Purification and Characterization of a β -Amylase from Soya Beans

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1. β -Amylase obtained by acidic extraction of soya-bean flour was purified by ammonium sulphate precipitation, followed by chromatography on calcium phosphate, diethylaminoethylcellulose, Sephadex G-25 and carboxymethylcellulose. 2. The homogeneity of the pure enzyme was established by criteria such as ultracentrifugation and electrophoresis on paper and in polyacrylamide gel. 3. The pure enzyme had a nitrogen content of 16.3%, its extinction coefficient, $E_{1\text{cm}}^{1\%}$, at 280 m μ was 17.3 and its specific activity/mg. of enzyme was 880 amylase units. 4. The molecular weight of the pure enzyme was determined as 61700 and its isoelectric point was pH 5.85. 5. Preliminary examinations indicated that glutamic acid formed the *N*-terminus and glycine the *C*-terminus. 6. The amino acid content of the pure enzyme was established, one molecule consisting of 617 amino acid residues. 7. The pH optimum for pure soya-bean β -amylase is in the range 5–6. Pretreatment of the enzyme at pH 3–5 decreases enzyme activity, whereas at pH 6–9 it is not affected.

The presence of amylase in soya beans has long been recognized (Stingl & Morawski, 1886; Street & Bailey, 1915). Newton & Naylor (1939) and Newton, Hixon & Naylor (1943) showed that the principal amylolytic activity of soya bean was β -amylase. The purification of soya-bean β -amylase free from Z-enzyme [an α -amylase according to Banks, Greenwood & Jones (1960) and Cunningham, Manners & Wright (1962)] by conventional ammonium sulphate precipitation methods has been reported by Peat, Pirt & Whelan (1952) and by Fukumoto & Tsujisaka (1954, 1955). As yet the pure enzyme has not been characterized with respect to composition, molecular weight or physico-chemical properties.

While purifying a trypsin inhibitor from soya beans on a CM-cellulose column, Birk, Gertler & Khalef (1963) separated a highly active soya-bean amylase. The present paper is devoted to purification of soya-bean β -amylase by successive fractionation on a calcium phosphate (hydroxyapatite) column and by anion- and cation-exchange chromatography, and to the characterization of the pure enzyme.

EXPERIMENTAL

Assay methods

Assay of amylase activity. The amylase activity was measured by the method of Bernfeld (1955) for the assay of sweet-potato β -amylase with dinitrosalicylic acid reagent, with these modifications: the substrate was dissolved in 0.2 M- instead of 0.016 M-buffer, and the reaction was

performed at 37° instead of 20° and at pH 5.5 instead of 4.8. The extinction of the solution containing the brown reduction product was determined at 550 m μ . One amylase unit (AU) was defined as 1 mg. of maltose liberated in the reaction mixture under the conditions described above.

Assay of accompanying activities. Trypsin- and α -chymotrypsin-inhibiting activity was determined by the casein-digestion method (Kunitz, 1947) and expressed in T.I.U. and Ch.I.U. respectively (Birk *et al.* 1963).

Phosphatase activity was determined by the following modification of the method of Garen & Levinthal (1960): 1 ml. of enzyme solution was added to 1 ml. of 3 mM-*p*-nitrophenyl disodium phosphate in 0.2 M-acetate buffer, pH 5.0. After 30 min. incubation at 37° the reaction was terminated by the addition of 1 ml. of 0.2 N-NaOH. The extinction of the reaction mixture was measured at 410 m μ with a Bausch and Lomb Spectronic 20 spectrophotometer. One unit of activity was defined as the amount of enzyme that causes an increase of 0.001 in the extinction/min.

Lipoxidase activity was assayed by the spectrophotometric method as described in the Worthington Biochemical Corp. (Freehold, N. J., U.S.A.) Descriptive Manual no. 11 (1961) (p. 31), and haemagglutinin as described by Liener (1955).

Urease activity was determined by the method of Sumner (1955) with the following modification: 1 ml. of enzyme was added to 1 ml. of 3% (w/v) urea in 0.01 M-phosphate buffer, pH 6.9, and the reaction mixture was incubated for 5 min. at 25°; 8 ml. of water and 0.5 ml. of Nessler's reagent were then added and the extinction was measured, after 15 min., at 480 m μ in a Bausch and Lomb Spectronic 20 spectrophotometer in a tube of 2.235 cm. path length.

The presence of Z-enzyme in fractions containing the bulk of β -amylase activity was detected by employing 1 unit of β -amylase as source of Z-enzyme for the hydrolysis of 1 ml. of 0.5% β -amyopectin limit-dextrin, prepared

according to the method of Applebaum (1964). The possible amylolytic activity was measured, after 72 hr. of incubation, by the method of Bernfeld (1955) and was expressed as mg. of maltose liberated from 100 mg. of substrate.

Determination of protein. Protein content was estimated by determining $E_{280\text{m}\mu}$. A protein solution giving $E_{280\text{m}\mu}^{1\text{cm.}}$ 1.00 was defined to possess one extinction unit (EU)/ml.

Isolation of the enzyme

Step 1: preparation of acidic extract. A sample (100g.) of ether-extracted soya-bean flour (Lincoln variety) was suspended in 1l. of 0.03N-HCl at room temperature, yielding pH 4.6–4.7, as measured in the suspension. Constant stirring was employed for 1 hr. and the suspension was centrifuged for 10 min. at 2000 rev./min. (1000g) in an International model V centrifuge. The clear supernatant, amounting to 800 ml., was collected and designated 'acidic extract'.

Step 2: fractionation with ammonium sulphate. To the acidic extract was added, with constant stirring, solid $(\text{NH}_4)_2\text{SO}_4$ (19.5g./100 ml. of soln.) to give 33% saturation. The precipitate was centrifuged and discarded. To the supernatant was added further solid $(\text{NH}_4)_2\text{SO}_4$ (19.5g./100 ml. of soln.) to give 60% saturation. The resulting precipitate was separated by centrifugation, dissolved in 200 ml. of water and designated 'ammonium sulphate solution'.

Step 3: dialysis. The ammonium sulphate solution was dialysed for 48 hr. against water at 5°. A precipitate, with very low amylolytic activity, that appeared during dialysis was removed by centrifugation. The clear supernatant, amounting to 230 ml., was designated 'non-diffusible extract'.

Step 4: fractionation on a calcium phosphate column. Calcium phosphate (hydroxyapatite) was prepared according to the method of Tiselius, Hjerten & Levin (1956). Small particles were removed by decantation at the different stages of adsorbent preparation to permit a fast rate of flow during the fractionation process. The calcium phosphate was poured into a column (3.6 cm. diam.) to the height of about 18 cm. The column was then washed with 2l. of 0.004M-phosphate buffer, pH 6.8. To 230 ml. of the non-diffusible preparation 0.8 ml. of 1.0M-phosphate buffer, pH 6.8, was added and the solution was applied to the column. Elution was performed with 700 ml. of 0.004M-phosphate buffer, pH 6.8, followed by stepwise increases in buffer concentration, namely 800 ml. of 0.028M-, 500 ml. of 0.06M- and finally 500 ml. of 0.5M-phosphate buffer. The rate of flow was 300–500 ml./hr. and 8 ml. fractions were collected. Every fifth fraction was assayed for amylase activity, and occasionally for accompanying activities. The successive eluate fractions corresponding to the bulk of amylolytic activity were designated 'calcium phosphate effluent'.

Step 5: anion-exchange chromatography. DEAE-cellulose (Carl Schleicher and Schüll) was equilibrated with 0.025M-phosphate buffer, pH 6.8 (the starting buffer). The suspension was poured into a column (1.7 cm. $\times$ 35 cm.) and allowed to settle under gravity. Calcium phosphate effluent (the combined contents of tubes 152–196 from the calcium phosphate column; see Fig. 1a) was applied to the column. Elution was primarily performed with the starting buffer and then with a gradient of NaCl by introducing 0.4M-NaCl

in the same buffer, through a 500 ml. mixing chamber containing the starting buffer. The rate of flow was adjusted to 200–250 ml./hr. and 8 ml. fractions were collected. Every second tube was assayed for protein content, and every third tube for amylolytic activity. Possible accompanying activities were assayed in the amylase-containing peak. The eluate corresponding to the peak with the bulk of amylolytic activity was designated 'DEAE-cellulose effluent'.

Step 6: desalting by gel filtration. DEAE-cellulose effluent (the combined contents of tubes 115–133 from the DEAE-cellulose column; see Fig. 1b) was applied to a Sephadex G-25 (A.B. Pharmacia, Uppsala, Sweden) column (4 cm. $\times$ 35 cm.), previously washed with water. Elution was performed with water and 8 ml. fractions were collected. The NaCl content of the effluent fractions was determined by titration with AgNO_3 , with K_2CrO_4 as indicator.

Step 7: cation-exchange chromatography. CM-cellulose (Cellex-CM; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) was equilibrated with 0.01M-phosphate-citrate buffer, pH 4.2 (starting buffer), and then poured into a column (1.7 cm. diam.) to the height of 13 cm. The desalted effluent from the Sephadex column (contents of tubes 27–41; see Fig. 1c) was brought to the pH and ionic strength of the starting buffer by adjusting the effluent with 0.4M-phosphate-citrate buffer, pH 4.2 (composed of 0.4M- Na_2HPO_4 and 0.2M-citric acid), and with 0.2M-citric acid to pH 4.2. The solution was applied to the CM-cellulose column and elution was performed by the starting buffer until the extinction of the eluate began to return to the base-line value. When this occurred, a gradient with respect to buffer concentration was started by introducing 0.4M-phosphate-citrate buffer, pH 4.2, through a 300 ml. mixing chamber containing the starting buffer. The flow rate was adjusted to 100–120 ml./hr. and 8 ml. fractions were collected. Each tube was assayed for protein content, and every third tube for amylolytic activity. The eluate corresponding to the peak with the bulk of amylase activity (contents of tubes 45–55; see Fig. 1d) was desalted on a Sephadex G-25 column, as described in step 6. The desalted effluent was designated 'CM-cellulose effluent'.

The purification achieved by these operations is summarized in Table 1.

The pure enzyme solution was finally concentrated by applying the CM-cellulose effluent to a small column (0.8 cm. $\times$ 4 cm.) of DEAE-cellulose, equilibrated with 0.025M-phosphate buffer, pH 6.8, and eluting the enzyme with 0.4M-NaCl in the same buffer. The effluent was then desalted by exclusion chromatography on a Sephadex G-25 column (1.7 cm. $\times$ 20 cm.) and kept either as a solution in the presence of $(\text{NH}_4)_2\text{SO}_4$ (66% saturation) or as a freeze-dried preparation.

The same purification procedure, throughout steps 1–7, was repeated in the presence of mercaptoethanol (1 mM). No differences of yield or activity were noticed between the two preparations, but the electrophoretic properties of the protein were slightly altered (see Fig. 3).

Characterization of the pure enzyme (CM-cellulose effluent)

Nitrogen content. Total N was determined by the micro-Kjeldahl procedure.

Specific extinction. $E_{1\text{cm}}^{1\%}$ was determined at 280 m μ .

Ultracentrifugational analyses. Measurements of sedimentation velocity were made at five 8-min. intervals in a Spinco model E ultracentrifuge, equipped with a phase-plate schlieren optical system, 12 mm. cell, operating at 59 780 rev./min. at 23°. The solvent employed was a solution of 0.1 M-NaCl in 0.01 M-phosphate buffer, pH 6.8. Three different concentrations of the enzyme (0.15, 0.3 and 0.6%) were examined. Measurements of diffusion were performed in the same Spinco model E ultracentrifuge with solutions containing 0.6, 0.3 and 0.15 g. of enzyme/100 ml. of the solvent employed for sedimentation measurements.

Paper electrophoresis. Samples of amylase were subjected to electrophoresis on Whatman 3MM paper in 0.07 M-collidine-acetate buffer (8.9 ml. of collidine, 3.06 ml. of acetic acid, and water to make the volume 1 l.), pH 6.9, with a potential gradient of 11 v/cm. for 18 hr. at 5°.

Electrophoresis in polyacrylamide gel. Samples (30, 60 and 100 μ g.) were subjected to disk electrophoresis in polyacrylamide gels (Ornstein & Davis, 1961; Reisfeld, Lewis & Williams, 1962). 0.35 M- β -Alanine-acetate, pH 4.5, or 0.37 M-glycine-tris, pH 8.9, were used as buffers and electrophoresis was carried out for 90–180 min. at approx. 100 v with a current of 3.5 mA/tube (7 cm. $\times$ 0.5 cm.). The gels were then stained with Amido Black 10 B.

Isoelectric point. The isoelectric point was determined from the electrophoretic mobilities of the enzyme as a function of pH. Paper electrophoresis was performed on Whatman 3MM paper in 0.07 M-collidine-acetate buffer, pH 5.2, 6.4, 6.9, 7.2 and 8.2, in a potential gradient of 11 v/cm. for 18 hr. at 5°.

Amino acid analysis. A sample of 5 mg. of the pure freeze-dried enzyme was hydrolysed in a sealed evacuated tube with 0.5 ml. of 5.7 N-HCl at 110° for 22 hr. and the

Fig. 1. Purification procedure of soya-bean β -amylase by successive column chromatographies. (a) Chromatography of 230 ml. of non-diffusible extract in 0.004 M-phosphate buffer, pH 6.8, on a calcium phosphate column (3.6 cm. $\times$ 18 cm.). Elution was performed successively with: A, 700 ml. of 0.004 M-phosphate buffer, pH 6.8; B, 800 ml. of 0.028 M-phosphate buffer, pH 6.8; C, 500 ml. of 0.06 M-phosphate buffer, pH 6.8; D, 500 ml. of 0.5 M-phosphate buffer, pH 6.8. The rate of flow was 300–500 ml./hr. and 8 ml. fractions were collected. (b) Chromatography of calcium phosphate effluent, the shaded area from procedure (a), on a DEAE-cellulose column (1.7 cm. $\times$ 35 cm.) equilibrated with 0.025 M-phosphate buffer, pH 6.8 (the starting buffer). Elution was first performed with the starting buffer and then with a gradient of NaCl concentration (E) by introducing 0.4 M-NaCl in the same buffer through a 500 ml. mixing flask containing the starting buffer. The rate of flow was 200–250 ml./hr. and 8 ml. fractions were collected. (c) Desalting of DEAE-cellulose effluent, the shaded area from (b), on a Sephadex G-25 column (4 cm. $\times$ 35 cm.). Elution was performed with water and 8 ml. fractions were collected. (d) Chromatography of the desalted effluent, the shaded area from (c), on a CM-cellulose column (1.7 cm. $\times$ 13 cm.) equilibrated with 0.01 M-phosphate-citrate buffer, pH 4.2 (the starting buffer). Elution was first performed with the starting buffer and then with a gradient of buffer concentration (F) by introducing 0.4 M-phosphate-citrate buffer, pH 4.2, through a 300 ml. mixing flask containing the starting buffer. The rate of flow was 100–120 ml./hr. and 8 ml. fractions were collected.

hydrolysates were analysed according to the method of Spackman, Stein & Moore (1958) in a Beckman amino acid analyser. Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952) and also by 'procedure L' described by Spies & Chambers (1949).

End-group analyses. An examination of the *N*- and *C*-termini was performed by the 1-fluoro-2,4-dinitrobenzene method (Sanger, 1945; Levy, 1954) and by hydrazinolysis (Akabori, Ohno & Narita, 1952; Niu & Fraenkel-Conrat, 1955).

Determination of thiol groups. Free thiol groups were determined by two different spectrophotometric titration methods. The method of Boyer (1954), with *p*-chloromercuribenzoate, as described by Benesch & Benesch (1962), was employed on the enzyme solution in 0.02 *M*-phosphate buffer, pH 6.8. The second method was that of Ellman (1959), with 5,5'-dithiobis-(2-nitrobenzoic acid), i.e. bis-3-carboxy-4-nitrophenyl disulphide, as titrating agent.

Effect of pH on enzymic activity. The β -amylase activity was determined in the range pH 3-9 by using 0.1 *M*-phosphate-citrate buffer for pH 3.0-7.0 and 0.1 *M*-phosphate buffer for pH 7.0-9.0. To study the effect of pH on the stability of the enzyme, samples of the enzyme were kept in solution at pH 3-9, at 37°, for different times before enzymic reaction.

RESULTS AND DISCUSSION

Isolation and purification of the enzyme

The fractionation procedure on calcium phosphate, DEAE-cellulose, Sephadex G-25, and CM-cellulose columns successively (steps 4, 5, 6 and 7) is illustrated in Fig. 1.

The small peak formed during the gel filtration (Fig. 1c) possessed only low specific β -amylase activity and was therefore discarded.

The whole purification procedure of soya-bean β -amylase from the acidic extract (as starting material) through the various steps of fractionation is summarized in Table 1.

Table 1 shows that, although the β -amylase in DEAE-cellulose effluent is about 90% pure, it is still accompanied by small amounts of trypsin inhibitor and phosphatase and traces of lipoxidase. The trypsin inhibitor and lipoxidase are completely removed by the CM-cellulose chromatography and only traces of phosphatase accompany the pure β -amylase. These traces can be removed by several successive rechromatographies on CM-cellulose, which result, however, in a decreased recovery of the β -amylase. The final β -amylase preparation was completely free of urease activity but was still accompanied by traces of Z-enzyme.

However, when we assayed the purest preparation of sweet-potato β -amylase (3 $\times$ crystallized; Worthington Biochemical Corp.) on β -amylopectin limit-dextrin as substrate it was found to contain traces of Z-enzyme to the same extent. Further, when the two preparations were assayed in the presence of 0.01 *M*-calcium chloride they were activated to

Table 1. *Yield, potency and accompanying activities of various fractions obtained from procedure for isolation of soya-bean β -amylase*

Fraction	Vol. (ml.)	<i>E</i> ₂₈₀	EU	Specific activity (AU/EU)	Total activity (AU)	Yield (%)	Accompanying activities (units/EU)			
							Trypsin inhibitor	Acid phosphatase	Lipoxidase	Haemagglutinin Z-enzyme
Acidic extract	800	19.500	15 600	8.8	137 280	100.0	0.32	226	1100	144.5
Ammonium sulphate solution	200	29.500	5900	21.6	127 440	92.8	0.42	316	4330	261.0
Non-diffusible extract	230	6.800	1 564	71.5	118 260	86.1	0.92	765	1710	388.0
Calcium phosphate effluent	330	0.830	274	325.0	89 050	64.9	1.06	2000	160	0
DEAE-cellulose effluent	130	0.820	107	480.0	51 360	37.4	0.51	2400	35	0
CM-cellulose effluent	105	0.570	60	510.0*	30 600	22.3	0	270	0	0

* The specific activity of the final preparation/mg. of enzyme was calculated, on the basis of the determined specific extinction, as 880 amylase units.

the same extent, thus agreeing with the findings of Cunningham *et al.* (1962) that Z-enzyme is an α -amylase.

Dialysis or freeze-drying of the pure enzyme resulted in a considerable loss in activity and solubility of the preparation. The process of desalting the eluates by exclusion chromatography on Sephadex G-25 was therefore a matter of necessity rather than of mere convenience.

The final preparation was kept as a concentrated solution with ammonium sulphate (66% saturation) and retained its full potency for at least 3 months.

The use of 1 mM-mercaptoethanol throughout the isolation and purification procedures did not affect at all the chromatographic patterns or the specific activities of the peaks with the bulk of β -amylase activity.

Homogeneity studies

The purity of the final enzyme preparation (CM-cellulose effluent), which seemed to be pure by criteria such as adsorption and anion- and cation-exchange chromatography, absence of accompanying activities, and a constant specific activity throughout the β -amylase peak, was further substantiated by the following tests.

Sedimentation and diffusion. Measurements of the sedimentation velocity of the pure enzyme (CM-cellulose effluent) at five 8 min. intervals showed a single symmetrical boundary (Fig. 2).

Sedimentation measurements were taken at three different enzyme concentrations and $S_{20,w}$, calculated by extrapolation, was 4.67 s. The mean diffusion coefficient, $D_{20,w}$, was determined to be $7.47 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. These sedimentation and diffusion coefficients, together with an assumed partial specific volume of 0.75 ml./g., indicate that the pure enzyme had a molecular weight of $61\,700 \pm 2130$.

Electrophoresis on paper and in polyacrylamide gel. The paper-electrophoretic analysis of the pure enzyme (CM-cellulose effluent) in collidine-acetate buffer, pH 6.9, indicated a main band accompanied by a 'shadow'. Since this 'shadow' completely

disappeared when the enzyme was purified in the constant presence of mercaptoethanol and since no difference in enzymic activity was noticed between the main band and its 'shadow', it may perhaps be assumed that the 'shadow' is a result of some conformational change of the molecule at a non-essential thiol group rather than an indication of charge difference. When the enzyme was subjected to disk electrophoresis in polyacrylamide gel at pH 4.5 it migrated as a single well-defined band (Fig. 3). When subjected to the same technique at pH 8.9, the main band was found to be accompanied by a 'shadow', similar to that which occurred in the paper-electrophoretic analysis under similar pH conditions; this 'shadow' was much smaller in the enzyme prepared in the presence of mercaptoethanol (Fig. 3).

Properties

Nitrogen content and specific extinction. The pure enzyme had a nitrogen content of 16.3%, and $E_{1\text{cm}}^{1\%}$ at 280 m μ was 17.3.

Fig. 3. Disk electrophoresis of soya-bean β -amylase (60 $\mu\text{g./tube}$) in 15% polyacrylamide gel at: (a) pH 4.5 with a current of 3.5 mA/tube for 3 hr.; (b) pH 8.9 with a current of 3.5 mA/tube for 1 hr.; (c) as for (b) but with the enzyme prepared in the presence of mercaptoethanol (1 mM).

Fig. 2. Sedimentation pattern of soya-bean β -amylase. Sedimentation is from right to left. The photographs were taken at 8 min. intervals. Sedimentation was performed in a Spinco model E ultracentrifuge operating at 59780 rev./min. The protein concentration was 6 mg./ml., the temperature 23° and the pH 6.8.

Table 2. *Amino acid composition of β -amylase*

Values are given in molar ratios; the molecular weight of β -amylase was taken to be 61700.

Constituent	Composition at 22 hr. of hydrolysis	No. of residues (to nearest integer)
Lysine	31.7	32
Histidine	11.6	12
Ammonia	62.1	62
Arginine	20.2	20
Aspartic acid	83.2	83
Threonine	22.7	23
Serine*	30.0	33
Glutamic acid	70.2	70
Proline	40.2	40
Glycine	52.8	53
Alanine	42.4	42
Cystine (half)	5.3	5
Valine	38.4	38
Methionine	9.3	9
Isoleucine	30.7	31
Leucine	61.6	62
Tyrosine	30.1	30
Phenylalanine	21.7	22
Tryptophan†	11.9	12
Total residues‡ ...		617

* Values corrected for 10% loss during hydrolysis.

† Estimated spectrophotometrically.

‡ Ammonia was not included.

Isoelectric point. This was calculated to be pH 5.85 from the plot of electrophoretic mobility, in 0.07 M-collidine-acetate buffer, against pH in the range 5.2–8.2.

Terminal group and amino acid analyses. A preliminary examination of the *N*- and *C*-termini indicated the presence of glutamic acid as the only *N*-terminal residue and glycine at the *C*-terminus. Table 2 shows the amino acid composition of a 22 hr. hydrolysate of the enzyme. One molecule of soya-bean β -amylase contains 617 amino acid residues.

The molecular weight of this enzyme, calculated on the basis of residual weights of the amino acids, was 69100, whereas that reported for sweet-potato β -amylase was 152000 (Englard & Singer, 1950).

Thiol groups. Five thiol groups were found/enzyme molecule, by using *p*-chloromercuribenzoate as titrating agent. Completion of the titration as well as almost complete loss of activity (99%) were achieved immediately. This number of thiol groups/molecule is in close agreement with the cysteine content of the enzyme. With the 5,5'-dithiobis-(2-nitrobenzoic acid) reagent only one thiol group was found/enzyme molecule; this reaction was completed after more than 1 hr. and

Table 3. *Effect of pH, and of pretreatment of the enzyme with buffers of different pH for 0–150 min., on enzyme activity.*

Enzyme activity is expressed in mg. of maltose liberated during 3 min. reaction by soya-bean β -amylase kept for the times indicated in buffer of the appropriate pH.

pH	Time (min.)...	Enzyme activity			
		0	30	75	150
3.0		0.35	0.11	0.05	0.03
4.0		0.53	0.35	0.32	0.31
5.0		0.64	0.65	0.66	0.66
6.0		0.64	0.66	0.66	0.65
7.0		0.54	0.55	0.52	0.54
8.0		0.30	0.29	0.30	0.31
9.0		0.10	0.11	0.11	0.10
10.0		None	—	—	—

no loss of enzyme activity was noticed even when excess of reagent was employed. It may therefore be assumed that at least one thiol group/molecule is not essential for enzyme activity and that the other four thiol groups of the enzyme molecule are not accessible to the 5,5'-dithiobis-(2-nitrobenzoic acid) reagent.

Effect of pH on enzyme activity. The activity of soya-bean β -amylase in the range pH 3–9, as well as the effect of keeping the enzyme in solutions of different pH at 37° for various times before activity determinations, is given in Table 3.

Table 3 shows that the pH optimum for soya-bean β -amylase is in the range pH 5–6. Pretreatment of the enzyme with buffer solutions of pH 3–5 results in decrease in activity, whereas keeping the enzyme in solution at pH 6–9 for more than 2 hr. does not affect its activity at all.

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REFERENCES

- Akabori, S., Ohno, K. & Narita, K. (1952). *Bull. chem. Soc. Japan*, **25**, 214.
- Applebaum, S. W. (1964). *J. Insect Physiol.* **10**, 897.
- Banks, W., Greenwood, C. I. & Jones, I. G. (1960). *J. chem. Soc.* p. 150.
- Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein. Chem.* **7**, 319.
- Benesch, R. & Benesch, R. E. (1962). *Meth. biochem. Anal.* **10**, 56.

- Bernfeld, P. (1955). In *Methods in Enzymology*, vol. 1, p. 149. Ed by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Birk, Y., Gertler, A. & Khalef, S. (1963). *Biochem. J.* **87**, 281.
- Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
- Cunningham, W. L., Manners, D. J. & Wright, A. (1962). *Biochem. J.* **85**, 412.
- Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.
- Englard, S. & Singer, T. P. (1950). *J. biol. Chem.* **187**, 213.
- Fukumoto, J. & Tsujisaka, Y. (1954). *Kagaku to Kôgyô, Osaka*, **28**, 282.
- Fukumoto, J. & Tsujisaka, Y. (1955). *Kagaku to Kôgyô, Osaka*, **29**, 124.
- Garen, A. & Levinthal, C. (1960). *Biochim. biophys. Acta*, **38**, 470.
- Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291.
- Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
- Liener, I. E. (1955). *Arch. Biochem. Biophys.* **54**, 223.
- Newton, J. M., Hixon, R. M. & Naylor, N. M. (1943). *Cereal Chem.* **20**, 23.
- Newton, J. M. & Naylor, N. M. (1939). *Cereal Chem.* **16**, 71.
- Niu, C. I. & Fraenkel-Conrat, H. (1955). *J. Amer. chem. Soc.* **77**, 5882.
- Ornstein, L. & Davis, B. J. (1961). *Disc Electrophoresis*, part 2, pp. 1-22. Distillation Products Industries.
- Peat, S., Pirt, S. J. & Whelan, W. J. (1952). *J. chem. Soc.* p. 714.
- Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962). *Nature, Lond.*, **195**, 281.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Spies, J. R. & Chambers, D. C. (1949). *Analyt. Chem.* **21**, 1249.
- Stingl, J. & Morawski, T. (1886). *Mh. Chem.* **7**, 176.
- Street, J. P. & Bailey, E. M. (1915). *Industr. Engng Chem.* **7**, 853.
- Sumner, J. B. (1955). In *Methods in Enzymology*, vol. 2, p. 379. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Tiselius, A., Hjerten, S. & Levin, O. (1956). *Arch. Biochem. Biophys.* **65**, 132.