130. *The Enzymic Synthesis and Degradation of Starch. Part XVII.* Z-Enzyme.*

By Stanley Peat, Gwen J. Thomas, and W. J. Whelan.

Z-Enzyme, which supplements the action of β-amylase or phosphorylase on amylose, has been isolated from the soya bean as a freeze-dried powder free from α- and β-amylases. Its function is the hydrolysis of the limited number of branch links in amylose which act as barriers to β-amylolysis or phosphorolysis. Z-Enzyme is shown to be a β-glucosidase and its action on amylose is simulated by the β-glucosidase of sweet almonds.

The form of the β-glucosidic links in amylose is discussed.

The evidence cited in the two preceding papers has shown that the complete conversion of amylose into maltose is effected by a mixture of two enzymes, namely, β-amylase and Z-enzyme. It is the function of the former enzyme to liberate maltose and of the latter to remove, by hydrolysis, anomalous structures within the amylose molecule which impede the endwise attack of β-amylase. In the absence of Z-enzyme, β-amylase converts only 70% into maltose. Z-Enzyme is associated with β-amylase in stock soya-bean β-amylase

* Part XVI, preceding paper.

and is probably to be found in β-amylase preparations from other plant sources. The present communication describes the isolation of Z-enzyme as a dry powder, entirely free from α- and β-amylases. Its properties are recorded and evidence is afforded which identifies it as a β-glucosidase. A preliminary account of some of the following observations has been given by Thomas, Whelan, and Peat (*Biochem. J.*, 1950, **47**, xl).

The possibility existed that the structures in amylose impeding β-amylolysis consisted of ester-phosphate groups. Posternak (*J. Biol. Chem.*, 1951, **188**, 317) has shown, for example, that ester-phosphate groups are present in the dextrins formed in the α-amylolysis of potato starch and that these obstruct the action of β-amylase on the dextrins. Although these dextrins were probably derived from the amylopectin component, potato amylose does contain esterified phosphorus in an amount corresponding to 1 atom of phosphorus per 2400 glucose residues. This quantity, although very small, would account for the observed effects if one-third of the amylose molecules contained an ester phosphate group situated at or near the non-reducing end. If this explanation were correct then Z-enzyme would be a phosphatase. Stock soya-bean β-amylase contains a phosphatase which can be detected by its action on sodium glycerophosphate. This phosphatase is not present in purified soya β-amylase, which is also free from Z-enzyme (see Part XVI). It was, however, demonstrated in the following manner that Z-enzyme is not a phosphatase. First, pre-treatment of potato amylose with bone phosphatase (which, according to Thoai, Roche, and Silkol-Bernère, *Compt. rend.*, 1946, **223**, 931, will dephosphorylate starch) had no effect on the limit of β-amylolysis (Table 3). Secondly, stock soya-bean β-amylase was fractionated on a paper chromatopile, from which Z-enzyme and phosphatase were eluted together by ammonium sulphate solution; the β-amylase was not extracted from the pile even by perfusion for 10 days. When the eluates were kept at room temperature for two weeks, they lost Z-enzyme activity, but phosphatase activity persisted. Finally, Z-enzyme was isolated, by the following procedure, as a freeze-dried powder which exhibited no phosphatase activity. The isolation of Z-enzyme free from associated enzymes was achieved as a result of an attempt to eliminate the α-amylase component of soya bean by a modification of the method of Blom, Bak, and Braae (*Z. physiol. Chem.*, 1936, **241**, 273), who effected the preferential destruction of α-amylase by the treatment of an impure malt β-amylase with acid at 0°. When the pH of the stock soya-bean preparation was adjusted to 3·0 and the solution kept at 35°, instead of at 0°, both α- and β-amylases were destroyed, but Z-enzyme activity was not appreciably diminished (Table 4, p. 728). The phosphatase was partly destroyed by this treatment and after the solution had been freeze-dried no phosphatase remained, although Z-enzyme was unaffected. Potato phosphorylase is similarly inactivated by being freeze-dried in the absence of buffer (Part VI, *J.*, 1950, 84).

Function of Z-Enzyme in the β-Amylolysis of Amylaceous Polysaccharides.—Having regard to their apparent similarity of function, particular emphasis is placed on the necessity to distinguish Z-enzyme from α-amylase. For this purpose a solution of salivary α-amylase was prepared and so diluted that it exerted only a slight action on amylose and amylopectin during several hours. Table 1 (p. 724) shows that Z-enzyme *per se* is without action on the α-1 : 4-glucosidic links of either amylose or amylopectin. Amylose was then incubated with crystalline β-amylase, alone and in the presence of Z-enzyme or the dilute α-amylase. Measurements of intensity of iodine stain [A.V. (680 mμ)] and reducing power were made after 6 and 19 hours and the results (Table 1) established that the arrest point of action had in each case been reached within 6 hours. It is thus obvious that, while Z-enzyme assists β-amylase in effecting the conversion of more than 90% of amylose into maltose, its action in this respect cannot be distinguished from that of dilute α-amylase. With amylopectin as the substrate in a similar series of experiments, the mixture of β-amylase and Z-enzyme, was, however, sharply differentiated from the mixture of α- and β-amylases (Table 1). It is clear also that the actions on amylopectin of (i) crystalline β-amylase, (ii) a mixture of Z-enzyme and crystalline β-amylase, and (iii) stock soya β-amylase are identical. None of these enzyme combinations brings about a conversion into maltose greater than 52·5%. The mixture of α- and β-amylases, on the other hand, effects a much more substantial hydrolysis of amylopectin, as is to be expected because, by fragmenting the "inner chains," α-amylase exposes new chain-ends to attack by β-amylase. That

Z-enzyme can act on amylose independently of the simultaneous action of β-amylase was shown by the fact that, when amylose was digested with Z-enzyme for 10 hours and the enzyme then destroyed by heat, the subsequent conversion of the amylose into maltose by crystalline β-amylase was 97·7%, compared with the value of 67·5% for the same sample of amylose before treatment with Z-enzyme. In an analogous experiment, in which Z-enzyme was replaced by dilute α-amylase, the subsequent % conversion into maltose effected by the β-amylase was lower, namely, 89·4%. α-Amylase fragments the linear chains of amylose and in acting for a limited period and in high dilution would be expected to yield some chains which were still protected by the anomalous linkages from endwise attack by β-amylase, thus accounting for the lower degree of conversion into maltose.

TABLE 1. *Action of α-amylase, β-amylase, and Z-enzyme on amylose and amylopectin.*

	Amylose, B.V. 1·45				Amylopectin, B.V. 0·158			
	6 hr.		19 hr.		2 hr.		7 hr.	
Enzyme(s)	Convn., %	A.V. (680 mμ)	Convn., %	A.V. (680 mμ)	Convn., %	A.V. (680 mμ)	Convn., %	A.V. (680 mμ)
Z	0·0	1·40	1·3	1·39	0·0	0·158	0·0	0·160
α	1·8	1·37	—	—	3·8	0·106	7·3	0·084
β	67·3	0·357	67·5	0·340	51·2	0·088	51·5	0·088
β + Z	90·5	0·079	93·4	0·038	52·3	0·088	51·5	0·088
β + α	96·0	0·010	—	—	58·3	0·032	64·9	0·023
Stock soya	91·5	0·055	92·0	0·042	50·9	0·095	52·5	0·073

All digests incubated at 35° and pH 4·8. Convn., % = conversion (%) into maltose; β = crystalline sweet-potato β-amylase; Z = Z-enzyme; α = freeze-dried salivary α-amylase; stock soya = stock soya-bean β-amylase.

With whole starch as substrate, the action of purified soya-bean β-amylase, with and without the addition of Z-enzyme, was compared with that of stock soya-bean β-amylase. The results given in Table 2 demonstrate, as in Part XV (p. 705), the different limits of action of purified β-amylase and stock soya-bean β-amylase, and also that when the action of pure soya-bean β-amylase is supplemented by that of Z-enzyme the limit of action of the enzyme mixture is identical with that of stock soya-bean β-amylase. The initial rate of action of the stock preparation is more rapid than that of the mixture, but the difference can be ascribed to a difference of Z/β ratio in the stock soya preparation and in the artificial mixture. The arrest point of action of the soya enzyme or of the mixture on starch, namely, 60% conversion into maltose, is that usually quoted as the hydrolysis limit by "β-amylase" preparations and there is no doubt that this hydrolysis is achieved by the agency of two enzymes. The arrest point of hydrolysis of starch by β-amylase alone is much lower (53%).

TABLE 2. β-*Amylolysis of potato starch* (B.V., 0·425) *in presence of Z-enzyme.*

	β-Amylase		β-Amylase + Z-enzyme		Stock soya-bean β-amylase	
Age of digest (hr.)	Conversion into maltose (%)	A.V. (680 mμ)	Conversion into maltose (%)	A.V. (680 mμ)	Conversion into maltose (%)	A.V. (680 mμ)
0·5	44·7	0·210	47·6	0·217	54·7	0·104
2·5	53·0	0·174	55·6	0·146	60·0	0·094
8·5	54·0	0·160	59·5	0·110	60·4	0·091
23	—	0·158	62·4	0·094	60·9	0·091
71	56·5	—	61·7	—	—	—

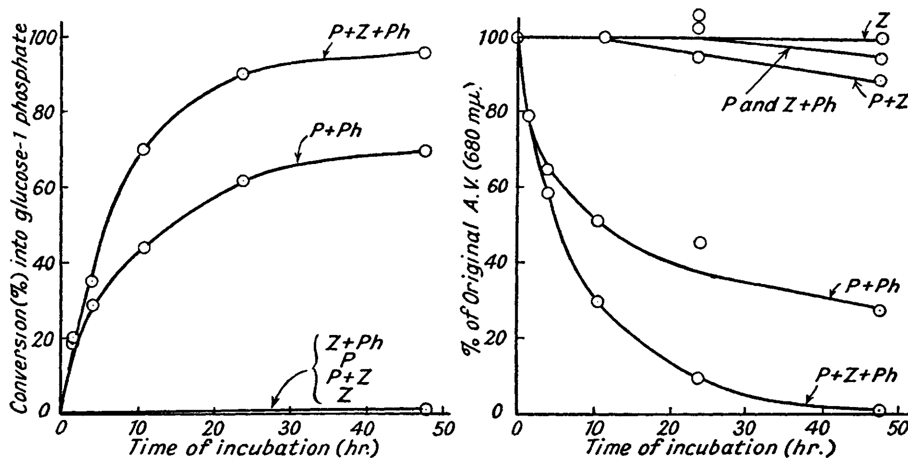
Z-Enzyme and the Phosphorolysis of Starch Polysaccharides.—Since phosphorylase, like β-amylase, degrades starch polysaccharides by an endwise attack on the terminal non-reducing glucose units it is reasonable to suppose that, unless the phosphorylase preparation contains Z-enzyme, phosphorolytic action on amylose should terminate at a limit of conversion similar to that found during the hydrolysis of amylose by pure β-amylase. Hestrin (*J. Biol. Chem.*, 1949, **179**, 943) found that the rate of action of crystalline muscle phosphorylase on maize amylose diminished markedly at a conversion into glucose-1 phosphate of 70%. The effect was ascribed to retrogradation (ageing) of the amylose. It would seem more probable that the incomplete conversion was due to the

absence of Z-enzyme, since it has been shown that Z-labile linkages are present also in maize amylose (Part XVI, preceding paper). Katz and Hassid (*Arch. Biochem.*, 1951, **30**, 272) find that phosphorolysis and arsenolysis of potato amylose by potato phosphorylase lead to substantially complete conversions into glucose-1 phosphate and glucose respectively, the rate curves showing no inflextion at 70% conversion. We cannot confirm this effect with our preparation of potato phosphorylase; its action on potato amylose terminates in the region of 70% (Fig. 1). When, however, Z-enzyme was also present the degree of conversion was 95·4%.

These observations on the effect of Z-enzyme on the phosphoroloysis of amylose parallel those of its effect on β-amylolysis. Furthermore, we have found that Z-enzyme is equally ineffective in extending the limit of conversion of amylopectin by potato phosphorylase or by β-amylase (Table 6, p. 730). It is reasonable to conclude therefore that potato amylose contains in its structure linkages which function as barriers to the progress of both phosphorolysis and β-amylolysis and that these barriers are removed by Z-enzyme.

The Nature of Z-Enzyme Action.—While this work was in progress, Dillon and O'Colla reported that an unfractionated specimen of wheat β-amylase contained an enzyme

FIG. 1. *Effect of Z-enzyme on the phosphorolysis of amylose* (B.V. 1·40).



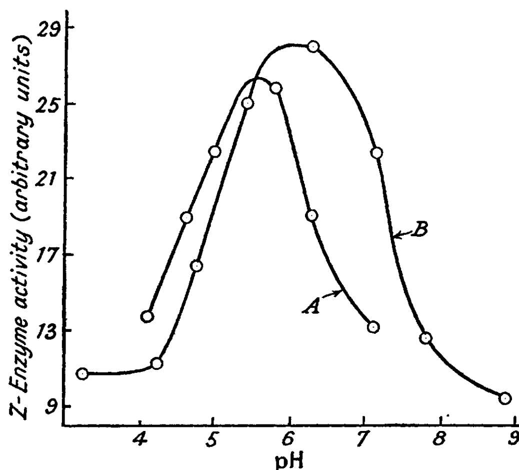
P = Potato phosphorylase ; Z = Z-enzyme ; Ph = phosphate buffer, pH 6·7.

(laminarinase) which brought about the hydrolysis of laminarin to glucose (*Nature*, 1950, **166**, 67). Laminarin, a seaweed polysaccharide, is a polyglucose containing β-1 : 3-linkages. We found that a sample of laminarin, kindly presented to us by Professor Dillon, was hydrolysed by stock soya β-amylase (Table 7, p. 730), the limiting yield of glucose (72·3%) agreeing closely with the value of 70% reported by Dillon and O'Colla for the wheat enzyme. Furthermore, pure Z-enzyme also hydrolysed laminarin. There is thus a strong indication that the Z-enzyme of soya bean and the laminarinase of wheat are identical in function. Supporting this view is the fact that the pH optima of the action of pure Z-enzyme on amylose and of the Z-enzyme in the stock soya β-amylase preparation on laminarin are similar (Fig. 2).

The action of Z-enzyme is not, however, confined to β-1 : 3-linkages since it also hydrolyses cellobiose, in which the linkage is β-1 : 4, gentiobiose, with a β-1 : 6-glucosidic link, and the β-1 : 2-linkages of crown gall polysaccharide (Putnam, Potter, Hodgson, and Hassid, *J. Amer. Chem. Soc.*, 1950, **72**, 5024). The only structural feature possessed in common by these polyglucoses is the glucosidic link (or links) which in each case has the β-configuration. It would appear therefore that Z-enzyme is a β-glucosidase, with group, rather than absolute, specificity. Confirmation of this view was forthcoming from the demonstration that the β-glucosidase of almonds (emulsin) could replace Z-enzyme as the factor required to supplement the hydrolysis of amylose by β-amylase (Table 9, p. 731)

and that the presence of emulsin did not influence the degree of β-amylolysis of amylopectin (Table 10, p. 731). Incidentally, Table 10 shows that the emulsin was not contaminated with α-amylase. The probable identity of Z-enzyme and emulsin was further supported by the results of the following experiments. First, emulsin is like Z-enzyme in not being dependent (for its action on amylose) on the presence of β-amylase; amylose, pre-treated with emulsin, was hydrolysed to a greater extent by β-amylase than was untreated amylose (Table 11, p. 731). Secondly, it was shown that mercuric chloride did not inhibit the hydrolysis of cellobiose by emulsin, nor did it inhibit the "debranching" actions of Z-enzyme or emulsin on amylose, as shown by the fact that the limits of phosphorolysis of an amylose treated, in presence of mercuric chloride, with either Z-enzyme or emulsin were 98·9 and 94·0% respectively (Table 12, p. 732). It is to be noted that this experiment sharply distinguishes Z-enzyme and emulsin from the α-1 : 4-glucosidases, α-amylase, β-amylase, and maltase, each of which is completely inhibited by mercuric chloride, and adds further to the evidence already presented that Z-enzyme exerts a true " debranching "

FIG. 2. *Optimum* pH *of Z-enzyme.*



A = Action of stock soya-bean β-amylase on laminarin.
B = Action of Z-enzyme and crystalline β-amylase on amylose limit β-dextrin.

action and is not merely a weakly active α-amylase. Z-Enzyme is also differentiated in this way from R-enzyme which " debranches " amylopectin (Part XIV; *J.*, 1951, 1451) and is inhibited by mercuric chloride. Finally, it was shown that emulsin, like Z-enzyme, hydrolyses laminarin (Table 13, p. 732; cf. Table 7, p. 730).

There is therefore substantial evidence that Z-enzyme is a β-glucosidase, and hence that the Z-labile linkages in potato amylose have the β-configuration. The location of these links in the amylose chains has not yet been determined, but several possible structures are being considered. Z-Enzyme appears to operate by an endwise attack on *terminal* β-glucosidic links inasmuch as paper chromatography of laminarin hydrolysates reveals that glucose is the only product of low molecular weight (cf. Dillon and O'Colla, *Chem. and Ind.*, 1951, 111) and, moreover, the action of Z-enzyme on amylose is independent of the presence of β-amylase. These facts suggest that the Z-labile links in amylose, unlike the branch linkages in amylopectin, join *single* glucose residues to the main chains.

EXPERIMENTAL

Analytical Methods.—All methods used, with the exception of the following, have been detailed in Part XVI of this series (preceding paper).

Determination of total phosphorus content of polysaccharides. The dried polysaccharide (100 mg.), contained in a " Pyrex " boiling-tube (7" × 1¼"), was moistened with a few drops of alcohol. Water (2 ml.) and 6N-sodium hydroxide (1·5 ml.) were added and the poly-

saccharide was dissolved by warming. Perchloric acid (60%; 4 ml.) was added, the tube was covered by a glass bulb, and the solution boiled on an electric hot-plate for 3—4 hours. A few drops of "AnalaR" hydrogen peroxide (100-vol.) were added to complete the combustion and when the solution was colourless the contents of the tube were washed into a 25-ml. standard flask. The phosphorus content was determined by Allen's method (*Biochem. J.*, 1940, **34**, 858). A control determination was carried out by the same method, the polysaccharide being omitted, to determine the phosphate content of the reagents. They were usually found to be completely devoid of phosphate. Results of typical determinations using this method are: P in potato amylose, 0·0086, 0·0085, in potato amylose β-limit dextrin, 0·0359, 0·0317, in maize amylose, 0·0061, 0·0059%.

Preparation of Enzymes.—Stock soya-bean β-amylase was prepared as in Part II (*J.*, 1945, 882), and purified soya β-amylase by the method of Part XVI (preceding paper). Crystalline sweet-potato β-amylase was the gift of Dr. A. K. Balls.

Bone phosphatase was prepared by Martland and Robison's method (*Biochem. J.*, 1929, **23**, 237); by the test described below, it was found to contain α-amylase which was removed by adsorption on starch grains (cf. Part XIV, *J.*, 1951, 1451); the resulting enzyme preparation was completely devoid of α-amylolytic activity but hydrolysed glycerophosphate rapidly. An aqueous solution (450 ml.) of bone phosphatase was prepared from the leg bones of two rabbits according to the above method and to it was added sodium sulphate (5 g.), ethanol (113 ml.), and potato starch (37·5 g.). The mixture was stirred for 24 hours at 2° and the enzyme isolated from the supernatant solution by precipitation with 2 volumes of acetone, washing with acetone, and drying over phosphoric oxide.

Emulsin was prepared from sweet almonds by Tauber's method (*J. Biol. Chem.*, 1932, **99**, 259). When dissolved in water it exhibited maltase activity, but a solution prepared by shaking the enzyme powder in 0·2*M*-acetate buffer (pH 4·8) and removing undissolved protein did not hydrolyse maltose.

Measurement of Enzyme Activity.—(a) *β-Amylase.* See Part XVI (preceding paper).

(b) *α-Amylase.* In order to detect small amounts of α-amylase in the presence of an excess of β-amylase, use was made of the fact that, whereas β-amylase brings about only slight hydrolysis of amylose glycollate (Peat, Bourne, and Thrower, *Nature*, 1947, **159**, 810), α-amylase degrades the amylose glycollate to achroic dextrin. A solution of amylose glycollate was incubated with crystalline α-amylase until the limit of hydrolysis (2—5% conversion) was reached. The resulting solution was used as substrate for the specimen being tested for α-amylase. α-Amylase activity was detected by measurement of fall in A.V. (680 mμ) of the iodine-stained polysaccharide. Under the conditions described below the rate of fall of A.V. (680 mμ) was uniform over an incubation period of 10 hours, and the change taking place in 5 hours was used as the measure of α-amylase activity. In some experiments amylopectin limit β-dextrin was used instead of the glycollate, the behaviour of the two polysaccharides with respect to α- and β-amylases being similar. The digests contained amylose glycollate (7 mg.; 3 ml.), 0·2M-acetate buffer (pH 7·0; 1 ml.), crystalline β-amylase (1000 units; 0·2 ml.), and test solution and water to 11·2 ml. The temperature of incubation was 35°. A portion (1 ml.) of the digest was withdrawn initially, and after 5 hours' incubation, for measurement of A.V. (680 mμ) after staining with standard iodine solution (0·02% in 0·2% KI; 1 ml.) and 6N-sulphuric acid (1 drop) in a total volume of 100 ml.

(c) *Phosphatase.* The optimum pH of soya-bean phosphatase, acting on sodium glycerophosphate, was found to be 5·5. A solution of this pH was prepared by dissolving sodium glycerophosphate (1·25 g.) and 0·2M-acetate buffer (pH 4·8; 30 ml.) in a total volume of 250 ml. The digests used in testing for phosphatase activity contained glycerophosphate solution (18 ml.) and enzyme solution (0·5 ml.). They were incubated at 35° for 45 minutes and then the mineral phosphate content of the digest was determined. Provided that the amount of phosphate liberated was less than 0·08 mg. the relation between degree of hydrolysis and enzyme concentration was linear.

(d) *Z-Enzyme.* The method used was to allow the Z-enzyme to act on amylose limit β-dextrin in the presence of an excess of β-amylase. Removal of the anomalous links in the amylose-dextrin by Z-enzyme enables the β-amylase to bring about further hydrolysis, which was detected by measurement of A.V. (680 mμ) after staining with iodine. The dextrin solution was prepared by dissolving amylose (B.V. 1·40; 110·2 mg.) in 0·1N-sodium hydroxide (50 ml.), neutralising the solution with N-sulphuric acid, and addition of 0·2M-acetate buffer (pH 4·8; 12 ml.), crystalline β-amylase (5000 units; 1 ml.), and water to 100 ml. The solution was incubated at 35° for 6 hours during which the limit of hydrolysis by the β-amylase was reached.

This dextrin solution (15 ml.) and the solution under test (1 ml.) were incubated together for 2 hours at 35°. A portion (2 ml.) was removed for staining with iodine solution (2 ml.) in a total volume of 100 ml. Under these conditions the extent of degradation of the limit dextrin was approximately proportional to the concentration of the enzyme until the A.V. (680 mμ) had decreased by more than 23%.

β-Amylolysis of Amylose treated with Bone Phosphatase.—Two digests were prepared containing amylose solution (B.V. 1·35; 25 mg.; 18·77 ml.), 0·2M-citrate buffer (pH 8·2; 6 ml.), and water to 50 ml. In addition bone-phosphatase solution (52·2 mg.; 13 ml.) was included in one of the digests. Both were incubated at 35° for 23 hours after which time the B.V. of the amylose in each digest was measured, on 2-ml. portions, and found to be unchanged. Both digests were then heated to 100° and, after cooling, the pH was adjusted to 5·2 with citric acid solution. Crystalline β-amylase (2500 units; 0·5 ml.) was added to the digests which were incubated at 35°. Measurements of A.V. (680 mμ) and reducing power were made at intervals as in Table 3.

TABLE 3. *Action of β-amylase on amylose treated with phosphatase.*

Age of digest (hrs.)	Untreated amylose: Conversion into maltose (%)	Untreated amylose: A.V. (680 mμ)	Phosphatase-treated amylose: Conversion into maltose (%)	Phosphatase-treated amylose: A.V. (680 mμ)
0·5	61·2	0·382	68·1	0·339
4	67·3	0·359	70·5	0·327
11	71·5	0·346	70·9	0·316
24	72·0	0·334	72·5	0·304

Fractionation of Stock Soya-bean β-Amylase on a Chromatopile.—The apparatus was essentially that described by Mitchell, Gordon, and Haskins (*J. Biol. Chem.*, 1949, **180**, 1071) but the procedure differed in that the ammonium sulphate solution, after passage through the filter papers, was collected in 6-ml. fractions by means of Phillips's automatic collector (*Nature*, 1949, **164**, 545). The lower disc holding the filter papers was constructed of " Perspex," being a hollow cylinder, perforated on the side in contact with the filter paper and channelled inside, the channels leading to a spout from which the solution dripped into the fraction collector. The filter-paper pile was contained within a close-fitting cylinder of " Perspex " to prevent evaporation of solution from the paper.

Stock soya β-amylase (800 mg.) was shaken with water (20 ml.), and the insoluble portion removed on the centrifuge. The supernatant solution was absorbed on 20 sheets of Whatman No. 1 9-cm. filter paper, dried in a desiccator over phosphoric oxide, and the papers were inserted at the top of a pile of 780 papers, covered by a further 20 papers, and clamped in the apparatus. The enzymes were eluted by the concentration-gradient method of the above authors, starting with half-saturated ammonium sulphate (pH 6·5). The fractions were tested for Z-enzyme, phosphatase, and β-amylase activity by the standard methods. In all, 68 fractions (6 ml. each) were collected, Z-enzyme and phosphatase being generally found in the same fractions. β-Amylase was, however, absent and, on dismantling of the chromatopile and elution of the separated sheets, was found to have moved to a position 200 sheets from the lower end, being spread over a thickness of 150 sheets. Some of the eluates containing Z-enzyme and phosphatase were combined and stored in the refrigerator for 2 weeks. After this time Z-enzyme activity had disappeared but the phosphatase activity was still present.

Isolation of Z-Enzyme.—Portions (50 mg.) of stock soya-bean β-amylase were shaken in 0·2M-acetate buffers (25 ml.) of pH 3·0 and pH 4·8, and the insoluble residues removed. The solutions were heated at 35° and at intervals portions were removed for determination of Z-enzyme, α-amylase, β-amylase, and phosphatase activity by the standard methods, the pH of each solution being adjusted to the appropriate value before determination of the enzyme activity. In Table 4 the enzyme activities remaining in the solutions treated at pH 3·0 are expressed as percentages of the activities remaining in the solutions treated at pH 4·8 for the same length of time.

TABLE 4. *Acid* (pH 3·0) *treatment of stock soya-bean β-amylase at* 35°.

Time of heating (hr.)	Enzyme activity remaining (%): Z-Enzyme	α-Amylase	β-Amylase	Phosphatase
1	133	0	3·9	47
2	129	0	—	—
3	95	—	—	—

Z-Enzyme was prepared by shaking stock soya β-amylase (600 mg.) with 0·2M-acetate buffer (pH 3·0; 300 ml.) and removing the insoluble residue on the centrifuge. The solution was kept for 90 minutes at 35°, then cooled, and the pH was adjusted to 4·8 by the addition of N-sodium hydroxide solution. The β-amylase activity had fallen to 0·6% of the original value. The solution was divided into three equal parts, one of which was freeze-dried and to the others were added 2 volumes of methyl alcohol and acetone respectively. The precipitates were centrifuged, washed with the appropriate solvent, and dried in a vacuum over phosphoric oxide. The weights of each preparation were, respectively, 2·37 g., 64·7 mg., and 31·9 mg., the high weight of the freeze-dried preparation being due to its consisting largely of acetate buffer. The units of Z-enzyme activity contained in the three preparations were, respectively, 1997, 769, and 110. It was evident that freeze-drying afforded the best method of isolating Z-enzyme from solution. The freeze-dried preparation was devoid of phosphatase activity. Attempts to modify this procedure by using a greater initial concentration of stock soya β-amylase were unsuccessful. The above method, incorporating the freeze-drying technique, has been used in all subsequent preparations of Z-enzyme.

β-Amylolysis in the Presence of Z-Enzyme or α-Amylase.—The technique used in the β-amylolysis of amylaceous polysaccharides has been described in Parts XV and XVI (preceding papers). In the experiments reported in Tables 1 and 2, the volume of each digest was 25 ml. and the earlier method was modified insofar as 300 units of β-amylase were used in each digest instead of 1300 units. The amounts of Z-enzyme and α-amylase, when present, were 75 mg. and 0·3 mg. respectively. The source of the α-amylase was saliva which had been diluted with an equal volume of water to precipitate mucins, the supernatant liquid being then freeze-dried. When amylose was treated successively with Z-enzyme (or α-amylase) and β-amylase, two identical digests were prepared and incubated at 35°, one of which was used to determine changes in A.V. (680 mμ) and reducing power caused by the Z-enzyme or α-amylase. After 24 hours the second digest was heated at 100° for 3 minutes, then cooled, crystalline β-amylase (300 units; 0·5 ml.) was added, and the incubation continued at 35°.

Phosphorolysis of Amylose and Amylopectin.—Phosphorylase was isolated from potatoes by precipitation and elution of the lead complex as in Part VI (*J.*, 1950, 84). The enzyme was then concentrated by successive fractionations with ammonium sulphate solution (pH 7·0; 50 g./100 ml.); the precipitate was retained which formed between the following limits of salt concentration (g./100 ml.): 25—35, 22—35, 21—35. It was finally freeze-dried in citrate buffer (pH 6·0) as in Part VI. The activity of the preparation was 106 units per g. In the phosphorolysis of amylose (B.V. 1·40) the following solutions were used: amylose (92·0 mg.; 50 ml.), phosphorylase (279 mg.; 13·5 ml.), Z-enzyme (300 mg.; 13·5 ml.), 0·2M-phosphate buffer (pH 6·8), and 0·5M-citrate buffer (pH 6·0). Six digests were prepared as indicated in Table 5 and diluted to 25 ml. with water. Digests 4, 5, and 6 were paired with digests 1, 2, and 3, but the last set contained phosphate buffer in addition. Digests 4, 5, and 6 were used for the detection of α-amylolytic activity in the phosphorylase and Z-enzyme preparations. Fig. 1 shows that α-amylase was absent from both.

TABLE 5.

Digest no.:	1	2	3	4	5	6
	Volume of solution (ml.)					
Amylose	7	7	7	7	7	7
Phosphorylase	3	3	—	3	3	—
Z-Enzyme	—	3	3	—	3	3
Phosphate buffer	8	8	8	—	—	—
Citrate buffer	2	2	2	2	2	2

At intervals portions (2 ml.) of digests 1, 2, and 3 were removed and treated with water (2 ml.) and magnesia reagent [2 ml., containing magnesium chloride (14 g.), ammonium chloride (17 g.), and aqueous ammonia (*d* 0·880; 30 ml.) in 250 ml. of solution]. The precipitated magnesium ammonium phosphate was removed on the centrifuge, and portions of the supernatant liquor (2 ml. each) were used for determination of mineral phosphate present before and after hydrolysis for 7 minutes at 100° in 10% perchloric acid. The difference between these values corresponds to the amount of glucose-1 phosphate formed. Control experiments had shown that glucose-1 phosphate was partly adsorbed by the precipitate, the recovery under the above conditions being 86·6%, and this value was used to correct the experimental data. At the same times, the reducing powers of the solutions were determined by means of the Shaffer–Hartmann reagent (*J. Biol. Chem.*, 1921, **45**, 377) in order to measure the amount of glucose liberated from the

phosphate ester by phosphatase action (see Bailey, Thomas, and Whelan, *Biochem. J.*, 1951, **49**, lvi). The Somogyi reagent (*J. Biol. Chem.*, 1945, **160**, 61) was used in all other determinations of reducing power, but could not be used in this instance because of the presence of the interfering phosphate ion (see Bailey and Whelan, *J.*, 1950, 3573). The amounts of glucose, calculated from reducing power, were assumed to be derived from glucose-1 phosphate and were incorporated in the values representing conversion of the polysaccharide by phosphorylase (Fig. 1). The phosphatase action was slight; in 47·75 hours 1·3% and 3·2% of the polysaccharide was present as glucose in digests 1 and 2 respectively. The changes in iodine stain were determined by treating portions (0·97 ml. ≡ 0·5 mg. of amylose) of the digests with standard iodine solution (0·5 ml.) in 50 ml. of solution containing one drop of 6N-hydrochloric acid, and measuring the A.V. (680 mμ). These concentrations correspond to the standard conditions of A.V. determination used throughout this series.

The phosphorolysis of amylopectin in the presence and in the absence of Z-enzyme was carried out in the same manner, and the numbered digests in Table 6 were of the same composition as the corresponding digests in Table 5 above, amylopectin being substituted for amylose.

TABLE 6. *Phosphorolysis of amylopectin* (B.V., 0·184) *in the presence and the absence of Z-enzyme.*

Digest no. :	1		2		3		4	5	6
Age of digest (hr.)	Conv.,* %	A.V. (680 mμ)	Conv., %	A.V. (680 mμ)	Conv., %	A.V. (680 mμ)	A.V. (680 mμ)	A.V. (680 mμ)	A.V. (680 mμ)
1·5	37·6	0·108	39·5	0·116	0·2	0·184	0·198	0·184	0·185
5·5	45·7	0·102	48·3	0·102	—	0·164	0·172	0·176	0·163
24·25	48·3	0·103	47·1	0·102	—	0·169	0·176	0·175	0·162

* Conv., % = Conversion (%) into glucose-1 phosphate.

It will be noticed that the limiting conversion of the amylopectin into glucose-1 phosphate, namely, 48%, is higher than the previously reported value of 39% (Part III, *J.*, 1949, 1448). The latter agrees with the findings of other workers. The degree of β-amylolysis of the amylopectin (58·8%) was also correspondingly higher than the normal value of 51·5%. This specimen was a sub-fraction of potato amylopectin purified as in Part XIII (*J.*, 1951, 801) and it possesses unusual properties, the significance of which will be discussed in a later paper. The abnormality of the specimen in no way affects the conclusions concerning the lack of action of Z-enzyme on amylopectin.

Action of Stock Soya-bean β-Amylase on Laminarin.—A digest containing laminarin solution (29·9 mg.; 20 ml.), stock soya β-amylase solution (238 mg.; 20 ml.), and water (10 ml.) was incubated at 35° and measurements of reducing power (as glucose) were made at intervals. A control digest without the laminarin was incubated at the same time to determine the reducing power of the enzyme preparation. The results are given in Table 7. After incubation for 175 hours, fresh enzyme solution, containing 150 mg. of enzyme, was added to each digest. No further hydrolysis took place. Similar digests were incubated in which stock soya β-amylase was replaced by crystalline β-amylase in equivalent amount or by freeze-dried salivary α-amylase (2·0 mg.; see above). In neither case did hydrolysis take place during 36 hours.

TABLE 7. *Action of stock soya-bean β-amylase on laminarin.*

Age of digest (hr.)	3	20	44	68	99	142	167
Conversion into glucose (%)	5·9	29·4	47·0	56·5	67·1	72·3	72·3

Action of Z-Enzyme on Laminarin, Cellobiose, and Gentiobiose.—Three digests containing Z-enzyme solution (600 mg.; 5 ml.), with laminarin, cellobiose, or gentiobiose (5 mg. each) in a total volume of 10 ml. were incubated at 35° for 146 hours, whereafter a further quantity of Z-enzyme solution (4·5 ml.; 407 mg.) was added to each digest. After a further 54 hours portions of the digests (1·5 ml.) were removed and after deproteinisation the reducing powers were determined. The conversions of the above substances into glucose were found to be 23·2, 5·3, and 35·4% respectively.

Effect of Z-Enzyme and Emulsin on the Hydrolysis of Amylose by β-Amylase.—The following solutions were used to prepare the digests shown in Table 8. Amylose (B.V. 1·40; 100 mg.; 50 ml.), 0·2M-acetate buffer (pH 4·8), Z-enzyme (152·4 mg.; 7 ml.), emulsin [401 mg. in 0·06M-acetate buffer (pH 4·8; 10 ml.)], and purified soya β-amylase (900 units/ml.). All digests were diluted with water to 25 ml. and incubated at 35°. At intervals, portions (2 ml.) were removed

for determination of reducing power and further portions (1 ml.) for determination of A.V. (680 mμ) under the standard conditions (see Table 9). Digest no. 4 was used for determination of the reducing power of the emulsin preparation.

TABLE 8.

Digest no.	Volume of solution (ml.)				
	Amylose	Buffer	Z-Enzyme	Emulsin	β-Amylase
1	6·25	3	3	—	0·5
2	6·25	2	—	3	0·5
3	6·25	2	3	3	0·5
4	—	2	—	3	0·5

TABLE 9. *Effect of Z-enzyme and emulsin on the β-amylolysis of amylose.*

Age of digest (hr.)	Z-Enzyme (digest no. 1)		Emulsin (digest no. 2)		Z-Enzyme + emulsin (digest no. 3)	
	Conversion into maltose, %	A.V. (680 mμ)	Conversion into maltose, %	A.V. (680 mμ)	Conversion into maltose, %	A.V. (680 mμ)
1	71·9	0·305	86·4	0·117	88·8	0·086
3	83·0	0·185	89·5	0·056	—	—
5·75	87·0	0·117	90·8	0·055	93·5	0·044
11·3	92·8	0·067	93·3	0·060	92·6	0·049
22·75	94·6	0·057	93·7	0·055	94·6	0·045

Effect of Emulsin on the β-Amylolysis of Amylopectin.—Two digests were prepared corresponding to nos. 2 and 4 in Table 8, and a third digest similar to no. 2 but containing 0·06M-acetate buffer (pH 4·8; 3 ml.) instead of emulsin solution. In each digest amylopectin (B.V. 0·196) replaced amylose. All digests were treated as above and the results of the experiment are given in Table 10.

TABLE 10. *Effect of emulsin on the β-amylolysis of amylopectin.*

Age of digest (hr.)	Emulsin + β-amylase		β-Amylase	
	Conversion into maltose, %	A.V. (680 mμ)	Conversion into maltose, %	A.V. (680 mμ)
1·5	47·6	0·118	50·7	0·124
4	48·9	0·118	51·9	0·119
11·5	49·7	—	51·6	0·119
24·75	51·3	0·120	—	—

β-Amylolysis of Emulsin-treated Amylose.—Amylose solution (B.V. 1·40; 25·05 mg.; 12·5 ml.), emulsin solution (92·7 mg.; 3 ml., prepared as above), and water (25 ml.) were mixed and incubated at 35° for 23·5 hours. After this time the B.V. of the amylose had fallen to 1·26, probably owing to partial precipitation of the iodine complex caused by the presence of emulsin protein. No visible retrogradation of amylose occurred in the digest. A similar digest, containing buffer instead of emulsin solution, was also incubated for 23·5 hours. Both digests were heated at 100° for 10 minutes, then cooled, and portions (15 ml.) removed and treated with purified soya β-amylase (500 units; 0·3 ml.) at 35°. Measurements of reducing power and A.V. (680 mμ) were made as in the previous experiment (see Table 11).

TABLE 11. *β-Amylolysis of emulsin-treated amylose.*

Age of digest (hr.)	Untreated amylose		Emulsin-treated amylose	
	Conversion into maltose, %	A.V. (680 mμ)	Conversion into maltose, %	A.V. (680 mμ)
0·5	59·8	0·397	—	—
1	67·7	0·368	79·5	0·173
3	70·0	—	82·2	0·150
5·5	72·0	0·313	—	—
22·5	72·5	—	86·3	0·142

Action of Z-Enzyme in Presence of Mercuric Chloride.—Two digests were prepared containing the following solutions: amylose (B.V. 1·40; 8·86 mg.; 10 ml.), 0·5M-citrate buffer (pH 6·0; 1 ml.), and 0·000154M-mercuric chloride (0·14 ml.). In addition one digest contained Z-enzyme solution (50 mg.; 2·5 ml.). Both digests were diluted to 14·14 ml. and incubated for 32·5

hours at 35°. They were heated at 100° for 10 minutes, then cooled, and the following solutions were added: phosphorylase (9 units; 3 ml.), 0·2M-phosphate buffer (pH 6·8; 7 ml.), and water to 25 ml. Incubation at 35° was then continued, and A.V. (680 mμ) and conversion into glucose-1 phosphate were determined as in earlier experiments. After 39·5 hours, when the arrest points of action had been reached, the A.V. (680 mμ) of the Z-treated amylose was 7·5% of the original value and the conversion into glucose-1 phosphate was 98·9%. The corresponding values for the untreated amylose were 32·0% and 73·4%. It follows that Z-enzyme is not inhibited by mercuric ion.

Effect of Mercuric Chloride on Emulsin.—Two digests of total volume 8·1 ml. were prepared, containing cellobiose (8·1 mg.) and emulsin solution (1·5 ml.), and were incubated at 35°. In addition one digest contained 0·000154M-mercuric chloride (0·08 ml.). The emulsin solution was prepared by dissolving the enzyme powder (300 mg.) in 0·083M-citrate buffer (pH 6·0; 6 ml.) and removing undissolved protein on the centrifuge. Portions (1 ml.) of the digests were used in the measurement of reducing power. In the absence of mercuric chloride the conversions of cellubiose into glucose after 4 and 21·75 hours were 35·6 and 45·4% respectively. In the presence of mercuric chloride the corresponding values were 36·5 and 55·8%. It is concluded that emulsin is not inhibited by mercury.

Effect of Emulsin on the Phosphorolysis of Amylose in the Presence of Mercuric Chloride.—The digest contained the following solutions: amylose (14 mg.; 7 ml.), 0·2M-phosphate buffer (pH 6·8; 8 ml.), phosphorylase (4 units; 1·2 ml.), emulsin (173 mg.; 4 ml.), 0·000154M-mercuric chloride (0·2 ml.), 0·5M-citrate buffer (1 ml.), and water to 25 ml. The temperature of incubation was 35° and after 34 hours a portion (15 ml.) of the digest was removed and mixed with fresh phosphorylase solution (4 units; 1·2 ml.). Measurements were made as before and the results, given in Table 12, confirm that emulsin is not inhibited by mercury and that it has no effect on the phosphorolysis of amylose.

TABLE 12. *Action of emulsin and phosphorylase on amylose in presence of mercuric chloride.*

Age of digest (hr.)	Conversion into glucose-1 phosphate (%)	% of original A.V. (680 mμ)
3·5	37·3	51·0
8·75	59·1	36·2
23·75	65·6	25·5
34	More phosphorylase added	—
48	94·0	7·7

Action of Emulsin on Laminarin.—The digest contained laminarin (25·2 mg.) and emulsin solution (320 mg.; 4 ml.) in a total volume of 25 ml. The temperature of incubation was 35°. and portions (1 ml.) were removed at intervals for measurement of reducing power as glucose (Table 13). A second digest, containing only emulsin solution, was used to determine the reducing power of the enzyme.

TABLE 13. *Hydrolysis of laminarin by emulsin.*

Age of digest (hr.)	3·75	19·25	44·75	123	167	263	331	527	1103
Conversion into glucose (%)	2·4	3·6	6·2	15·8	18·2	28·7	36·1	50·4	72·8

Optimum pH *of Z-Enzyme.*—Amylose (B.V. 1·40; 109·2 mg.) was dissolved in 0·3N-sodium hydroxide (20 ml.), and neutralised with 6N-sulphuric acid. 0·2M-Acetate buffer (pH 4·8; 0·1 ml.) and crystalline β-amylase (1000 units; 1 ml.) were added and the solution was diluted to 100 ml. The digest was incubated at 35° for 2 days and the resulting solution of amylose β-limit dextrin was used in the determination of Z-enzyme activity as described above. The activity was determined at eight different values of pH, which was controlled by the use of 0·13N-sodium veronal buffer (see Fig. 2).

Optimum pH *of Hydrolysis of Laminarin.*—Stock soya-bean β-amylase (315·3 mg.) was shaken with water (30 ml.) and filtered. To portions (3 ml.) of the solution were added portions (3 ml.) of 0·13M-sodium veronal buffer of various hydrogen-ion concentrations. The enzyme buffer mixtures (5 ml.) were incorporated in digests containing laminarin solution (7·47 mg.; 3·75 ml.) and water (3·75 ml.), which were incubated at 35°. After 67·5 hours the reducing power (after deproteinisation) and pH of each digest were determined (see Fig. 2).

Action of Z-Enzyme on Crown-gall Polysaccharide.—The polysaccharide (7·7 mg.) was dissolved in warm water (10 ml.). Z-Enzyme solution (500 mg.; 5 ml.), 0·2M-acetate buffer (pH 4·8; 3 ml.), and water to 25 ml. were added, and the digest was incubated at 35°. After

166 hours the degree of conversion into glucose was 7·4%, and after 185 hours was 8·0%. These figures should be compared with those given earlier for the action of Z-enzyme on laminarin, cellobiose, and gentiobiose.

We thank Professors T. Dillon and E. L. Hirst, F.R.S., for gifts of laminarin, Dr. W. Z. Hassid for the specimen of crown-gall polysaccharide, and Mrs. Olive Owen (Colonial Products Research Council) for technical assistance. We are also grateful to Imperial Chemical Industries Limited for financial assistance and to the Department of Scientific and Industrial Research for a grant to one of us (G. J. T.).

University College of North Wales, Bangor. [*Received, September* 21*st*, 1951.]