29. *Physicochemical Studies on Starches. Part XXI.* Observations on Z-Enzyme.*

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The action pattern of the Z-enzyme associated with preparations of β-amylase has been critically studied by viscosity and light scattering. Solutions of the enzyme were obtained by selective inhibition of the β-amylase with mercuric chloride. The enzyme degraded, in a manner consistent with a random hydrolytic action, both linear amylose and amylose containing a barrier to β-amylase. Limited hydrolysis of amylopectin β-limit dextrin occurred, whilst there was no action on the β-limit dextrin of glycogen. The hydrolytic action of Z-enzyme on amylose and amylopectin is indistinguishable from that of an α-amylase. The implication of these findings regarding the nature of the barrier to β-amylase in amylose is discussed.

The nature of the barrier to the complete β-amylolysis of amylose is not yet known. We have shown, however, that the barriers may be due to an artefact as they can be introduced by treating a heated solution of amylose with oxygen.[1] Such artificially induced barriers can be effectively removed [2]—as can those which normally occur in amylose—by the Z-enzyme described by Peat, Pirt, and Whelan.[3] These results suggested that Z-enzyme was either specific for the removal of oxidised anhydroglucose residues or had a random hydrolytic action. In a effort to characterise amylose completely, as a preliminary to detailed studies of hydrodynamic behaviour, we have now investigated the action of Z-enzyme. The nature of this enzyme has been in dispute.[3–7] Peat, Pirt, and Whelan [3] have shown that it is heat-labile, is inactivated at pH 3·6, and has optimum activity about pH 6 at which pH the barriers to β-amylase present in amylose are effectively removed. The original evidence [4] that Z-enzyme had no α-amylolytic activity but was a β-glucosidase has been questioned.[5,6] As we were unable to repeat the isolation [4] of Z-enzyme from impure preparations of soya-bean β-amylase, we have employed *selective inhibition* of β-amylase in samples containing both enzymes. This technique has proved to be both exceptionally simple and effective. Z-Enzyme was present in only small amounts, therefore physicochemical methods were essential to study the enzyme action pattern;

* Part XX, *J.*, 1959, 3436.

[1] Banks, Greenwood, and Thomson, *Chem. and Ind.*, 1959, 928.
[2] Banks and Greenwood, *Stärke*, 1959, in the press.
[3] Peat, Pirt, and Whelan, *J.*, 1952, 705.
[4] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[5] Hopkins and Bird, *Nature*, 1953, **172**, 492.
[6] Neufeld and Hassid, *Arch. Biochem. Biophys.*, 1955, **59**, 405.
[7] Cowie, Fleming, Greenwood, and Manners, *J.*, 1957, 4430.

chemical methods dependent on measurements of reducing power and iodine-stain were insensitive to detect the limited changes involved.

Experimental

Isolation of Substrates.—Amylose samples I and II were isolated from dispersions of potato starch (vars. Epicure and Redskin) as described earlier.[8] Amylose IIa was a linear subfraction of potato amylose obtained by aqueous leaching.[9] Amylose III was leached at 85° from oat starch (var. Milford). Amylose IV was a composite sample of subfractions of wheat amylose (var. Victor II).[9] Amylopectin was prepared from potato starch (var. Redskin).[8] Glycogen was isolated from brewers' yeast.[10]

Enzyme Preparations.—Purified and crude soya-bean β-amylase were prepared as described by Peat *et al.*[3,11] Commercial samples A, B, and C were obtained from Nutritional Biochemicals Corporation, Lights Ltd., and Wallerstein Laboratories Inc., respectively. (Sample C was kindly provided by Dr. Manners.) Maltase activity was negligible in all preparations. The percentage conversion into maltose of amylose I and potato amylopectin is shown in Table 1 for these enzymes in digests of various values of pH, using *ca.* 50 units of β-amylase [12] per mg. of substrate.

Effect of Various Inhibitors on Enzymic Activity.—Digests were prepared with crude soya-bean β-amylase (50 units [12] of enzyme/mg. of amylose; pH 4·6) in the presence of the inhibitors shown below. After 48 hr. at 35°, the residual polysaccharide was isolated as the butan-1-ol complex and its β-amylolysis limit determined with purified soya-bean β-amylase. The results were as shown.

	β-Amylolysis limit [a]		Inhibition [b] of	
Inhibitor	original	final	β-amylase	Z-enzyme
Formamide (30% aq.; v/v)	82	83	+	+
Formamide (40% aq., v/v)	82	80	+	+
Ascorbic acid (1×10^{-2}M)	82	84	+	+
Mercuric chloride ($1{\cdot}5 \times 10^{-6}$M)	82	89	+	−

[a] Expressed as percentage conversion into maltose. [b] + = Complete inhibition; − = non inhibition.

Experiments involving Inhibition by Mercuric Chloride.—Digests were prepared as follows: enzyme solution (10 ml.; 2500 units of β-amylase [12]) and 0·2M-acetate buffer (10 ml.) were diluted to *ca.* 60 ml. with water, and $1{\cdot}5 \times 10^{-5}$M-mercuric chloride (10 ml.) was added. Amylose solution (10 ml.; *ca.* 100 mg. of amylose) was then added, and the whole diluted to 100 ml. and incubated at 37°; toluene was added to prevent bacterial action. Control experiments without enzyme were carried out similarly. After 48 hr. samples were withdrawn for estimation of reducing power,[9] and then the polysaccharide was precipitated as a complex with butan-1-ol. A small, variable amount (<5%) of amylose which retrograded from solution during this time was removed by centrifugation before the addition of alcohol.

Characterisation of Amylose.—The limiting viscosity number was determined on the butan-1-ol complex.[8] β-Amylolysis limits after treatment with Z-enzyme were obtained with purified soya-bean β-amylase at pH 3·6; details of this procedure have been given elsewhere.[9]

Light Scattering.—Amylopectin β-limit dextrin (*ca.* 10 mg.) was dissolved in water (25 ml.) and clarified by filtration under gravity through a G4 sintered-glass filter.[13] Enzyme powder (*ca.* 100 units of β-amylase/mg. of substrate) was dissolved in 0·2M-acetate buffer (3 ml., of appropriate pH), any undissolved material being removed by filtration. (This procedure avoided an immediate increase in turbidity observed when the enzyme was brought to an acid pH.) The enzyme solution was then added to the solution of the dextrin, and measurements was immediately made of the light scattered at 90° $[(R_{90})_0]$, a Brice–Phœnix photometer being used.[13] Measurements were then made after incubation for varying times to give $(R_{90})_t$.

Similar experiments were carried out on glycogen β-limit dextrin, the only difference being that the solutions were rather more concentrated (*ca.* 18 mg./25 ml.).

[8] Cowie and Greenwood, *J.*, 1957, 2862.
[9] Banks, Greenwood, and Thomson, *Makromol. Chem.*, 1959, **31**, 197.
[10] Greenwood and Jones, unpublished results.
[11] Bourne, Macey, and Peat, *J.*, 1945, 882.
[12] Hobson, Whelan, and Peat, *J.*, 1950, 3566.
[13] Bryce, Greenwood, and Jones, *J.*, 1958, 3845.

Results were expressed as $(R_{90})_t/(R_{90})_0$, and this ratio was plotted as a function of time (t) (see Figure). Variations in angular scattering were neglected and hence *absolute* values of molecular weights are not available, but changes in the Rayleigh ratio indicate changes in molecular weight, *i.e.*, any decrease in $(R_{90})_t/(R_{90})_0$ must represent a decrease in molecular weight of the polysaccharide, particularly as this ratio is liable to cause underestimation of any changes involved.

Z-Enzyme Activity.—The measurement of Z-enzyme activity is difficult. A relative measure was adopted in this work. Digests containing amylose, enzyme, and mercuric chloride at pH 5·5 were incubated for 1 hr. at 37°. After denaturation of the Z-enzyme by heat, the amylose was isolated as the butan-1-ol complex. The resultant decrease in limiting viscosity number of the amylose was taken as a measure of Z-enzyme activity. The results quoted below are the relative activities of Z-enzyme for equal amounts of β-amylase activity:

Sample	Commercial A	Commercial B	Commercial C	Crude soya-bean
Relative activity	1	2	3	6

Results and Discussion

The β-amylolysis results in Table 1 show that all the enzyme preparations, with the exception of purified soya-bean β-amylase, contained Z-enzyme. The crude soya-bean preparation apparently contained α-amylase as shown by an increase in the β-amylolysis limit for amylopectin at pH 5·5. A preparation from another batch of soya-beans behaved similarly. However, this enzyme did not attack glycogen β-limit dextrin, and hence was not an α-amylase (see below).

The nature of the essential thiol groups in β-amylase was first established by Weill and Caldwell.[14] These authors [15] also showed that the enzyme could be inhibited by iodine, hydrogen sulphide, iodomercuric compounds, and iodoacetamide. More recently, non-competitive inhibition by ascorbic acid [16] has been studied. Other inhibitors which have been suggested include formamide [17] and mercuric chloride.[3] However, no reports

Table 1. *Properties of β-amylase samples.*

	β-Amylolysis limit [a] for Amylose			Amylopectin		
pH of digest	3·6	4·6	5·5	3·6	4·6	5·5
Purified soya-bean	80	82	83	56	57	57
Crude soya-bean	83	98	99	55	57	63
Commercial enzyme A	81	98	100	56	56	57
Commercial enzyme B	83	100	99	57	56	57
Commercial enzyme C	82	100	100	56	57	57

[a] Expressed as percentage conversion into maltose.

of the inhibition of β-amylase in the presence of Z-enzyme appear to have been made. We therefore studied the effect of some of the above inhibitors on a mixture of β-amylase and Z-enzyme to see whether a preferential inhibition of β-amylase could be achieved. This was found to occur with mercuric chloride; the inhibition of β-amylase was complete, but Z-enzyme was unaffected (cf. ref. 4). This inhibitor was therefore used in all subsequent work to provide solutions of Z-enzyme.

The Action of Z-Enzyme on Amylose.—Table 2 shows the properties of various amyloses after treatment with Z-enzyme at pH's of 3·6, 4·6, and 5·5. The control experiments—in which amyloses were incubated without enzyme and also with inhibited purified β-amylase—show that the physical conditions caused no fundamental change in either β-amylolysis limit or limiting viscosity number. However, with the exception of those at pH 3·6, in all digests containing Z-enzyme, the limiting viscosity numbers of the isolated amyloses were considerably smaller than of the controls, whilst the β-amylolysis limits tended

[14] Weill and Caldwell, *J. Amer. Chem. Soc.*, 1945, **67**, 212.
[15] Weill and Caldwell, *J. Amer. Chem. Soc.*, 1945, **67**, 214.
[16] Rowe and Weill, *Cereal Chem.*, 1958, **35**, 289.
[17] Husemann and Lindemann, *Stärke*, 1954, **6**, 141.

to 100% conversion. At pH 3·6, where Z-enzyme is inhibited, the properties of the treated and control amylose were comparable.

The decrease in viscosity was largest in those digests buffered to pH 5·5, in which region Z-enzyme has maximum activity (cf. ref. 4). Even at this pH, however, reducing sugars could not be detected in the digests by the alkaline ferricyanide reagent. When the enzyme : substrate ratio was substantially increased, by incubating *ca.* 20 mg. of amylose with 3,000 units [12] of commercial enzyme B in the presence of inhibitor, reducing sugars were still not apparent; the residual polysaccharide could not be precipitated with butan-1-ol, indicating considerable hydrolysis, but it still gave a typical blue stain with iodine.

It is difficult to postulate any arrangement of barriers in amylose such that a *specific* attack by Z-enzyme, at the site of these barriers, could result in the large decreases in viscosity reported in Table 2. The non-specific nature of Z-enzyme is further emphasised by its ability to attack sample IIa, a linear amylose, *i.e.*, one which is completely degraded by *pure* β-amylase and cannot therefore contain any barriers.

The large decrease in viscosity suggests, in fact, that random hydrolytic action of an α-amylolytic type is occurring. This will necessarily result in an increase in the β-amylolysis limit. If it is assumed that random degradation of amylose chains—having not more than one barrier per molecule—is occurring, then

$$L = 100 - \{(100 - L_0)[\eta]/[\eta]_0\}$$

where L_0 and $[\eta]_0$ are the original β-amylolysis limit and limiting viscosity number, respectively, and L and $[\eta]$ are the corresponding values for the degraded amylose. This derivation assumes that $[\eta]$ varies as the molecular weight, which has been shown for our experimental conditions. Values for such theoretical β-amylolysis limits for the amylose

TABLE 2. *The action of Z-enzyme on amylose.**

Amylose	pH	$[\eta]_0$ †	$[\eta]$ †	L_0 ‡	L ‡	$L_{calc.}$ ‡	Amylose	pH	$[\eta]_0$ †	$[\eta]$ †	L_0 ‡	L ‡	$L_{calc.}$ ‡
		Purified soya-bean							Commercial enzyme B				
II	3·6	440	430	83	82	83	II	3·6	440	440	83	84	83
	4·6	430	420	84	83	83		4·6	430	110	84	97	96
	5·5	420	435	84	83	83		5·5	420	30	84	100	99
		Crude soya-bean					IV	3·6	190	190	71	72	71
I	3·6	360	370	81	81	81		4·6	195	80	74	92	89
	4·6	370	60	83	96	97		5·5	195	30	73	96	96
	5·5	370	20	83	100	99			Commercial enzyme C				
III	3·6	160	165	74	74	74	II	3·6	440	430	83	84	83
	4·6	170	75	74	88	89		4·6	430	145	84	95	95
IV	3·6	190	185	71	73	71		5·5	420	45	84	99	98
	4·6	195	80	74	93	89	IV	3·6	190	185	71	72	71
	5·5	195	25	73	98	97		4·6	195	115	74	85	85
		Commercial enzyme A						5·5	195	45	73	94	94
II	3·6	440	440	83	83	83							
	4·6	430	290	84	89	89							
	5·5	420	120	84	94	95							
IV	3·6	190	190	71	73	71							
	4·6	195	160	74	77	79							
	5·5	195	80	73	87	89							
IIa	4·6	250	150	98	99	99							

* For digest conditions see text.

† Limiting viscosity number of amylose in control solution $[\eta]_0$, and amylose after treatment with Z-enzyme $[\eta]$.

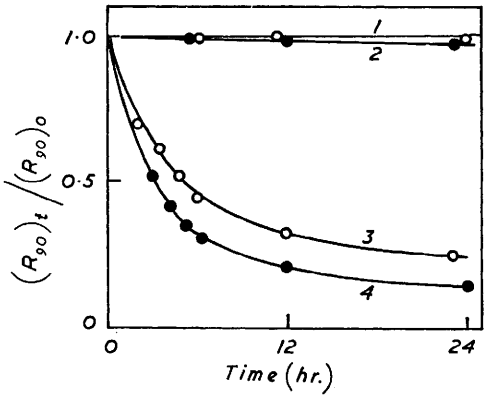
‡ β-Amylolysis limit of amylose in control solution (L_0), after treatment with Z-enzyme (L), and calculated value, random degradation being assumed ($L_{calc.}$) [see text].

samples treated with Z-enzyme show excellent agreement with the observed values (see Table 2), thus again suggesting that the action of Z-enzyme on amylose is of an α-amylolytic type. On this basis, β-amylolysis of the amylose samples treated with Z-enzyme is not necessarily expected to be complete; the extent will depend simply on the concentration

of enzyme (cf. activity measurements quoted in the Experimental section) and the pH of the digest.

The difference between the concurrent action of the two enzymes and the effect of β-amylase following Z-enzyme must be related to the high turnover number of the β-amylase.[18] Amylose is thought to consist [9] of a mixture of (i) completely linear molecules and (ii) those containing a randomly situated barrier; the percentage of the latter being *ca.* 45% for potato starch. When both enzymes are present, the β-amylase will rapidly degrade all the linear molecules completely and those with a barrier to *ca.* 50%. Further β-amylolysis will then occur as these resistant portions are concurrently hydrolysed by Z-enzyme. As the effective concentration of the resistant material will rapidly decrease, β-amylolysis of the amylose will soon be effectively complete. However, when Z-enzyme acts alone, it will hydrolyse a random selection of the amylose molecules present. Hydrolysis of a linear molecule will cause no change in the observed β-limit, whilst hydrolysis of a molecule with a barrier will cause an increase in subsequent β-amylolysis; the conversion will depend simply on the extent of hydrolysis.

The Action of Z-Enzyme on Amylopectin and Glycogen.—If Z-enzyme is an α-amylase, it should attack amylopectin and glycogen even though the extent of such reaction under our conditions will necessarily be very small. Previous claims [3] that Z-enzyme had *no* effect on these substrates were based on evidence of reducing power and iodine-staining measurements. However, neither of these methods is sensitive to limited degradation, and some physical technique for measuring changes in molecular size is required. The light-scattering method is the most convenient and accurate of these for following con-



Action of Z-enzyme on amylopectin and glycogen β-limit dextrins as shown by light scattering.

Variation of $(R_{90})_t/(R_{90})_0$ [see text] as a function of time of incubation with Z-enzyme for (1) glycogen β-limit dextrin at pH 5·5, (2) amylopectin β-limit dextrin at pH 3·6, (3) amylopectin β-limit dextrin at pH 4·6, (4) amylopectin β-limit dextrin at pH 5·5.

tinuous changes and we have used it here. Typical results of such studies are shown in the Figure.

It is obvious that, whereas the amylopectin β-limit dextrin is degraded at those pH values at which Z-enzyme is active, the glycogen β-limit dextrin is unaffected. Furthermore, the enzyme present in the crude soya-bean preparation which degraded amylopectin at pH 5·5 (see Table 1) had also no effect on the molecular size of glycogen. This suggests that true α-amylase is absent, the increase in hydrolysis being brought about by the Z-enzyme. As the crude soya-bean β-amylase contained much more Z-enzyme than any other preparation (see Experimental section) a measurable increase in β-amylolysis limit would be expected when amylopectin is incubated with high concentrations of commercial enzyme. This was, in fact, found to be the case.

Earlier Observations on the α-Amylolytic Nature of Z-Enzyme.—Peat *et al.*[4] suggested that Z-enzyme could not be a weak α-amylase because (1) α-amylases are inhibited by mercuric ions, whereas Z-enzyme is not, and (2) when Z-enzyme was replaced by salivary α-amylase, the β-amylolysis limit of the amylose did not reach 100% conversion. Hopkins

[18] Englard, Sorof, and Singer, *J. Biol. Chem.*, 1951, **189**, 217.

and Bird[5] have already pointed out that the first argument is fallacious (cf. ref. 19). Further, our results suggest that comparison of Z-enzyme with a true α-amylase is impossible.

In an earlier paper,[20] we found that the mean sedimentation coefficient of amylose did not change appreciably during the action of β-amylase alone, or under the concurrent action of β-amylase and Z-enzyme. It was therefore concluded that not only did β-amylase degrade by a single chain mechanism, but that the constancy of molecular weight during degradation in the presence of Z-enzyme suggested that random degradation was not occurring. However, measurement of the change in mean sedimentation coefficient is not sufficient to detect changes in molecular-weight distribution. The mean value measures, in fact, only the sedimentation coefficient of the most abundant species present. As the digests were not carried out at the optimum pH of Z-enzyme, the number of bonds broken by this enzyme would obviously be small. In the initial stages of the attack by β-amylase and Z-enzyme, any hydrolytic action exerted by the latter would result in a change in the molecular-weight distribution without necessarily affecting the value of the mean sedimentation coefficient. At rather higher percentage conversions into maltose, the most abundant species present is still likely to be the limit dextrin, which has the same sedimentation coefficient as the original amylose. [This reasoning is based on the assumption that, at high conversions, the rate-controlling factor will be hydrolysis by Z-enzyme (cf. above).]

Conclusions.—We have shown that four different samples of β-amylase contain a second amylolytic enzyme. This enzyme is not inhibited by mercuric chloride, has no apparent effect on the β-amylolysis limit of amylopectin under normal digest conditions, and is inactivated by digestion at pH 3·6. Further, the enzyme exerts a random hydrolytic action on amylose, a limited hydrolysis of a few bonds in amylopectin, but is unable to attack glycogen.

Thus the action pattern of Z-enzyme on amylose and amylopectin is indistinguishable from that of an α-amylase. Its apparent inability to attack glycogen, however, differentiates it from normal α-amylases.

The usual source of the enzyme is the ungerminated seed.[21] In the dormant state seeds do not contain α-amylase,[22] but there is no doubt that it appears on germination. Z-Enzyme could therefore be the dormant form of α-amylase. (It is of interest that Baba and Kojima [23] suggested that the Z-enzyme present in preparations of crude emulsin is an α-amylase.)

As Z-enzyme is non-specific in character, the nature of the barrier in amylose to the action of β-amylase remains unknown. It could be an artefact introduced during isolation or fractionation of the starch, or a natural modification of the amylose chain, or a mixture of both. The barrier may indeed vary according to the botanical source of the amylose. Elsewhere,[2] we have discussed these problems in detail and concluded that a combination of both a natural and an artificial barrier is most likely; artificial barriers can be inadvertently introduced during fractionation,[1] whilst phosphate groups [24] or branching may well occur naturally. These problems are being studied.

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Edinburgh, 9. [*Received, July 7th*, 1959.]

[19] Caldwell, Weill, and Weill, *J. Amer. Chem. Soc.*, 1945, **67**, 1079.
[20] Cowie, Fleming, Greenwood, and Manners, *J.*, 1958, 697.
[21] Whelan in Ruhland, " Encyclopedia of Plant Physiology," Springer-Verlag, Berlin, 1958, Vol. VI, p. 154.
[22] Myrbäck and Neumuller in Myrbäck and Sumner, " The Enzymes," Academic Press, New York, 1950, Vol. I, p. 653.
[23] Baba and Kojima, *Chem. Abs.*, 1958, **52**, 17,337.
[24] Banks and Greenwood, *Biochem. J.*, 1959, **73**, 237.