β -Amylase-Resistant Amylose

EFFECT OF UREA ON THE LIMITED HYDROLYSIS OF AMYLOSE BY β -AMYLASE

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Amylose prepared from starch dispersed in 10M-urea, pH 6.2, was found to be resistant to the action of β -amylase and phosphorylase, though it was degraded by α -amylase. Amylose isolated by conventional methods was similarly refractory after urea treatment, and was hydrolysed by β -amylase to the extent of 32–35%; it had no inhibitory effect towards β -amylase. The physical and chemical properties of the modified amylose were in general comparable with those of normal amylose with a β -amylolysis limit of 94–98%. Starch and amylopectin were unaffected by urea treatment, i.e. the presence of amylopectin protected amylose against changes induced in it by urea. It is speculated that urea treatment 'freezes' amylose molecules in a conformation that renders non-reducing termini inaccessible to the active site of the exo-enzymes. Such changes may limit the degradative action of β -amylase and phosphorylase.

Complete dispersion of starch granules (defatted and wet) is achieved by prolonged contact with buffered 10M-urea, pH 6.2, at 26–28°C under extremely mild conditions (Patil *et al.*, 1974a). The amylose isolated from this dispersed starch by fractional precipitation with ethanol and complexing with butan-1-ol and thymol shows a β -amylolysis limit lower than that of amylopectin purified from the same source. It was decided therefore to investigate the action of β -amylase on amylose preparations isolated by different procedures to understand the structural changes involved in the resistance acquired by amylose molecules on treatment with urea.

Materials and Methods

All the reagents used were of AnalaR grade. β -Amylase (0.8 mg/ml, specific activity, 1490 units/mg) was prepared from sweet potatoes, purchased locally, by the method of Nakayama & Amagase (1963), followed by chromatography on a column of DEAE-Sephadex (Marshall & Whelan, 1973) and was devoid of α -amylase and maltase activity. One unit of β -amylase activity is defined as the amount which liberates 1 μ mol of maltose/min at 37°C. Salivary α -amylase was prepared from human saliva by the method of Bernfeld (1955). The purified α -amylase had a specific activity of 860 units (1 unit liberates 1 mg of maltose/3 min at 37°C).

Defatted wet potato starch was dispersed in

buffered urea (10M, pH 6.2) solution at room temperature (26–28°C) by gentle stirring for 70–80 h. Amylose and amylopectin were isolated from dispersed starch by fractional precipitation with ethanol and complexing with butan-1-ol and thymol. Trace quantities of amylose were removed from the amylopectin fraction by adsorption on defatted cellulose (Patil *et al.*, 1974b). The purified products were recovered by precipitation with ethanol and characterized by 'blue value' (Bourne *et al.*, 1948), iodine-binding capacity (Patil, 1971) based on the procedure of Lansky *et al.* (1949) and β -amylolysis limit expressed as percentage conversion of polysaccharide (2–4 mg/ml) (37°C, 16 h) into maltose by β -amylase (0.2 ml, 24 units) in 0.025M-acetate buffer, pH 4.8 (2 ml). Total carbohydrate content was determined with thymol/FeCl₃/HCl reagent (Patil *et al.*, 1973). Intrinsic viscosities of amylose samples were determined by the methods of Schoch (1957) and Greenwood (1964).

α -Amylolysis was measured in a system containing polysaccharide (2–4 mg/ml) and α -amylase (125 units/ml, 0.2 ml) in phosphate buffer, pH 6.9, in final volume of 2.0 ml after incubation at 37°C for 16–18 h. Maltose in the enzyme digest was determined by the 3,5-dinitrosalicylic acid procedure (Bernfeld, 1955).

Results and Discussion

It is evident (Table 1) that potato amylose, isolated by dispersion of starch granules with 10M-urea, pH 6.2, at 26–28°C and by using different fractionating agents, is of high purity, and the molecular size, as judged from iodine binding, blue value and intrinsic-viscosity data, compared

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favourably with preparations isolated by leaching procedure (Baum & Gilbert, 1954), dispersion with dimethyl sulphoxide (Killion & Foster, 1960) and mixed-solvent system (4M-guanidine hydrochloride + 2M-LiBr) (Erlander & Tobin, 1967).

Surprisingly, however, the purified amylose exhibited a refractory behaviour towards the action of β -amylase, whereas amyloses isolated by more conventional procedures (Schoch, 1957; Killion & Foster, 1960) have β -amylolysis-limit values of 96.0% and 94.0% respectively (Table 1).

Amylose, a linear polymer of α -D-glucose units linked through α -D(1 $\rightarrow$ 4) bonds, is expected to be hydrolysed completely by crystalline β -amylase to maltose and maltotriose; the latter on prolonged action gives glucose plus maltose. The incomplete hydrolysis of purified amylose preparations (iodine-binding capacity, 18.50–19.96; 'blue value', 1.41–1.43; $\eta = 3.56$ –4.2) by crystalline β -amylase is attributed to the presence of barriers or anomalous linkages, which can be native in character or introduced artificially (Cowie & Greenwood, 1957; Banks *et al.*, 1959; Greenwood & Thomson, 1962).

Starch dispersed in 10M-urea, pH 6.2, made urea-free and fractionated by Schoch's procedure as modified by Killion & Foster (1960), shows a β -amylolysis-limit value of 85%, which is enhanced to 96% after alkali treatment (Table 1, A). This result indicates that the alterations in the structure of the amylose molecule occur during fractionation in the presence of 2M-urea at 26–28°C or during the process of removal of urea [by repeated washing with

60% (v/v) ethanol or dialysis], but not during dispersion by 10M-urea, pH 6.2, at 26–28°C.

Further, it was noticed (Table 2) that amylose preparations with high β -amylolysis-limit values (>94%) acquired a refractory nature towards the action of β -amylase on treatment with 10M-urea at 26–28°C, the process being accelerated at higher temperature (98°C). Related bases such as L-arginine, L-ornithine and guanidine hydrochloride do not decrease the β -amylolysis limit of amylose under these conditions.

The β -amylolysis-limit values of starch dispersed in 10M-urea (pH 6.2, 26–28°C), in dimethyl sulphoxide (26–28°C) and in 1M-NaOH (26–28°C) in the inert atmosphere of N₂ are similar (60–62%). The β -amylolysis-limit values of amylopectin isolated from these starches are also similar (50–52%), and are unaffected by the urea treatment. This suggests that the presence of amylopectin in starch provides some protection for amylose against structural changes caused by the action of urea. Further, the resistance acquired by the amylose cannot be attributed to simple chemical modification of D-glucose units, because such changes would equally affect amylopectin, at least its outer chains. The possibility of chemical bonding between the urea molecules and amylose is remote, since the amylose fractions, on analysis (Concon & Soltess, 1973) were devoid of nitrogen. Further, the amylose samples on hydrolysis with dilute mineral acids (0.25M-H₂SO₄, at 70–80°C for 4–5 h) yield a product which was chromatographically identified as D-

Table 1. *Characterization of potato amylose*

The β -amylolysis limit is expressed as a percentage conversion into maltose. X, Conventional alkali treatment, 1M-NaOH at room temperature (26–28°C) for 5 min in the inert atmosphere of N₂. Y, β -Amylolysis limit of 'X' on treatment with NaOH (1M) at 98°C for 5 min in the inert atmosphere of N₂ and neutralized, and the β -amylolysis limits determined, since these samples exhibited a refractory nature towards the action of β -amylase.

Dispersing agent for the starch granules	Temperature (°C)	pH	Fractionation procedure [temperature (°C) in parentheses]	Iodine- binding capacity	Blue value	Intrinsic viscosity (η) at 30 $\pm$ 0.05°C in 0.5M-KOH (cm ³ ·g ⁻¹)	β -Amylolysis limit	
							X	Y
A 10M-Urea	26–28	6.2	Butan-1-ol (70)	19.80	1.42	3.53	85.0	96.0
B 10M-Urea	26–28	6.2	Thymol (26–28)	19.80	1.41	3.51	34.0	92.0
C 10M-Urea	26–28	6.2	Ethanol (25)	19.96	1.43	3.56	35.0	96.0
D 10M-Urea	26–28	6.2*	—	18.50	—	1.79	81.3	93.0
E 10M-Urea	26–28	6.2†	Ethanol (25)	19.90	1.43	4.32	27.0	96.0
F 2M-Urea	110	—	Ethanol (25)	19.85	1.43	3.33	20.6	98.0
G Dimethyl sulphoxide	26–28	—	Butan-1-ol (70)	19.86	1.42	3.55	94.0	94.0
H Water/phos- phate buffer	95–98	6.8	Butan-1-ol (95–98)	19.80	1.42	2.56	96.0	96.0

* Amylose fraction diffused during the early stages of dispersion of starch granules in urea (10M, pH 6.2) at room temperature (26–28°C).

† Residual amylose fraction from *.

Table 2. Effect of urea treatment on the β -amylolysis limit of some potato amylose preparations

The β -amylolysis limit is expressed as a percentage conversion into maltose.

Fractionation procedure	Poly-saccharide	Dispersing agent for the starch granules	β -Amylo-lysis limit before treatment	Treatment [temperature ($^{\circ}$ C) in parentheses]	β -Amylo-lysis limit after treatment (R)	β -Amylolysis of (R) on treatment (for 5 min in the inert atmosphere of N_2) with 1 M-NaOH at 98° C
Butan-1-ol ($95-98^{\circ}$ C)	Amylose	Water/phosphate buffer, pH 6.8 ($95-98^{\circ}$ C)	96.0	10M-Urea (26-28)	32.0	96.0
	Amylose		96.0	2M-Urea (98)	20.5	97.0
Butan-1-ol (70° C)	Amylose	Dimethyl sulphoxide ($26-28^{\circ}$ C)	94.0	10M-Urea (26-28)	35.0	94.0
	Amylose		94.0	2M-Urea (98)	21.0	94.0
	Amylose		94.0	10M-Urea [contain- ing 0.5M-(NH_4) $_2$ SO $_4$] (26-28)	42.0	94.0

glucose. The carbohydrate content (Patil *et al.*, 1973) of these biopolymers, expressed as glucose, was 105% (w/w) confirming the product as polyglucose.

The resistance towards the action of β -amylase, acquired by the amylose molecules on treatment with urea, can be progressively overcome (20-30% conversion into maltose becomes 95-96%) by treatment with 1M-NaOH in an inert atmosphere of N_2 , as the temperature is raised from 25° to 98° C. The possibility exists that under these conditions amylose might suffer a partial degradation, resulting in the formation of new non-reducing end groups to re-initiate β -amylolysis. Interestingly amylose samples exhibiting low β -amylolysis-limit values were completely digested by an endoenzyme α -amylase (the α -amylolysis-limit values were 96-98%), showing that extensive structural changes of urea-treated amylose had not occurred.

From the results of the present investigation it is postulated that the amylose molecules aggregate on removal of urea by rapid dialysis or extraction with aqueous ethanol, forming a peculiar associated structure(s) perhaps stabilized by intramolecular H-bonds; such associated molecules can acquire resistance to the action of exo-enzymes such as β -amylase and phosphorylase, owing to the inaccessibility of non-reducing end groups to the active site of these enzymes. The polysaccharide is readily hydrolysed by α -amylase, an endo-glucosidase which attacks the substrate molecules in a random fashion.

It is noteworthy, however, that intrinsic-viscosity data, which should provide some information about this aggregated form of amylose, show no significant difference in the intrinsic-viscosity values for amylose (Table 1, G; β -amylolysis limit = 94%; η = 3.55) and amylose (Table 1, C; β -amylolysis limit = 35%; η = 3.56).

One cannot therefore rule out the possibility of

some chemical modification, perhaps very limited, of amylose prepared by the urea method. Freshly prepared 10M-urea solution from recrystallized urea followed by acidification (pH 6.2) gave a negative result when tested for cyanate (Werner, 1923). However, urea slowly does equilibrate with ammonium cyanate on heating (Stark *et al.*, 1960). The production of such an active cyanate might lead to some carbamoylation of hydroxyl groups:

and this substitute might be alkali labile. Hence the alkali treatment would remove the barrier to β -amylolysis. The addition of (NH_4) $_2$ SO $_4$ (0.5M) to 10M-urea solution, pH 6.2, should diminish the concentration of cyanate, and the amylose isolated under such conditions should increase the β -amylolysis limit. Such a treatment, however, does not significantly increase the β -amylolysis limit (Table 2). The results, therefore, favour a non-covalent physical change in amylose affected by urea.

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