3 volumes of acetone. The precipitate was centrifuged out, dissolved in water, and extensively dialysed against water until free from NaCl. Finally, the CMS solution was lyophilised to a white, fluffy powder. The yield was 30–40% (w/w). The available H^+ equivalents of the derivatives were estimated by titration. The intrinsic viscosity was determined by measuring the specific viscosity (η_{sp}) of aqueous *O*-(carboxymethyl)starch solutions at various concentrations at 25°, using an Ostwald capillary viscometer. Specific viscosity (η_{sp}) $= \frac{t - t_0}{t_0}$, where t_0, is the efflux time of water and t is the efflux time of aqueous CMS.

The glucose content of CMS was determined with the orcinol–H_2SO_4 reagent[10], using glucose as the standard.

Consumption of periodate. — After the addition of periodate to starch or CMS, the decrease in the periodate concentration of the reaction mixture was followed by measuring the absorbancy at 225.5 nm and correcting for the absorbancy of iodate ion

at the same wavelength[11]. Various amounts (2–25 mg) of carefully dried samples of starch and CMS (sodium salt) were dissolved in 10 mL of 0.015M sodium periodate solution either in 0.1M phosphate (pH 7.2) or in 0.2M borate buffer (pH 9.0). The mixtures were incubated at 35° in the dark[12]. Aliquots (0.02 mL) were withdrawn at various times and diluted 250 times with water. The absorbance at 225 nm of the samples and of similarly diluted original periodate and 0.015M sodium iodate solutions was measured. Total periodate consumed in the reaction mixture was determined after 24 h of incubation at 35°. Molar periodate consumption by starch or CMS was calculated as moles of periodate disappearing per mole of glucose residue.

Sources of enzymes. — α-Amylase from *Aspergillus oryzae* (type X-A, cat. no. A 0273), *Bacillus subtilis* (type II-A, cat. no. A 6380), β-amylase from sweet potato (type I-B, cat. no. A 7005), and amyloglucosidase from *Aspergillus niger* (cat. no. A 3514) were obtained from the Sigma Chemical Company, St. Louis, MO, U.S.A. Pancreatic amylase (hog) was a commercial preparation from TTK Pharma Limited, Madras, India. Human saliva was used as the source of salivary amylase. Amylases of microbial origin were dissolved in 0.1M acetate buffer (pH 5.0) and others in 0.1M phosphate buffer (pH 7.0). Enzyme solutions were suitably diluted with the respective buffers.

Assay of amylase activity. — The amount of reducing group liberated from soluble starch or CMS was measured by the method of Nelson[13] and Somogyi[14], as described earlier[15]. Substrate (2 mg) was mixed with enzyme in 0.4 mL of either 0.1M acetate buffer, pH 5.0 (for microbial enzymes), or 0.1M phosphate buffer, pH 7.0 (for mammalian enzymes), and the reaction mixture was incubated 30 min at 40°. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μmol of glucose equivalent per min under the assay conditions.

Determination of K_m and V_{max} data for each enzyme, for both substrates, were obtained using the reaction mixture just described, with variable amounts of substrate. Incubation was for 30 min at 40°. Values of K_m and V_{max} were obtained from Lineweaver–Burk plots. Protein was estimated according to Lowry *et al.*[16], using bovine serum albumin as standard.

REFERENCES

1 K. Aunstrup, *Appl. Biochem. Bioeng.*, 2 (1979) 27–69.
2 G. Ashwell, *Methods Enzymol.*, 3 (1957) 73–105.
3 J. E. Hodge and B. T. Hofreiter, *Methods Carbohydr. Chem.*, 1 (1962) 380–394.
4 B. W. Smith and J. H. Roe, *J. Biol. Chem.*, 179 (1949) 53–59.
5 H. Suzuki, Y. Tadokoro, and N. Taketomi, *Denpun Kogyo Gakkaishi*, 9 (1961) 33–42; *Chem. Abstr.* 57 (1962) 8784e.
6 Y. Sakaguchi and I. Ishizu, *Sen-i Gakkaishi*, 14 (1958) 814–817; *Chem. Abstr.* 53 (1959) 1796a.
7 D. C. Leegwater and J. B. Luten, *Staerke*, 23 (1971) 430–432.
8 M. Fujinaga-Isemura, T. Ikenaka, and Y. Matsushima, *J. Biochem. (Tokyo)*, 64 (1968) 73–80.
9 D. C. Leegwater, *Staerke*, 24 (1972) 11–15.
10 W. Brown and Ö. Andersson, *J. Chromatogr.*, 57 (1971) 255–263.
11 J. S. Dixon and D. Lipkin, *Anal. Chem.*, 26 (1954) 192–196.
12 G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 357–360.
13 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375–380.
14 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
15 A. K. Ghosh, P. C. Banerjee, and S. Sengupta, *Biochim. Biophys. Acta*, 612 (1980) 143–152.
16 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.