STUDIES ON STARCH-DEGRADING ENZYMES
PART II* THE Z-ENZYME FROM SOYA BEANS; PURIFICATION AND PROPERTIES

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INTRODUCTION

Z-Enzyme was first found to be associated with soya-bean β-amylase when amylose, which was incompletely hydrolysed by crystalline sweet-potato β-amylase, was completely degraded into maltose by the "purified" soya-bean enzyme1,2. Most samples of amylose are now known to contain some type of barrier to the action of pure β-amylase3-5. Peat et al.2,6 initially suggested that Z-enzyme specifically removed this structural feature. However, by preferentially inhibiting the β-amylase, we established that Z-enzyme was α-amylolytic in character, and consequently non-specific with regard to its action on the barrier in amylose7. This general conclusion has been confirmed by later workers8,9. In this paper, we extend our earlier observations on Z-enzyme, and report a convenient method for isolating and extensively purifying this enzyme from soya beans. This has enabled us to make the first detailed studies of the properties of the purified enzyme.

EXPERIMENTAL

General analytical methods

Routine determinations of protein concentrations were made from absorption measurements at 280 mμ, the method being calibrated by micro-Kjeldahl estimations. The concentration of polysaccharide solutions was estimated by hydrolysis, and titration of the liberated glucose with alkaline ferricyanide3. Concentrations of reducing sugar in enzymic digests were estimated by the same reagent. β-Amylolytic limits, [β], were carried out as described earlier3, except that crystalline sweet-potato β-amylase (Worthington Biochemical Corporation, New Jersey, U.S.A.) was used. (This enzyme was shown to be free from Z-enzyme activity.) The technique for measuring the limiting-viscosity number, [η], has been detailed elsewhere10; 0.2 M potassium hydroxide was used as solvent, and measurements were made at 25°.

* For Part I, see ref 31.
Substrates

Soluble starch (‘Analar’, B.D.H.) was used for estimation of \( \beta \)-amylase activity. Amylose \( ([\eta] = 500, [\beta] = 80) \) and amylopectin \( ([\eta] = 180, [\beta] = 56) \) were prepared\(^8\) from a dispersion of potato starch (var. Redskin). Linear amylose \( ([\eta] = 260, [\beta] = 100) \) was obtained by aqueous leaching of potato-starch granules\(^3\). Glycogen \( ([\beta] = 45) \) was extracted from rabbit livers with cold trichloroacetic acid\(^11\). \( \beta \)-Limit dextrans from amylopectin and glycogen were obtained by dialysis and freeze-drying of the appropriate digests.

Digest conditions

Unless otherwise stated, digests were carried out at 35\(^\circ\), and the pH was controlled by acetate buffer (0.2 M, pH 5.5).

Measurement of enzymic activity

\( \beta \)-Amylase. Measurement was made of the amount of maltose produced in a 1 ml portion of a digest containing starch solution (0.6%, 25 ml), buffer (4 ml, pH 3.6), and enzyme (1 ml), after incubation for 30 min. Activities were then expressed as mg of maltose produced per mg of protein.

Z-Enzyme. A modification of the procedure devised by Briggs\(^12\) was employed. The \( \beta \)-limit dextrin from amyllopectin was used as substrate, and the “time reference point\(^{12}\)” was chosen as the time when the corrected absorption value (A.V.) had fallen to 2.00 colorimeter units.

Digests were prepared from buffer (1 ml, pH 5.5), dextrin (amylopectin \( \beta \)-limit) solution (2 ml, 0.6%), enzyme solution, and water to give a total volume of 8 ml. Aliquot portions (2 ml) were removed at intervals and treated with iodine (1 ml, 0.2% in 2% potassium iodide) and hydrochloric acid (0.2 ml, 5 M), in a total volume of 50 ml. The A.V. was then measured at 540 m\(\mu\) in an EEL-colorimeter (filter No. 625). Activities were expressed\(^{12}\) as iodine–dextrin-colour units/mg of protein/ml of digest.

Preparation of Z-enzyme

(a) Initial extraction and fractionation. Dry, defatted, finely-ground, soya-bean flour (300 g) was shaken with calcium chloride solution (0.2%, 1500 ml) for 4 h at 18\(^\circ\). After centrifugation at 900 g, the resultant supernatant liquor (ca. 40 mg of protein/ml) was cooled to 0\(^\circ\). Acetone at \(-5^\circ\) was added to a concentration of 10\% (v/v). The temperature of the mixture was then lowered to \(-5^\circ\) and cold acetone was added slowly, with continuous stirring. Protein fractions obtained by centrifugation (at \(-5^\circ\), 1100 g) were air-dried to remove excess of acetone, and suspended in water at 2\(^\circ\); any insoluble residue was removed by centrifugation. The fractions were characterized, and a typical result is shown below:

<table>
<thead>
<tr>
<th>Acetone concentration (v/v)</th>
<th>0</th>
<th>20</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>45</th>
<th>50</th>
<th>55</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Specific activity of Z-enzyme</td>
<td>6.4</td>
<td>5.0</td>
<td>5.3</td>
<td>7.8</td>
<td>15.4</td>
<td>20.0</td>
<td>35.0</td>
<td>36.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Specific activity of ( \beta )-amylase</td>
<td>7.6</td>
<td>6.7</td>
<td>7.0</td>
<td>8.5</td>
<td>13.0</td>
<td>12.0</td>
<td>12.0</td>
<td>9.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Carbohydrate Res.*, 1 (1965) 229–241
(b) Removal of β-amylase; preliminary experiments. Portions of fraction 5 were heated on a water-bath at 70° (i) in the presence of added calcium acetate (2 mg/ml), and (ii) without additional calcium ions. The protein content, β-amylase, and Z-enzyme activities of aliquot portions of the cooled, centrifuged (1500 g) digests were then measured at appropriate intervals. The results in Fig. 1 indicate that, although the activity of the crude Z-enzyme preparation was remarkably stable at pH 5.5 and 70° (curve 1), the stability was further increased by the addition of calcium ions (curve 2). In the presence of this metal ion, the β-amylase was almost completely deactivated in 10 min under these conditions (curve 4).

Removal of β-amylase; final procedure. Uniform heat-treatment on the water-bath was ensured by heating standard portions (10–15 ml) of the enzyme fractions. To such portions of fraction 7 were added calcium acetate (20 mg), and acetate buffer to bring the pH to 5.5. The mixture was then maintained at 70° for 20 min, cooled, and centrifuged at 1500 g. Resultant supernatant liquors were combined. Typical specific activities were as below:

<table>
<thead>
<tr>
<th>β-Amylase activity</th>
<th>Z-Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initially</td>
<td>9</td>
</tr>
<tr>
<td>After heat treatment</td>
<td>0</td>
</tr>
</tbody>
</table>

This heat treatment was shown to irreversibly deactivate the β-amylase.

(c) Fractionation with acetone. The heat-treated enzyme solution was subfractionated with acetone at –5°. Typical results for the protein fractions obtained were as below:

<table>
<thead>
<tr>
<th>Acetone concentration (v/v)</th>
<th>0</th>
<th>35</th>
<th>42</th>
<th>47</th>
<th>52</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>7</td>
<td>7.1</td>
<td>7.2</td>
<td>7.3</td>
<td>7.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Specific activity of Z-enzyme</td>
<td>51</td>
<td>6.8</td>
<td>33</td>
<td>90</td>
<td>450</td>
<td>92</td>
</tr>
</tbody>
</table>

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(d) Glycogen-complex formation. The acetone-precipitated fractions were finally purified as the glycogen complex, by the method of Loyter and Schrammls. Ethanol to 40% v/v was slowly added with stirring to the enzyme solution at 2°. After 15 min, any precipitated protein was removed at 1100 g. Phosphate buffer (0.5 ml/10 ml of enzyme solution, pH 8.0, 0.2 M) and glycogen (2%, 0.2 ml/10 ml of enzyme) were then added to the enzyme in 40% v/v ethanol. The suspension was stirred for 10 min, and then centrifuged at 1100 g to yield a precipitate which was suspended in phosphate buffer (pH 6.7, 0.02 M). The suspension was maintained at 35° for 6 h to aid digestion of contaminating glycogen, and centrifuged at 1500 g. The resultant solution was cooled to 2° and treated with excess of acetone, and the precipitated material redissolved in water at 2°. Enzyme solutions were found to retain their specific activity (ca. 800) at this temperature for several weeks.

**Effect of temperature and pH**

In experiments where the effect of temperature on activity was studied, digests at pH 5.5 were pre-incubated to temperature equilibrium before enzyme was added. In stability experiments at pH 5.5, the enzyme solution and buffer were maintained at the appropriate temperature for 1 h, and then cooled to 35° before addition of the dextrin. Resultant activities were compared with those obtained at 35°, without prior incubation. The pH dependence of activity at 35° was obtained using McIlvaine's standard buffer solutions. The effect of pH on enzyme stability was determined by maintaining the enzyme and buffer at 20° for 75 min. Digests were then brought to pH 5.5 and incubated with dextrin at 35°, and the activities determined.

**Activity at pH 3.6.** Digests were prepared as follows:

(a) Buffer (2 ml, pH 5.5) + enzyme (0.2 ml) + amylose (20 ml, 3 mg/ml)
(b) Buffer (2 ml, pH 5.5) + amylose (20 ml, 3 mg/ml)
(c) Buffer (2 ml, pH 3.6) + enzyme (0.2 ml) + amylose (20 ml, 3 mg/ml)
(d) Buffer (2 ml, pH 3.6) + enzyme (0.2 ml); incubated for 2 h at 20° before amylose (20 ml, 3 mg/ml) was added.

After incubation at 35° for 24 h, the digests were heated on a boiling water-bath for 5 min, cooled, and filtered, and the amylose product precipitated with excess of butan-l-ol. No butan-l-ol complex was obtained in digest (a). For the products from digests (b)–(d), [η] was measured.

**Effect on amylose-viscosity of Z-enzyme pretreated with mercuric and calcium chlorides**

Digests of enzyme and buffer (pH 5.5) were prepared containing (e) 10^{-3} M mercuric chloride, (f) 10^{-4} M mercuric chloride, (g) 10^{-3} M calcium chloride, and (h) no additional salts. After incubation at 20° for 2 h, the solutions were added to equal volumes of the same amylose solution (3 mg/ml) and incubated at 35°.
Butan-1-ol was added to digests (g) and (h) after 1 h, and to digests (e) and (f) after 24 h; \([\eta]\) of the amylose products was then determined.

**Effect of ethylenediaminetetra-acetate (EDTA) and trypsin**

Digests (i)-(o) were prepared by adding to 10 ml portions of digest at pH 6.6 (McIlvaine's buffer); for (i) and (j), EDTA (0.1 ml, 10^{-1} M) and trypsin (0.1 ml, 0.4 mg); for (k) and (l), EDTA (0.1 ml, 10^{-1} M); for (m) and (n), trypsin (0.1 ml, 0.4 mg). A control digest (o) was also prepared. After incubation at 20^\circ for 12 h, calcium chloride (1 ml, 1 M) was added to digests (i), (k), and (m), and coagulated protein removed by centrifugation. Equal volumes of the same amylose solution (3 mg/ml) were added to all of the digests, which were then incubated at 35^\circ for 24 h. Excess of butan-1-ol was then added; no amylose was precipitated from digests (m) and (o). For the amylose products from the other digests, \([\eta]\) was determined.

**Modification of Z-enzyme**

Coupling with p-diazobenzenesulphonic acid\(^{14}\). Phosphate buffer (1 ml, pH 8.2) and p-diazobenzenesulphonic acid (0.1 ml, 0.6\%) were added to Z-enzyme (2 ml, activity = 2 units/ml). A control digest was set up containing sulphanilic acid (0.1 ml, 0.6\%). The mixtures were left at 18^\circ for 1 h, dialysed at 2^\circ against calcium acetate (0.2\%, 3 \times 200 ml), and centrifuged. Activities were then determined in the usual way.

**Acetylation\(^{14}\)**. Sodium acetate (250 mg) was added to Z-enzyme (1 ml, activity = 2 units/ml) at 0^\circ, and then acetic anhydride (0.03 ml) was added. After 1 h, the mixture was dialysed as above. A control was prepared without the anhydride, and the activities of both digestes were determined.

**RESULTS AND DISCUSSION**

**Isolation and purification of Z-enzyme**

Z-Enzyme was first characterized\(^{11,12}\) as the enzyme which — associated with, and acting in conjunction with, soya-bean \(\beta\)-amylase — would completely degrade any amylose at pH 4.6, but which was itself inhibited completely at pH 3.6. This latter behaviour, in conjunction with the fact that classical reducing-power tests were negative, led to the suggestion that Z-enzyme was not an \(\alpha\)-amylase. The more sensitive, physical techniques of viscosity and light-scattering were necessary to establish the \(\alpha\)-amylolytic character of the enzyme\(^7\). In this work, therefore, we have used procedures applicable to \(\alpha\)-amylases to isolate and purify the Z-enzyme in soya beans.

Initially, a method for measuring Z-enzyme activity in the presence of the contaminating \(\beta\)-amylase had to be developed. Methods involving estimation of reducing power\(^{15}\), fall in viscosity\(^{16}\), or decrease in starch–iodine stain\(^{17}\) are all influenced by concurrent \(\beta\)-activity. However, a modification of Briggs' method\(^{12}\),

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in which we used β-limit dextrin from amylopectin as the substrate, was satisfactory. Any fall in iodine-staining power of this substrate must be due to α-amylolysis. (Although in the presence of excess of β-amylase, α-amylolytic activity will be followed by β-amylolysis, the effect of this on the iodine-staining ability of the dextrin was shown to be small). This method, which is based on the assay of Sandstedt et al.18, measures activity as the reciprocal of the time taken to decrease the dextrin–iodine stain by a standard amount. Here, the standard graph, from which subsequent Z-enzyme activities were calculated, was constructed by measuring the effect of salivary α-amylase on the limit dextrin. (It was later found that hydrolyses of the dextrin by salivary α-amylase and the purified Z-enzyme were very similar in the initial stages).

Preliminary experiments showed that fractionation of the soya-bean protein by ammonium sulphate and alcohol was not successful, little separation of β-amylase and Z-enzyme activities being achieved. (All β-amylase activities were determined at pH 3.6 to inhibit the Z-enzyme.) A successful separation was made, however, by the use of acetone at low temperatures. Heat-treatment was attempted to remove the contaminating β-amylase, as it has been shown10,80 that β-amylase activity can be preferentially removed from malted-barley α-amylase preparations by heating the enzyme mixture at 70°, in the presence of calcium ions. Experiments showed (Fig. 1) that a comparable heat-treatment at 70° and pH 5.5 preferentially, and irreversibly, deactivates the β-amylase in the Z-enzyme/β-amylase fractions. After this removal of the β-amylase, further inert protein was removed by a second fractionation with acetone. Finally, the specific activity of the Z-enzyme was nearly doubled by the formation of a glycogen complex. The latter procedure has been suggested18,21 as a general method for preparing α-amylases of very high activity.

The purification procedure increased the overall specific activity of Z-enzyme by a factor of ca. 150. Maltase, laminarase, and cellobiase were absent, as shown by digestion with the appropriate substrate, followed by paper chromatographic analysis. Similarly, incubation of the enzyme preparation with maltotriose for 72 h showed the presence of trace amounts of glucose and maltose, probably arising from the slow action of Z-enzyme itself; D-enzyme was absent.

Effect of temperature on activity and stability
The temperature of maximal activity of Z-enzyme is ca. 55° (see Fig. 2a). The purified enzyme lost only ca. 10% of its original activity after 1 h at 50°, but there was then a very rapid decrease between 55 and 60°. In the heat-treatment stage of the preparation procedure, the Z-enzyme appears to be stabilized by contaminating protein. An Arrhenius plot of the temperature dependence of the activity is shown in Fig. 2b; the apparent heat of activation varies from 14 kcal at 9°, to 6 kcal at 25°, and is zero at 55°.

Effect of pH on activity and stability
The effect of pH on the enzymic activity is shown in Fig. 3b, where the ratio Carbohydrate Res., 1 (1965) 229–241
(activity at a given pH, $V_{H^+}$)/(maximum activity at the optimum pH, $V_{max}$) is plotted against the pH. A variation in substrate concentration did not affect the shape and position of the curve. The results have been analysed, using the scheme shown in

Fig. 2. (a) Effect of temperature on the activity (- O -) and stability (- ● -) of Z-enzyme; (b) Arrhenius plot of temperature dependence of activity.

Fig. 4, for an enzyme with two ionizable groups $^{22-25}$. If (i) the form EHS is assumed to be the only one of the three enzyme-substrate complexes capable of reacting to give the products, and (ii) the ionization of the two groups concerned is considered to be unaffected by substrate binding, i.e. $K_a' = K_a$ and $K_b' = K_b$, it can be readily shown $^{25}$ that

$$\frac{V_{H^+}}{V_{max}} = \frac{1 + 2 \sqrt{K_a/K_b}}{1 + K_a/[H^+] + [H^+/K_b]}.$$  

i.e. $V_{H^+}/V_{max}$ is a function which is independent of the substrate concentration. As our experimental data showed such independence, the function and experimental
points in Fig. 3b were used to calculate $K_a$ and $K_b$. Values of $pK_a = 8.15$, and $pK_b = 4.3$, were found. The solid line in Fig. 3b represents the function $V_H^+/V_{\text{max}}$ calculated from these values. Essentially, the experimental points lie on this theoretical curve, except at pH 4.0 and below, where irreversible denaturation of the enzyme may be occurring.

![Diagram](image)

**Fig. 4.** Hypothetical scheme for the ionization and reaction of the enzyme; E = enzyme, H = proton, S = substrate. Velocity constants ($k$) are as indicated. Ionization constants of the enzyme ($K_a, K_b$) and the enzyme/substrate complex ($K'_a, K'_b$) are defined as:

$$K_a = k_a/k_a, \quad K_b = k_b/k_b$$

$$K'_a = k'_a/k'_a, \quad K'_b = k'_b/k'_b.$$

The stability of Z-enzyme at various pH values is shown in Fig. 3a. Although these results suggest that all experimental values obtained below pH 5.5 in Fig. 3b should lie beneath the theoretical curve, the two sets of data are not directly comparable; in the stability experiments, the enzyme was incubated in the absence of substrate before the activity was determined, and hence the stabilizing effect of an enzyme–substrate complex was not present, as it was for the results in Fig. 3b.

**Nature of the active centres in Z-enzyme**

The nature of the ionizing groups under consideration may be inferred from the pK-values; the group with pK 4.3 is probably a carboxylic acid, whilst that with pK 8.15 is likely to be an ammonium group. However, there is the possibility that interaction with an anion may have displaced the pH-activity curve, in which case the ionizing group might be an imidazolium group (as in histidine). As the behaviour of the enzyme can be explained in terms of the fact that the ionization is unaffected by binding with substrate, these groups may be involved in the breakdown of the enzyme–substrate complex to give the reaction products, rather than in the formation of a complex.

In order to investigate the nature of the group with pK = 8.15, digests were prepared incorporating (i) iodine ($3 \times 10^{-6} \text{ M}$), and (ii) sodium p-chloromercuribenzoate ($3 \times 10^{-5} \text{ M}$). In digest (i), inhibition was complete, whilst in (ii) there was no change in enzyme activity. Since iodine reacts preferentially with histidine, tyrosine, and sulphhydryl groups, whilst the sodium salt only reacts with sulphhydryl groups.

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groups, the results suggest that histidine and/or tyrosine are necessary for activity of Z-enzyme. This was confirmed when coupling of the enzyme with p-diazobenzene-sulphonic acid — a reagent which combines primarily with tyrosine and histidine residues in proteins — diminished the activity by 95%. Another reagent, which is fairly specific for free amino groups in proteins, although it also reacts with free sulphhydryl groups and, in some cases, free phenolic groups, is acetic anhydride. Acetylation reduced the activity of the Z-enzyme by 75%, suggesting that free amino groups are necessary. However, insufficient enzyme was available for an estimation of the degree of acetylation, or of the types of group acetylated. Thus diminished activity of the enzyme may have been caused by partial acylation of tyrosine residues.

**TABLE I**

**Activity of Z-enzyme at pH 3.6**

<table>
<thead>
<tr>
<th>Digest</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions&lt;sup&gt;a&lt;/sup&gt;</td>
<td>pH 5.5</td>
<td>Control</td>
<td>pH 3.6; directly</td>
<td>pH 3.6; pre-incubation</td>
</tr>
<tr>
<td>[η] of amylose, after incubation</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>510</td>
<td>400</td>
<td>500</td>
</tr>
</tbody>
</table>

<sup>a</sup>See Experimental section (p.232).
<sup>b</sup>[η] too small to be measured accurately.

**TABLE II**

**Effect of various reagents on activity of Z-enzyme**

% inhibition from iodine-staining measurements is quoted

<table>
<thead>
<tr>
<th>Molarity</th>
<th>10&lt;sup&gt;-3&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-4&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-5&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric chloride</td>
<td>100</td>
<td>100</td>
<td>42</td>
<td>15</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100</td>
<td>90</td>
<td>70</td>
<td>42</td>
</tr>
</tbody>
</table>

**Activity of Z-enzyme at pH 3.6**

As the behaviour of Z-enzyme at pH 3.6 is of critical consequence, we made a careful study of the effect of this pH on the enzyme's activity by following changes in [η] of an amylose. This technique yields an extremely sensitive measure of any hydrolytic action. The results of these experiments are shown in Table I, where the negligible [η] of digest (a) shows the high rate of amylolytic degradation at pH 5.5. The difference in [η] between samples (b) and (d) is within experimental error, and shows that pre-incubation at pH 3.6 for 2 h completely destroys the Z-enzyme activity. There was, however, a significant decrease in the viscosity of sample (c), showing that the substrate had been hydrolysed before complete inhibition of the
enzyme had been achieved. Pre-incubation for at least 2 h at pH 3.6 is essential, therefore, before β-amyloysis limits can be obtained using β-amylase preparations which contain Z-enzyme.

**Effect of various reagents on activity**

The effect of various reagents on the activity of Z-enzyme is shown in Table II. Potassium cyanide and tryptophane do not affect the activity, whilst the negligible effect of ammonium molybdate shows that the activities of Z- and R-enzyme are distinct. Ascorbic acid ($10^{-3}$ M) and mercuric chloride ($10^{-4}$ M) are very efficient inhibitors of Z-enzyme activity. Table II also shows that quite extensive inhibition of Z-enzyme must have occurred at the concentration of mercuric chloride ($1.5 \times 10^{-6}$ M) used in our original work. Complete inhibition by mercuric chloride is shown by the [η]-values for digests (e) and (f) in Table III. This Table also shows, from a comparison of the [η]-values for digests (g) and (h), that calcium chloride ($10^{-3}$ M) did not activate the enzyme.

**TABLE III**

**EFFECT ON AMYLASE-VISCOSITY OF Z-ENZYME PRETREATED WITH MERCURIC AND CALCIUM CHLORIDES**

<table>
<thead>
<tr>
<th>Digest</th>
<th>(e)</th>
<th>(f)</th>
<th>(g)</th>
<th>(h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment conditions at pH 5.5</td>
<td>$\text{Hg}^{2+} = 10^{-3} \text{ M}$</td>
<td>$\text{Hg}^{2+} = 10^{-4} \text{ M}$</td>
<td>$\text{Ca}^{2+} = 10^{-3} \text{ M}$</td>
<td>—</td>
</tr>
<tr>
<td>[η] of amylose, after incubation</td>
<td>500</td>
<td>500</td>
<td>80</td>
<td>75</td>
</tr>
</tbody>
</table>

*aSee Experimental section (p. 232).

*b[η] of amylose = 510.

The α-amylolytic nature of Z-enzyme suggested that its action might be very dependent on the presence of calcium ions. The importance of this ion to α-amylase activity has been extensively studied by Fischer and his collaborators. Calcium ions may be effectively removed from aqueous solution by the chelating action of ethylenediaminetetra-acetate (EDTA). In our experiments, the effect of the presence of EDTA on Z-enzyme activity was followed by measuring the amylolysis of amylose by changes in [η], as this method again provides the most sensitive measure of assay. The fall in viscosity [45%; Table IV, digest (l)] shows that although the enzyme is inhibited to some extent [compare, digest (o)], hydrolysis has taken place in the EDTA/Z-enzyme/amylose digest. This inhibition is largely reversible, because on addition of excess of calcium ions, the extent of hydrolysis is greatly increased [digest (k)]. The protease, trypsin, has little effect on the enzyme activity as shown by the large decrease in [η] for the amylose in digest (n), although this fall is even larger in the presence of calcium ions [digest (m)]. However, under the combined action of EDTA and trypsin there was only a 10% fall in [η] for the amylose sample. Thus a mixture of EDTA and trypsin is a more efficient inhibitor than EDTA by itself. This inhibition is not completely reversible as, on the addition of excess of calcium ions, the amylose in digest (l) was not degraded to the same
extent as that in \( k \). This effect may be due to protease attack on calcium-deficient protein molecules.

TABLE IV

<table>
<thead>
<tr>
<th>Digest</th>
<th>(i)</th>
<th>(j)</th>
<th>(k)</th>
<th>(l)</th>
<th>(m)</th>
<th>(n)</th>
<th>(o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial conditions</td>
<td>EDTA/trypsin</td>
<td>EDTA/trypsin</td>
<td>EDTA</td>
<td>EDTA</td>
<td>trypsin</td>
<td>trypsin</td>
<td>control</td>
</tr>
<tr>
<td>Final conditions</td>
<td>+Ca(^{2+})</td>
<td>—</td>
<td>+Ca(^{2+})</td>
<td>—</td>
<td>+Ca(^{2+})</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>([\eta]) of amylose(^a)</td>
<td>75</td>
<td>455</td>
<td>33</td>
<td>280</td>
<td>10(^e)</td>
<td>40</td>
<td>10(^e)</td>
</tr>
</tbody>
</table>

\(^a\)See Experimental section (p. 233).
\(^b\)[\([\eta]\) of amylose = 510.]
\(^c\)[\([\eta]\) too small to be measured accurately.

Action of Z-enzyme on various substrates

In our earlier work\(^7\), the hydrolysis of linear amylose by Z-enzyme was indicated by viscosity results, whilst attack on amyllopectin and amylopectin limit-dextrin was shown by light-scattering measurements. The preparation of purified enzyme has now enabled the action on these substrates to be studied by classical iodine-staining and reducing-power measurements. Fig. 5 shows that all three substrates can be hydrolysed to the “achroic limit”. In particular, it should be noted that the action of Z-enzyme on the \( \beta \)-limit dextrin from amyllopectin is comparable to that of salivary \( \alpha \)-amylase. This justifies the method of estimation of activity, whilst showing the similar nature of the two enzymes.

It is apparent that there are two distinct stages in the hydrolysis of linear amylose by Z-enzyme. There is first a rapid decrease in size of the amylose molecules, as shown by the fall in colour of the iodine stain. This is accompanied by an increase in reducing power of the solution. The second part of the reaction begins at the achroic limit of the amylose solution, and is characterized by a slow increase in reducing power of the solution. The discontinuity occurs at ca. 30% apparent conversion into maltose, and the disappearance of the iodine stain indicates that there are only small maltodextrins present at this stage in the reaction. However, it has to be stressed that the achroic point during \( \alpha \)-amylolysis of amylose is not invariant, but depends entirely on the amylose–enzyme ratio\(^30\).

The effect of Z-enzyme on the \( \beta \)-limit dextrin from glycogen is not yet known with certainty. Our earlier light-scattering measurements indicated that there is no attack on this substrate, whilst Cunningham et al.\(^9\) obtained an increase in reducing power of the digest, using very large quantities of enzyme and prolonged incubation. If limited \( \alpha \)-amylolytic attack is occurring, a more sensitive measure of this can be obtained from the concurrent action of \( \beta \)-amylase and the purified Z-enzyme. Under our normal digest conditions, with the addition of \( \beta \)-amylase, we found a
2% apparent conversion into maltose after 24 h. This result indicates that purified Z-enzyme does cause degradation of glycogen limit-dextrin.

![Graph of % of original A.V. versus apparent conversion into maltose.](image)

Fig. 5. Graph of % of original A.V. versus apparent conversion into maltose. (1) amylose/Z-enzyme; (2) amylopectin/Z-enzyme; (3) amylopectin β-limit dextrin/salivary α-amylase; (4) amylopectin β-limit dextrin/Z-enzyme.

**The character of Z-enzyme**

The above experiments have extended our original observations regarding the α-amylolytic character of Z-enzyme. The variation of activity and stability with temperature and pH are comparable to those of other α-amylases. The enzyme is irreversibly deactivated by pre-incubation at pH 3.6, but we have found that this behaviour is a characteristic of several other plant α-amylases. Inhibition occurs in the presence of mercuric chloride, whilst calcium ions are essential for activity. As with other α-amylases, hydrolytic degradation of amylose, amylopectin, and glycogen occurs.

Earlier, we suggested that Z-enzyme might be a dormant form of α-amylase. However, when soya beans were germinated, we found that the increase in Z-enzyme activity was not significant, compared to that which occurs in barley. It would appear, therefore, that Z-enzyme is similar to other plant α-amylases, but is normally present in extremely small quantities in the soya bean.

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**SUMMARY**

A method for the isolation and purification of the Z-enzyme in soya beans is described. This procedure involves the formation of the glycogen–enzyme complex.

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A method for assay of activity is presented. The properties of the purified enzyme have been studied; in particular, the variation of activity and stability with temperature and pH has enabled the nature of the active sites in the enzyme to be investigated. Characterization of the activity of Z-enzyme at pH 3.6, and in the presence of a variety of reagents, has been achieved by viscometric techniques. Both linear and branched glucans are attacked by the purified enzyme. It is concluded that the properties of Z-enzyme are similar to those of other plant α-amylases.

REFERENCES

9 W.L. Cunningham, D.J. Manners, and A. Wright, Biochem. J., 85 (1962) 408.