129. *The Enzymic Synthesis and Degradation of Starch. Part XVI.* The Purification and Properties of the β-Amylase of Soya Bean.*

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A method is described for the purification of the β-amylase of soya bean, the product of which is amorphous but is virtually free from Z-enzyme as well as from α-amylase and maltase. The elimination of Z-enzyme has been studied in detail and it is shown that for the structural analysis of amylaceous polysaccharides the purified soya-bean β-amylase is exactly equivalent to the crystalline enzyme of sweet potato.

It is established that the amyloses of sago, tapioca, and maize are branched in the same way as potato amylose and that the branch points are attacked by Z-enzyme.

It was shown in Part XV * that soya-bean β-amylase as ordinarily prepared is a mixture of an enzyme similar to the crystalline β-amylase of sweet potato, and Z-enzyme, which supplements the β-amylolysis of amylose. The purpose of the present investigation was to isolate the β-amylase of soya bean in a form free from all other starch-degrading enzymes, and to compare its action with that of the crystalline enzyme from sweet potato

Initial Purification of Soya-bean β-Amylase.—An initial concentrate of β-amylase was prepared by the successive steps summarised in Table 1, namely, (*a*) aqueous extraction of defatted bean flour, (*b*) isoelectric precipitation of inactive protein, (*c*) heat treatment to remove Z-enzyme (see below), (*d*) precipitation of β-amylase by means of acetone (Newton, Naylor, and Hixon, *Cereal Chem.*, 1939, **16**, 71; Meyer, Fischer, and Piguet, *Helv. Chim. Acta*, 1951, **34**, 316), and (*e*) reprecipitation with ammonium sulphate followed by dialysis (Balls, Walden, and Thompson, *J. Biol. Chem.*, 1948, **173**, 9). Precipitation with acetone served to concentrate the enzyme but not to increase its purity and was therefore omitted in later work. The factor of purification from aqueous extract to final concentrate was 5·8. The product contained negligible amounts of α-amylase and maltase and at 0° the dialysed solution retained its activity over several months.

Table 1. *Initial purification of soya-bean β-amylase.*

Operation	Recovery of β-amylase (%)	Units of enzyme/mg. of protein-N
Ground, defatted soya beans (955 g.) extracted for 2 hours with H_2O (4775 ml.). Residue discarded	100	429
pH adjusted to 4·8 with N-H_2SO_4 (82·5 ml.). Ppt. discarded	81	794
Temperature held at 60—61° for 30 minutes. Ppt. discarded	61	1060
pH adjusted to 6·3 with N-aq. NH_3 (50 ml.), solution cooled to 0°, acetone (2 volumes) added, and ppt. dissolved in H_2O (650 ml.)	48	1060
Aq. $(NH_4)_2SO_4$ (saturated; pH 6·0; 2 vols.) added at room temp. Ppt. centrifuged and dissolved in H_2O (120 ml.; 0°). Soln. dialysed against distilled H_2O for 48 hr.	35	2510

Removal of Z-Enzyme.—Z-Enzyme is partly separated from β-amylase when the method of purification given in Table 1, but without the heat treatment, is used. When amylose (B.V. 1·24) was hydrolysed by such a preparation the conversion limit was 75%, compared with 86% for the unpurified enzyme preparation. To achieve complete removal of Z-enzyme from β-amylase, use was made of the fact that Z-enzyme is more heat-labile than β-amylase (see Part XV) and, in order to follow the inactivation of Z-enzyme by this process, a method of measuring its activity was devised. Amylose was hydrolysed to the point of limiting conversion with crystalline β-amylase and to the resulting solution of amylose limit β-dextrin at pH 4·8 (still containing active β-amylase) was added the preparation under test. If the latter contained Z-enzyme, the Z-labile links of the limit β-dextrin were removed and the β-amylase present was enabled to effect further hydrolysis

* Part XV, preceding paper.

and consequent further diminution in the iodine absorption. The effect on amylose limit β-dextrin of the addition of a stock soya-bean preparation is shown in Fig. 1. The experiments to determine the conditions for the removal of Z-enzyme were carried out on a bean preparation after isoelectric precipitation (Table 1). The solution was heated at 63° for varying times and at various pH values, and the amounts of β-amylase and Z-enzyme remaining were estimated. The results show that at pH 5·5 β-amylase and Z-enzyme are equally thermostable.

FIG. 1. *Action of crystalline and stock soya-bean β-amylases on amylose limit β-dextrin at* pH 4·8.

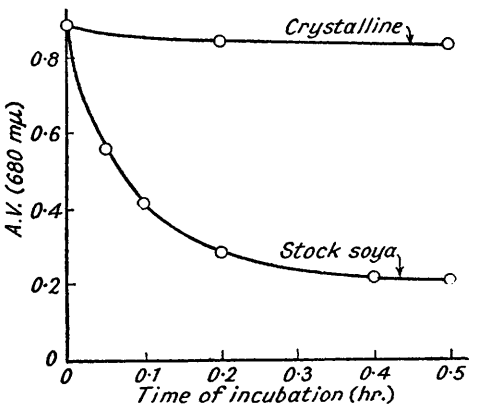


FIG. 2. *Relative heat stabilities of Z-enzyme and β-amylase at* pH 4·8. *Time of heating* = 30 *min.*

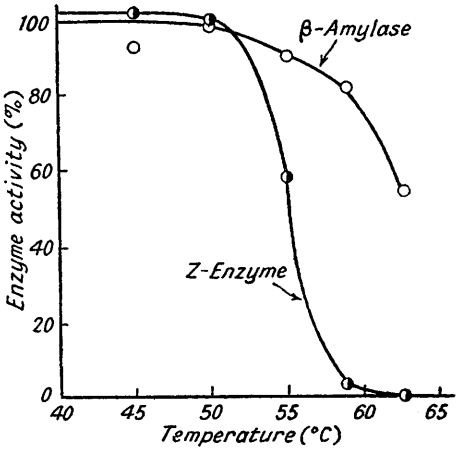


FIG. 3. *Optimum time of heating at* 62° *and* pH 4·8 *for inactivation of Z-enzyme.*

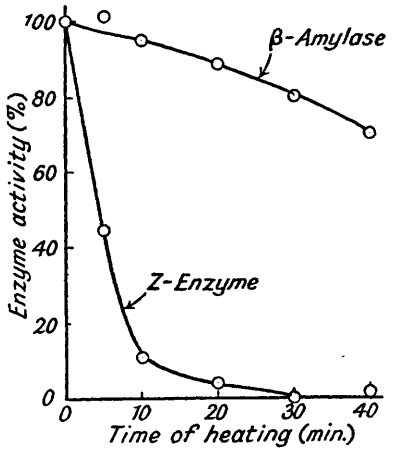
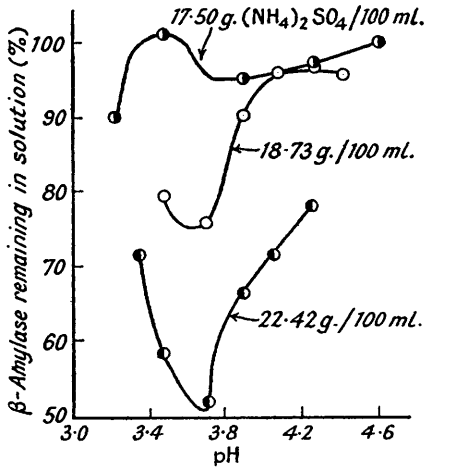


FIG. 4. *The* pH *of minimum solubility of β-amylase in ammonium sulphate solution.*



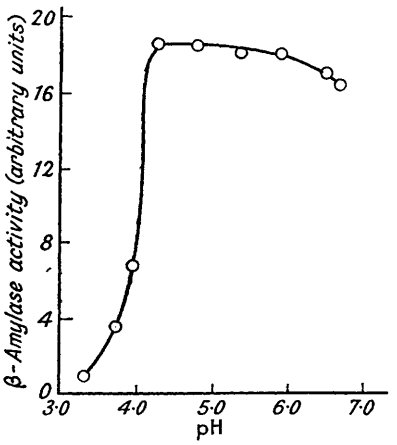
Inactivation of Z-enzyme is therefore best effected at a pH value removed from 5·5, and pH 4·8 was chosen since this is also the pH of the solution after isoelectric precipitation. The optimum temperature (60°) and optimum time (30 minutes) for removal of Z-enzyme were deduced from the data of Figs. 2 and 3 respectively, and these conditions were observed in the large-scale purification.

Balls *et al.* (*loc. cit.*) included a short heat treatment in their method of purification but did not state the reason for doing so. This treatment, it now seems, was of considerable importance in freeing the preparation from Z-enzyme. It was also reported that it reversibly inactivated the sweet potato β-amylase. This was also true of the soya-bean

enzyme before isoelectric precipitation (Table 7, p. 720), but the inactivation was irreversible at a later stage in the purification and under the conditions used for the removal of Z-enzyme.

Final Purification of the β-Amylase Concentrate.—At an early stage in the purification of soya-bean β-amylase an attempt was made to crystallise the enzyme from a concentrated solution under the conditions used for the crystallisation of sweet potato β-amylase, *i.e.*, by ammonium sulphate fractionation at pH 3·6—3·7. As the attempt was unsuccessful the modification was introduced of precipitating the β-amylase at its pH of minimum solubility in ammonium sulphate solution, since this method was applied with success by Distèche in crystallising several of the enzymes in a muscle extract (*Biochim. Biophys. Acta*, 1948, **2**, 265), and by Schwimmer and Balls in the crystallisation of malt α-amylase (*J. Biol. Chem.*, 1949, **179**, 1063). The pH of minimum solubility of soya-bean β-amylase was found to be 3·70 by Distèche's method with ammonium sulphate buffered to constant ionic strength with sodium acetate (Fig. 4). The further purification of the initial concentrate (Table 1) was achieved by repeated precipitation with ammonium sulphate (17·0—27·0 g. per 100 ml.) at pH 3·70. This process did not progressively increase the purity of

FIG. 5. *Optimum* pH *of purified soya-bean β-amylase. Temperature of incubation* = 35°.



the enzyme; the value rose to about 9000 β-amylase units per mg. of protein-nitrogen and thereafter remained constant (Table 2). At first, this appeared to be the upper limit of purification of the soya-bean β-amylase since the same limit was reached independently of variations in the initial purification process or in the range of ammonium sulphate concentration used. A preparation which had attained this limiting value was, however, further fractionated by the addition of ammonium sulphate to the enzyme solution, until a precipitate began to form, followed by cooling to −1·5°. A fraction was thus isolated containing the β-amylase in high yield (74%) and having an activity of 14 900 units per mg. of protein-nitrogen (Table 8, p. 721) representing an overall purification factor of 35. This is the purest preparation obtained in the present work. None of the fractions could be induced to crystallise by methods in which the precipitant (ammonium sulphate or alcohol) was added slowly to a concentrated solution of β-amylase, either by dropwise

TABLE 2. *Fractionation of soya-bean β-amylase with ammonium sulphate at* pH 3·7.

Operation	Limits of $(NH_4)_2SO_4$ concentration (g./100 ml.)	β-Amylase units/ml.	β-Amylase units/mg. of protein-N	Recovery of enzyme (%)
Concentrate prepared as in Table 1 (337 ml.)...	—	10 200	3440	100
Precipitation 1. Ppt. dissolved in H_2O (81 ml.)	16·5—27·0	20 200	5950	75
Precipitation 2. Ppt. dissolved in H_2O (18 ml.)	17·0—24·0	49 000	8100	51
Precipitation 3. Ppt. dissolved in H_2O (9 ml.)	17·5—24·0	83 500	8930	43
As 3	17·5—24·0	55 000	8600	35
As 3	17·5—24·0	61 000	9350	26

addition or by diffusion through a Cellophane membrane. An approximate determination of specific activity carried out on the sample of crystalline β-amylase in our possession gave a value of 41 000 units per mg. of protein-nitrogen. It appears therefore that the soya-bean preparation is still capable of further purification. It was, however, decided at this stage to examine the purified enzyme with respect to its action on starch polysaccharides.

Properties of Purified Soya-bean β-Amylase.—A sample having an activity of 8700 units, prepared as in Tables 1 and 2, was used. The pH dependence of the activity of the enzyme is depicted in Fig. 5. Soya-bean β-amylase exhibits the same broad range of optimum activity as does the sweet-potato enzyme. The action of the enzyme on potato starch and its fractions was studied at pH 4·8 (Table 3). The progress of reaction was followed by means of the A.V. (680 mμ) of the iodine-stained digest determined at intervals until the arrest point was reached, the percentage conversion into maltose being measured at this point. Table 3 shows that the arrest point in the case of amylose is reached within

TABLE 3. *Amylolytic action of purified soya-bean β-amylase at* pH 4·8.

	Amylose (B.V. 1·47)		Potato starch (B.V. 0·470)		Amylopectin (B.V. 0·193)	
Age of digest (hours)	A.V. (680 mμ)	Conversion into maltose, %	A.V. (680 mμ)	Conversion into maltose, %	A.V. (680 mμ)	Conversion into maltose, %
1·0	0·419	—	0·209	—	0·128	—
2·0	0·411	—	0·205	—	0·119	—
3·0	0·425	67·7	0·199	53·0	0·108	51·0
20·0	0·397	—	0·183	54·5	0·119	53·3
44·0	0·362	70·4	—	—	—	—

an hour. The freedom of the sample from Z-enzyme is indicated by the very slow fall in A.V. (680 mμ) after the arrest point has been reached. These results, when compared with those of Figs. 1 and 2 in Part XV (preceding paper) demonstrate the complete parallelism in action between purified soya-bean β-amylase and the crystalline sweet-potato enzyme. The results in Table 4 defined the arrest points of action on potato starch and

TABLE 4. *Amylolytic actions of sweet potato, purified and stock soya-bean preparations.*

	Amylose (B.V. 1·47)		Potato starch (B.V. 0·470)		Amylopectin (B.V. 0·193)	
Enzyme prepn.	A.V. (680 mμ) at arrest point	Conversion into maltose, % at arrest point	A.V. (680 mμ) at arrest point	Conversion into maltose, % at arrest point	A.V. (680 mμ) at arrest point	Conversion into maltose, % at arrest point
Crystalline sweet potato	0·41	68	0·20	53	0·11	52
Purified soya	0·42	68	0·20	52	0·11	51
Stock soya	0·06	97	0·12	61	0·11	53

its components of these two preparations of β-amylase and also of stock soya-bean preparation which contains Z-enzyme in addition. It would seem probable that all β-amylase preparations which bring about the complete conversion of potato amylose will be found to contain Z-enzyme as well as β-amylase.

It may well be that the supposed linear components of other starches also possess these Z-labile anomalous linkages. We have in fact shown that the amyloses of sago, tapioca, and maize are incompletely hydrolysed by pure β-amylase whereas the stock soya-bean enzymes (β-amylase + Z-enzyme) effect almost complete conversions of these samples of amylose (Table 5). Furthermore, the presence of these anomalous linkages may be detected by phosphorylase action. In the following paper (Part XVII), it is shown that potato phosphorylase can, in the absence of Z-enzyme, convert only 70% of amylose into glucose-1 phosphate; in the presence of added Z-enzyme the conversion is almost 100%.

The data given in Table 3 indicate the virtual absence from the purified soya-bean β-amylase of Z-enzyme and α-amylase action at pH 4·8. Maltase was present in traces; approximately 1% of maltose was hydrolysed at pH 4·8 in each 10 hour period of incubation.

TABLE 5. *β-Amylolysis of amyloses from different plant sources.*

Plant source of amylose	Blue value	Purified soya-bean β-amylase: A.V. (680 mμ) at arrest point	Purified soya-bean β-amylase: Conversion into maltose, % at arrest point	Stock soya-bean enzymes: A.V. (680 mμ) at arrest point	Stock soya-bean enzymes: Conversion into maltose, % at arrest point
Potato	1·47	0·42	68	0·06	97
Sago	1·41	0·36	70	0·09	90
Tapioca	1·37	0·33	70	0·07	90
Maize	1·26	0·31	68	0·08	86

The values of percentage conversion, into maltose, of the polysaccharides given in Table 3 are corrected for this maltase activity.

Influence on the Conversion Limit of the Method used to dissolve Amylose.—A series of control experiments were made with a view to determining the influence of various methods of dissolution on the conversion limit of amylose. The four methods chosen were (*a*) dissolution in 0·25N-sodium hydroxide followed by neutralisation (HCl) and buffering to pH 4·8; (*b*), as (*a*) except that acid and buffer were mixed before being added to the alkaline amylose solution; (*c*) dissolution in water at 100°; and (*d*) autoclaving at 126° in unbuffered aqueous solution. The dissolved amyloses were then hydrolysed at pH 4·8 by the purified and the stock soya-bean preparations (Table 6).

TABLE 6. *β-Amylolysis of amylose dissolved by different methods.*

Method of dissolving amylose *	Blue value †	Purified soya-bean β-amylase: A.V. (680 mμ) at arrest point	Purified soya-bean β-amylase: Conversion into maltose, % at arrest point	Stock soya-bean β-amylase: A.V. (680 mμ) at arrest point	Stock soya-bean β-amylase: Conversion into maltose, % at arrest point
(*a*)	1·45	0·371	68·5	0·058	92·0
(*b*)	1·47	0·363	68·0	—	—
(*c*)	1·49	0·420	66·6	0·060	89·0
(*d*)	1·49	0·423	66·4	0·055	88·5

* See text. † After buffering of amylose to pH 4·8 (see Part XV, Experimental section).

It is seen that the method used in dissolving the amylose has but small influence on the amounts of maltose liberated by either purified or stock soya-bean preparations. The possibility that the anomalous linkage in the amylose molecule which arrests the progress of β-amylase is an artifact introduced by alkali treatment is therefore ruled out. That precautions are necessary when using alkali is indicated by the data in Table 9 (p. 722), from which it is seen that prolonged treatment of amylose with alkali at 100° *raises* the conversion limit of amylose attained with the purified enzyme, probably because of alkali-induced fragmentation of the amylose chains. The fall in blue value of the amylose which accompanies the treatment with alkali and the decrease in glucose recoverable on acid hydrolysis are indications of such degradation.

This investigation serves to establish (i) the occurrence in the amylose chains of anomalous structures which impede β-amylolysis, and (ii) the presence in a stock β-amylase preparation of an enzyme (Z-enzyme) which eliminates these barriers. Discussion of the related problems of the nature of these anomalous linkages in amylose and of the action of Z-enzyme upon them is reserved for the paper which follows (Part XVII) since in the latter is described the isolation of Z-enzyme free from both α- and β-amylase.

EXPERIMENTAL

Analytical Methods.—(a) *General.* The methods by which starch fractions have been prepared, together with details of the technique used in β-amylolysis are given in Part XV (preceding paper). Purified soya β-amylase was stored as a suspension in ammonium sulphate solution (pH 3·7; see below), drops of which were diluted with water for measurement of the activity of the solution before use. The enzyme protein did not interfere with measurement of reducing power; deproteinisation was therefore unnecessary.

(b) *Determination of protein-nitrogen.* Soya-bean flour was extracted with water and divided into two portions. One of these was taken to dryness and the nitrogen content was determined by Ma and Zuazaga's method (*Ind. Eng. Chem., Anal.*, 1942, **14**, 280) with the apparatus described by Redemann (*ibid.*, 1939, **11**, 635). The other portion was used to calibrate the biuret reagent of Gornall, Bardawill, and David (*J. Biol. Chem.*, 1949, **177**, 751). From the results of both determinations a curve was plotted relating the colour developed by the biuret reagent to the nitrogen content of the protein. It was confirmed that interference with the colour reagent by ammonium sulphate was suppressed by the addition of sodium hydroxide solution.

(c) *Determination of density of ammonium sulphate solution.* The gradient-tube method of Buchanan and Arfinsen (*ibid.*, 1949, **180**, 47), which requires one drop of the solution under test, was used.

Initial Purification of Soya-bean β-Amylase (Table 1).—(a) *Extraction of soya flour.* Control experiments showed that the maximum yield of β-amylase was achieved by shaking the ground and ether-defatted soya beans (see preceding paper) with water for 2 hours. Extracts prepared by shaking for longer periods showed a progressively diminishing activity.

(b) *Isoelectric precipitation of inactive protein.* An aqueous extract of soya beans was prepared as in (a), the pH of which was 6·3. Portions of this extract were acidified to various pH values with 0·1N-sulphuric acid. The weights of protein precipitate and the β-amylase activity remaining in solution were measured. The results showed that precipitation was optimal at pH 4·8 and that a constant recovery of β-amylase (88%) was obtained at pH values greater than 4·6; at more acid pH values the recovery decreased steadily, being 47% at pH 3. Removal of inactive protein and recovery of β-amylase were therefore both maximal at pH 4·8.

(c) *Elimination of Z-enzyme.* Amylose (B.V. 1·24; 425 mg.) was hydrolysed under standard conditions by crystalline β-amylase at pH 4·8 in a digest of total volume 500 ml. Portions were removed at intervals for the measurement of A.V. (680 mμ) and reducing power. When the conversion limit had been reached the digest was heated on a boiling water-bath for 20 minutes, cooled, and rediluted to 500 ml. with 0·015M-acetate buffer (pH 4·8). The solution of amylose limit β-dextrin so prepared was stored under toluene.

The method of determining Z-enzyme activity comprised the preliminary measurement of the β-amylase activity of the solution under test, adjustment of the concentration of enzyme to 660 units of β-amylase per ml., and the addition of this solution (1 ml.) to the amylose limit dextrin solution (25 ml.) at 35·4°. Measurements of A.V. (680 mμ) were made at intervals by diluting a portion (5 ml.) of the digest to 100 ml. with standard iodine solution (1 ml.), 5N-hydrochloric acid (3 drops), and water. The activity of Z-enzyme was expressed in terms of the fall in A.V. (680 mμ) which occurred during the first 30 minutes' incubation of the above digest. It should be noted that this method of testing for Z-enzyme does not distinguish it from α-amylase. The original aqueous bean extract did not, however, cause any significant degradation of amylopectin limit β-dextrin at pH 4·8, a sensitive test for α-amylase with which Z-enzyme does not interfere. The assumption that, under the above conditions, the hydrolysis of amylose limit β-dextrin is initiated by Z-enzyme is therefore justified.

An aqueous extract of defatted soya flour was purified by isoelectric precipitation, and portions (25 ml.) of this extract were treated by the various methods indicated below and cooled to 15°, and, if necessary, the pH was adjusted to 6·1—6·5. After the mixture had been kept for 3 days at +2°, 0·2M-acetate buffer (pH 4·8) was added to 0·033M-concentration, and any precipitate was discarded. The β-amylase and Z-enzyme activities remaining in solution were then determined and compared with the activities in control portions of the original extract which had been stored for the same time but were otherwise untreated. It was always observed that heat-treated solutions of soya β-amylase caused an immediate and reproducible fall of 0·2 in the A.V. (680 mμ) of the amylose limit β-dextrin. The fall was due to precipitation of the dextrin by protein since part of the blue iodine complex could be filtered off from the iodine solution. The apparent Z-enzyme activity was due entirely to this precipitation of polysaccharide since, after the initial fall, the A.V. (680 mμ) remained constant. Allowance has been made for this factor in calculating the results given in Figs. 2 and 3. The co-precipitation effect was peculiar to enzyme solutions at this stage in the purification. It was not observed with soya β-amylase after final purification. To determine the effect of pH on the enzyme activities portions of the extract were adjusted to various pH values by the addition of either N-sulphuric acid or N-ammonia, and the solutions were heated for different times at 63°. After 15 minutes' heating at pH 4·45, 6·43, and 7·46, Z-enzyme activity had disappeared. Only at pH 5·53 did the Z-enzyme activity persist for more than 30 minutes; and even in this

case it was eliminated after storage at 63° for 50 minutes. The effect of temperature was determined by heating portions of the extract on a water-bath for 30 minutes over a range of temperatures (Fig. 2); the time of heating at 62° was also varied (Fig. 3).

TABLE 7. *Reversible heat inactivation of soya-bean β-amylase.*

Time of heating at 60° (min.)	1	4·7	9·3	15	19·8
Enzyme activity recovered immediately after heating (%)	102	80·5	61	55	56
Enzyme activity recovered after 4 days at 3° (%)	99	98	94	93	96

Heat inactivation of soya-bean β-amylase. An aqueous extract of defatted soya flour was prepared and a portion (150 ml.; pH 6·3) heated on a water-bath at 60°. Portions (25 ml.) of this heated solution were removed at intervals, then cooled rapidly, and the β-amylase activities determined immediately and after storage for 4 days at 3°. The activity of an unheated portion was determined at the same times and the percentage recoveries of the enzyme estimated (Table 7). When a bean extract (pH 4·8), purified by isoelectric precipitation, was heated at 60° for 15 minutes the loss in activity was much less than in the above experiment, being 10% compared with 45%. No regeneration of activity was, however, observed when this solution was stored at 3°.

(d) *Precipitation with acetone.* To portions (10 ml.) of aqueous bean extracts before and after isoelectric precipitation were added varying amounts of acetone up to 65% by volume, the whole operation being conducted at 0°. The suspensions were centrifuged and the precipitates dissolved in water (25 ml.) for estimation of β-amylase activity (see below). The results show that before isoelectric precipitation the enzyme is precipitated at a much lower concentration of acetone. Presumably the enzyme is co-precipitated with the inactive protein removed by isoelectric precipitation.

Concn. of acetone (%)	20	30	40	50	60	70	80	89·5
Enzyme pptd. (%) Before isoelectric pptn.	76	91	87	99	99·5	104	100	99·5
Enzyme pptd. (%) After isoelectric pptn.	3·5	—	26·1	—	92	—	89	—

(e) *Precipitation with ammonium sulphate.* An aqueous extract of soya β-amylase was purified by isoelectric precipitation and acetone precipitation as in Table 1. To portions of this purified extract were added increasing amounts of saturated ammonium sulphate solution (pH 6·0), and after removal of the precipitate the amount of enzyme remaining in solution was determined.

Concn. of $(NH_4)_2SO_4$ (g./100 ml.)	6·4	12·7	19·1	25·4	31·9	37·3
Enzyme pptd. (%)	9·6	10·0	11	15	86	86

(f) *Final method.* The method finally adopted differed in two respects from that given in Table 1. First, lack of adequate refrigeration facilities caused inactivation of β-amylase when the acetone precipitation was carried out on a large scale. This stage was therefore omitted. Secondly, the use of large volumes of ammonium sulphate in the succeeding stage of purification was avoided by adding the required amount of solid ammonium sulphate to the enzyme solution with stirring until dissolved. The modified method may therefore be summarised as follows. Ether-defatted soya flour was shaken with water (5 ml./g. of flour) for 2 hours, octyl alcohol (0·1 ml./l.) being added to prevent foaming. The suspension was centrifuged and to the supernatant solution was added N-sulphuric acid (3·23 ml./100 ml.) to bring the pH to 4·8. The precipitate was removed on the centrifuge and the solution heated at 60—61° for 30 minutes. After cooling, the precipitate was centrifuged and rejected. Ammonium sulphate ("AnalaR"; 41·8 g./100 ml.) was added slowly with stirring. The precipitate was centrifuged and dissolved, 10 ml. of water being used for each 250 ml. of original ammonium sulphate solution. The enzyme solution was dialysed against running distilled water for 36—48 hours, and then stored under toluene in the refrigerator. All operations, except where otherwise stated, were carried out at room temperature. In a typical preparation by this method the overall recovery of β-amylase activity was 78% and the specific activity was 2610 units per mg. of protein-nitrogen (cf. Table 1).

Final Purification of β-Amylase.—(a) *Determination of* pH *of minimum solubility.* The method is based on that of Distèche (*loc. cit.*). Ammonium sulphate solutions containing acetate buffer of ionic strength 0·0725 were prepared, the amounts of acetic acid and sodium acetate required being calculated from the nomogram prepared by Boyd (*J. Amer. Chem. Soc.*,

1945, **67**, 1035). The pH values of portions diluted to half strength were determined. A solution of soya β-amylase was prepared by the process described above. To portions (4 ml.), contained in 10-ml. centrifuge tubes at 20°, were added with stirring portions (4 ml.) of the buffered ammonium sulphate solutions. The precipitates were removed on the centrifuge and the activity of β-amylase remaining in solution was determined. By comparison with the initial enzyme activity the amount of enzyme in the precipitates was calculated. It was verified that the pH of the ammonium sulphate solution did not change on dilution with the enzyme. The curves depicting the amounts of β-amylase remaining in solution at various values of pH and ammonium sulphate concentration are given in Fig. 4, which shows that the pH of minimum solubility is 3·7.

(b) *Fractional precipitation of β-amylase.* A solution of pH 3·70 was prepared containing ammonium sulphate (490 g.), hydrated sodium acetate (9·86 g.), and glacial acetic acid (29·2 ml.) per litre. This was used as the precipitant for β-amylase in the fractionation procedure outlined in Table 2. To the enzyme solution was added ammonium sulphate to the lower limit of concentration given in Table 2, any precipitate being rejected. The enzyme was precipitated by the further addition of precipitant to the stated concentration. The precipitant was always added dropwise, with stirring, during 30 minutes, at 0—5°. The precipitated β-amylase was centrifuged immediately and redissolved in the minimum of ice-cold water. The concentration of ammonium sulphate, arising from that occluded on the precipitate, was determined by the gradient-tube method. Control experiments had shown that at the first and second precipitations by this method the enzyme began to appear in the precipitate at 17 g. of ammonium sulphate/100 ml. and precipitation was complete at 27 g./100 ml. The following results relate to a once-precipitated enzyme.

Concn. of $(NH_4)_2SO_4$ (g./100 ml.)	16	17·5	19	22	24	26
Enzyme pptd. (% of initial activity)	5	8	17	49	73	91·5

(c) *Fractionation by cooling a saturated enzyme solution.* The end product of the fractionation procedure given in Table 2 was sub-fractionated as follows. The precipitate was dissolved in water (5 ml.) at 0° and a small insoluble residue removed in a centrifuge. A turbid solution was obtained which did not become clear on addition of water (4 ml.). The ammonium sulphate concentration was now 11·3 g./100 ml. The temperature of the solution was raised to 9° and buffered ammonium sulphate solution added with stirring until a permanent precipitate was produced. The solution was cooled slowly to −1·5° and after 24 hours the precipitate was centrifuged, yielding a clear solution which was further fractionated in the same manner. Four precipitated fractions and a supernatant solution were obtained which were analysed for β-amylase and protein content (Table 8).

TABLE 8. *Sub-fractionation of soya-bean β-amylase.*

Approx. limits of $(NH_4)_2SO_4$ concn. (g./100 ml.)	Precipitate	
	Units of enzyme/mg. of protein-N	% of original activity
11—16	4580	4
16—18	3970	3
18—19	4480	3
19—21	6850	16
>21	14 900 (supernatant)	74 (supernatant)

Optimum pH of Purified Soya-bean β-Amylase.—The activity of the enzyme was determined in the presence of 0·2M-sodium acetate–acetic acid buffers. The total volume of the activity digest was 20·4 ml., containing 2·4 ml. of buffer and other reagents in proportion to the standard amount (Fig. 5).

Action of Purified Soya-bean β-Amylase on Starch Polysaccharides.—The technique was identical with that adopted for crystalline β-amylase (see Part XV, preceding paper). The sample of enzyme used was prepared by the above method of initial purification and was refractionated as in Table 2.

Comparison of Methods of Dissolving Amylose.—The same sample of amylose (B.V. 1·49) was used in the following experiments. (*a*) Amylose (71·3 mg.) was dissolved in the usual way in 0·25N-sodium hydroxide (20 ml.), cooled, and diluted to 50 ml. A portion (25 ml.) was neutralised with N-hydrochloric acid (phenolphthalein), and 0·2M-acetate buffer (pH 4·8; 6 ml.) was added, followed by water to 50 ml. To the remainder of the amylose solution the acid, indicator, and buffer were added as a mixture before dilution to 50 ml. The amyloses were then

submitted to hydrolysis by the stock and the purified preparation of soya-bean β-amylase. The arrest points, attained in 2 hours, are given in Table 6. (*b*) Amylose (66·9 mg.) was wetted with benzene (0·45 ml.) and dissolved in water (60 ml.) on a boiling water-bath. After 10 minutes' heating the amylose had dissolved to give a faintly turbid solution; the heating was continued for a further 20 minutes. The solution was diluted to 100 ml. with water and acetate buffer to 0·024M-concentration and the amylose was then hydrolysed as in (*a*). (*c*) An amylose solution (pH 6·7), prepared as in (*b*), was placed in an autoclave which was raised to 20 lb./sq. in. pressure (126°) in 25 minutes and kept at this pressure for 30 minutes. After cooling, the solution was treated as in (*b*) (see Table 6).

Action of Alkali on Amylose.—Amylose (B.V. 1·49; 111 mg.) was dissolved in water (45 ml.) at 100° as under (*b*) above, cooled, and diluted to 50 ml. Portions (4·5 ml.) were added to 10-ml. flasks, followed by 2N-sodium hydroxide (0·645 ml.), giving a final concentration of 0·25N-alkali. The flasks were heated on a boiling water-bath for varying times and then cooled rapidly. The solutions were neutralised (N-hydrochloric acid); 0·2M-acetate buffer (pH 4·8; 1·2 ml.) and water to 10 ml. were then added. A portion (1 ml.) was used for determination of B.V. and the remainder for incubation with purified soya β-amylase, the arrest points, attained within 2 hours, being determined (see Table 9).

TABLE 9. *Treatment of amylose with* 0·25N-*sodium hydroxide at* 100°.

Time of heating in alkali (min.)	B.V.	Conversion into maltose (%) at arrest point	A.V. (680 mμ)	Polysaccharide remaining * (%)
0	1·47	63·5	0·420	100
1·5	1·45	66·5	—	—
3	1·45	67·2	0·369	—
5	1·48	67·8	0·350	100·9
8	1·45	69·1	0·325	—
10	—	—	—	98·1
12	1·37	72·7	0·220	—
15	—	—	—	93·2
17	1·35	74·0	0·170	—
20	—	—	—	95·8
30	—	—	—	88·2
40·3	—	—	—	82·5

* See Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.

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