297. *The Enzymic Synthesis and Degradation of Starch. Part XXIV.* The Purification of D- and Q-Enzymes.*

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Details are given of a method for the isolation of D-enzyme and Q-enzyme from the same sample of potato juice. Both enzyme preparations are free from amylases and phosphorylase, and neither contains a detectable amount of the other.

It is established that Q-enzyme, when free from D-enzyme, exerts no appreciable " transferase " action on natural amylopectin.

In a previous communication [1] we described the preparation of D-enzyme from the potato. This enzyme preparation was free from phosphorylase but contained traces of other carbohydrases, notably Q-enzyme. These impurities were of little consequence in many studies but interfered with experiments designed to elucidate the mode of action of D-enzyme. Further methods of purification have now been developed and this communication describes the method finally adopted to give a purified D-enzyme and, at the same time, a purified Q-enzyme.

Baum and Gilbert [2] used partly retrograded amylose suspensions as adsorbents for phosphorylase and Q-enzyme in the purification of these two enzymes and found that in the presence of 11% (w/v) ethanol, the Q-enzyme of potato juice was quantitatively adsorbed on the amylose. When the amylose precipitate was washed with buffer solution a portion of the Q-enzyme was eluted. The remainder, however, was more firmly held and could be eluted only by the natural substrate, starch or its components, dissolved in water.[2,3] No details being available, the study of the behaviour of D-enzyme under these conditions was undertaken. As starting material we have used (*a*) charcoal-clarified potato juice [1] and (*b*) the enzyme mixture obtained from the juice by precipitation with ammonium sulphate in presence of copper sulphate (to inactivate phosphorylase [4]). As in Baum and Gilbert's work [2,3] each preparation (*a*) and (*b*) was treated first with 0·15% and then with 0·3% amylose suspensions to give precipitates A and B respectively, from which the enzymes were recovered by elution with buffer.[2,5] Table 1 shows that, with each starting material, *a* and *b*, 70—80% of the D-enzyme was recovered from precipitate A and none from precipitate B.

Table 1. *Adsorption of potato enzymes on retrograded amylose.*

	Recovery as % of original activity			
	(*a*) Charcoal-clarified juice		(*b*) Juice precipitated with ammonium sulphate	
Enzyme	Precipitate A	Precipitate B	Precipitate A	Precipitate B
Q-Enzyme	21	5	13	0
D-Enzyme	70	0	78	0
Phosphorylase	10	70	0	0
Amylase	5	5	0	0
R-Enzyme	0	0	0	0

The eluate from precipitate A also contained 10—20% of the Q-enzyme (together with traces of other enzymes when charcoal-clarified juice, *a*, was used).

While this method gave considerable purification of D-enzyme with respect to protein

* Part XXIII, *J.*, 1957, 2490.

[1] Peat, Whelan, and Rees, *J.*, 1956, 44.
[2] Baum and Gilbert, *Nature*, 1953, 171, 983.
[3] Baum, Ph.D. Thesis, Birmingham, 1953.
[4] Peat, Whelan, and Jones, *J.*, 1957, 2490.
[5] Whelan, " Methods in Enzymology," Vol. I, Academic Press, New York, 1955, p. 194.

and other enzymes, it did not give a product free from Q-enzyme. Consequently we directed our attention towards the fraction of the D-enzyme (20—30%) which was not eluted from the amylose by buffer.

Inasmuch as firmly adsorbed Q-enzyme is eluted by solutions of its substrate,[2,3] it was argued that the firmly held D-enzyme might be desorbed by a solution of maltodextrins of low molecular weight which would not be expected to desorb the firmly bound Q-enzyme since they are not substrates for the latter enzyme. The D- and the Q-enzyme of potato juice were therefore adsorbed on amylose, and the loosely held fractions removed by elution with buffer solution. The amylose residue was then extracted with 0·05% maltotriose dissolved in buffer solution (pH 7·0), and the extracts were examined for D- and Q-enzyme activities. The extract contained 18% of the original D-enzyme activity of the potato juice but no detectable Q-enzyme. After dialysis to remove maltotriose, the D-enzyme was precipitated with ammonium sulphate, redissolved in citrate buffer solution, and freeze-dried.

The amylose precipitate left after extraction of the D-enzyme still held the strongly adsorbed fraction of Q-enzyme and we have confirmed that this fraction can be extracted by dilute solutions of starch or starch components.[2] Q-Enzyme obtained in this way is free from D-enzyme, but if the extraction with maltotriose is omitted both D- and Q-enzymes are eluted together.

Properties of Purified D-Enzyme.—The enzyme prepared as described could be stored for periods of at least 3 months without loss of activity. It was free from detectable amounts of amylases, Q-enzyme, or phosphorylase. It appeared to be without action, as judged by diminution of intensity of iodine stain (A.V.)[6] or by liberation of reducing groups, on either of the separated components of starch, amylose, and amylopectin. It is not, however, to be expected that the transfer by D-enzyme of a fragment of one amylose chain to another would become manifest by either a fall of A.V. or an increase in reducing power. If an acceptor such as glucose is present, the transferring action of D-enzyme is at once detected by a diminution of A.V. Despite the failure of these methods to detect an action of D-enzyme on amylose or amylopectin, separately, nevertheless when whole potato starch was used as the substrate, a fall in blue value[6] was observed from 0·44 to a constant value of 0·29 in 25 hr. It may be that the difference is ascribable to the transfer in whole starch of amylose fragments to the non-reducing ends of the amylopectin, with a consequent diminution of A.V. It is perhaps worthy of note that no change of reducing power is observed in the action of D-enzyme on whole starch.

Properties of Purified Q-Enzyme.—The enzyme prepared as described is free from D-enzyme, phosphorylase, and amylases. We confirm the unpublished observation by Dr. G. A. Gilbert that the purified Q-enzyme is best stored as the enzyme–amylose complex which is freeze-dried and can be reconstituted when required. With this preparation we have reinvestigated the action of Q-enzyme on amylopectin.

Although it is generally agreed that the rapid action of Q-enzyme on amylose ceases when a branched polysaccharide similar to natural amylopectin is produced, it is by no means established that Q-enzyme has any action on natural amylopectin. Most of the Q-enzyme preparations previously obtained have in fact exhibited a slow action on amylopectins, manifested by a diminution in intensity of the iodine stain of the substrate.[7,8] Larner[8] has shown that Q-enzyme preparations exert a branching action on synthetic polysaccharides which in respect of the length of the outer chains are amylopectins in type. At the same time is was claimed that the amylopectin of maize was attacked by Q-enzyme. It may be premature to ascribe this slow action on amylopectin specifically to Q-enzyme since it is now known that the earlier preparations contained D-enzyme in addition.

[6] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[7] Barker, Bourne, and Peat, *J.*, 1949, 1712; Peat, Whelan, and Bailey, *J.*, 1953, 1422; Rees, Ph.D. Thesis, Cambridge, 1953.
[8] Larner, *J. Biol. Chem.*, 1953, **202**, 492.

Q-Enzyme was extracted from the amylose precipitate with a dilute solution of waxy-maize amylopectin and the extract added to a digest containing a sample of the same amylopectin. There appeared to be no effect on the amylopectin as judged by iodine stain in the course of 48 hr. In a similar experiment with potato amylopectin there was no change in the iodine stain during 60 hr. although tests indicated that the enzyme was still active. Estimation, by periodate oxidation, of the basal chain length (proportion of non-reducing end glucose units) of the potato amylopectin at intervals during this experiment gave the results listed in Table 2. The decrease in chain length is so small

TABLE 2. *Action of Q-enzyme on potato amylopectin.*

Time (hr.)	0	12	24	60	96	108
A.V.*	18·0	18·0		17·9		
Chain length †	26·0		26·2	25·2	25·2	24·4

* Intensity of iodine stain measured in arbitrary units [1] with an E.E.L. colorimeter. † By periodate oxidation.

over the prolonged incubation period that it is reasonable to conclude that Q-enzyme has no appreciable " transferase " action on natural amylopectin.

EXPERIMENTAL

General Methods.—Starch fractions were prepared as by Hobson *et al.*[9] For estimation of reducing sugars, blue value (B.V.), and absorption value (A.V.), see Bourne *et al.*[6] In the isolation of enzymes, all operations were performed at 0°, with reagents previously cooled to 0°. Periodate oxidation of polysaccharides was carried out on samples (50 mg.) dissolved in 3% (w/v) sodium chloride (10 ml.) with 0·37M-sodium metaperiodate (10 ml.). The formic acid produced was estimated by the micro-method of Peat *et al.*[10] Charcoal-clarified potato juice was prepared as by Peat *et al.*[1] and phosphorylase-free enzymes were prepared from this juice by precipitation with ammonium sulphate in presence of copper sulphate as by Peat *et al.*,[4] and redissolution in 0·01M-citrate buffer (pH 7·0).

Measurement of Activity of Enzymes.—(i) For D-enzyme, two methods were used. (*a*) A maltodextrin mixture was prepared and fractionated on a charcoal–Celite column as by Whelan *et al.*[11] The fractions eluted from the column between 20% and 50% (v/v) ethanol were pooled and evaporated to dryness. The maltodextrin mixture so obtained had maltotetraose as its lowest member. The digest in an E.E.L. colorimeter cell (1·3 cm.) contained 4% (w/v) of maltodextrin (0·1 ml.) and enzyme solution (0·1 ml.) and was incubated at 35° for 30 min. Iodine solution (7 ml., containing 5 mg. of iodine and 25 mg. of potassium iodide per 100 ml.) was added, and the iodine stain measured in the E.E.L. colorimeter at 510 mμ (Ilford filter No. 404). A digest containing no enzyme was used as control. The increase in the iodine stain was taken as a measure of activity. (*b*) Two digests, each containing 1% (w/v) of waxy-maize amylopectin (1 ml.), 0·2M-citrate buffer (pH 7·0; 2 ml.) and enzyme solution (2 ml.), were prepared. Glucose (20 mg.) was dissolved in one digest, and both were incubated at 20°. After 30 min. samples (1 ml. each) were withdrawn and stained with iodine (2 mg.) and potassium iodide (40 mg.) in a final volume of 50 ml., and the absorption values measured at 680 mμ. The difference in A.V. between the two digests was used as a measure of D-enzyme activity. (ii) Q-Enzyme and α-amylase were determined as by Gilbert and Patrick.[12] Qualitative tests for Q-enzyme activity were made by comparing visually the iodine stains at the beginning and after digestion for 30 min. A perceptible change of the stain towards red was taken as evidence of activity provided that no amylase was present. (iii) Phosphorylase was determined by Whelan and Bailey's method [13] and (iv) R-enzyme was detected by its action on amylopectin.[14]

[9] Hobson, Pirt, Whelan, and Peat, *J.*, 1951, 801.
[10] Peat, Whelan, and Turvey, *J.*, 1956, 2317.
[11] Whelan, Bailey, and Roberts, *J.*, 1953, 1293.
[12] Gilbert and Patrick, *Biochem. J.*, 1953, **51**, 181.
[13] Whelan and Bailey, *Biochem. J.*, 1954, **58**, 560.
[14] Hobson, Whelan, and Peat, *J.*, 1951, 1451.

Adsorption of Potato Enzymes on Retrograded Amylose.—Butanol–amylose complex,[5] equivalent to 0·5 g. of amylose, was dissolved in water (200 ml.) and boiled until the volume was reduced to 100 ml. After cooling, 0·5M-citrate buffer (pH 6·0; 2 ml.) was added, and the solution cooled slowly to 0° with constant stirring. The retrograded amylose appeared as a flocculent suspension which was used directly for the adsorption of enzymes. For the preparation of " 50% ethanol " and " 11% ethanol " see Whelan.[5]

The charcoal-clarified potato juice (50 ml.) was adjusted to pH 6·0 with 0·1N-hydrochloric acid and then brought to 11% (w/v) ethanol concentration by addition of " 50% ethanol." It was then poured slowly, with stirring, into amylose suspension (15 ml.), and the ethanol concentration readjusted to 11% by addition of " 50% ethanol." After 15 minutes' stirring the amylose precipitate (precipitate A) was separated on the centrifuge and washed three times with portions (100 ml. each) of " 11% ethanol." The loosely bound enzymes were finally extracted from precipitate A by gently stirring it for 15 min. with 0·05M-citrate buffer (pH 7·0; 25 ml.), centrifuging the mixture, and filtering the supernatant solution through a grade 3 sintered-glass filter. The residue was similarly extracted with a second portion of buffer, and the extracts were combined for determination of enzyme activities (Table 1). The solution remaining after removal of precipitate A was added slowly to a further quantity of the amylose suspension (125 ml.), and the ethanol concentration again adjusted to 11%. After 15 minutes' stirring the mixture was separated on the centrifuge to give precipitate B. This precipitate was washed and the enzymes were extracted as for precipitate A.

The enzyme solution (50 ml.) obtained from potato juice by precipitation with ammonium sulphate was submitted to the same procedure, and the recovery of enzyme activity determined (Table 1).

Elution of firmly Bound Enzymes from Retrograded Amylose.—Potato juice (200 ml.) was precipitated with ammonium sulphate and redissolved in 0·01M-citrate buffer (pH 6·0; 150 ml.) as described above. The enzymes were then adsorbed on to retrograded amylose (0·5 ml. per ml. of enzyme solution) in presence of " 11% ethanol," as in the preceding experiment, to give precipitate A which was washed five times with portions (250 ml. each) of " 11% ethanol." Precipitate A was then extracted with five portions (30 ml. each) of 0·05M-citrate buffer (pH 7·0) to remove loosely bound enzymes. Only the first three extracts were active, containing Q-enzyme and 74% of the original D-enzyme activity of the potato juice. The residual precipitate was then eluted four times with portions (30 ml. each) of 0·05% (w/v) maltotriose in 0·05M-citrate buffer (pH 7·0), and the extracts were filtered through a grade 4 sintered-glass filter and tested for enzymic activity. Extracts 1 and 2 contained 11% and 8% respectively of the original D-enzyme activity, and extracts 3 and 4 were inactive. No Q-enzyme, R-enzyme, or α-amylase could be detected in any of these extracts.

The amylose precipitate A remaining after elution of the D-enzyme was extracted with 0·05% (w/v) potato amylose (blue value 1·45) in 0·05M-citrate buffer (pH 7·0; 20 ml.). After centrifugation and filtration through sintered glass, the extract was incubated at 20° and portions (0·5 ml. each) were removed at intervals and examined for Q-enzyme activity by measurement of absorption value (680 mμ) after staining with iodine (1 mg.) and potassium iodide (10 mg.) in a total volume of 50 ml.:

Time of incubation at 20° (hr.)	0	1	2·5	4	25
A.V. (680 mμ)	0·555	0·120	0·050	0·049	0·049

There was no detectable D-enzyme or α-amylase activity.

In a similar experiment in which the extraction with maltotriose was omitted, the extract obtained with amylose solution contained both D- and Q-enzyme.

Final Method Adopted for Preparation of D- and Q-Enzyme.—The enzyme solution (150—200 ml.) obtained by precipitation of potato juice with ammonium sulphate in the presence of copper sulphate was treated with " 50% ethanol " and amylose suspension (0·3 ml. per ml. of enzyme solution) as described above, to give precipitate A. The precipitate was washed and loosely adsorbed enzymes were extracted with citrate buffer as in the preceding experiment. D-Enzyme was extracted from the residual amylose precipitate A with four portions (20 ml. each) of 0·05% (w/v) maltotriose in 0·05M-citrate buffer (pH 7·0). The first two extracts were filtered and dialysed against three changes of 0·05M-citrate buffer (pH 7·0; 1 l.) for 24 hr. To the impermeate solution was added 50% (w/v) ammonium sulphate (pH 7·0) to a final concentration of 23% (w/v), and the precipitate removed on a grade 4 filter and washed on the filter

with a mixture of 50% (w/v) ammonium sulphate (pH 7·0; 1 vol.) and 0·05M-citrate buffer (pH 7·0; 1 vol.). The precipitate was dissolved in 0·05M-citrate buffer (pH 7·0; 10 ml.) and freeze-dried. The product usually contained 10—15% of the original D-enzyme activity. After the removal of D-enzyme, precipitate A was suspended in 0·05M-citrate buffer (pH 7·0; 50 ml.) and freeze-dried. The freeze-dried product was stored at 0° over phosphoric oxide until required for elution of the Q-enzyme. In this form the Q-enzyme remained active for periods up to 3 months.

Action of D Enzyme on Starch Fractions.—Each digest contained 0·2M-citrate buffer (pH 7·0; 3 ml.), D-enzyme solution (2 ml.), and polysaccharide solution (5 ml.). The polysaccharide solutions were: 0·1% (w/v) potato amylose (B.V. 1·45); 0·5% (w/v) waxy-maize amylopectin; and 0·4% (w/v) potato starch (B.D.H. soluble starch). After incubation at 20° for various times, portions (1 ml. each, but 0·5 ml. for potato starch) were removed and stained with iodine (1 mg.) and potassium iodide (10 mg.) in 100 ml., and the absorption values were measured at 680 mμ. The absorption values of the amylose and amylopectin digests remained constant at 0·720 and 0·372 respectively during 24 hr., while that of the potato starch underwent the following changes:

Time (hr.)	0	2	4	7	20	25
A.V. (680 mμ)	0·440	0·412	0·376	0·348	0·291	0·290

No reducing sugars were detected in any digest.

Action of Q-Enzyme on Amylopectins.—(i) *Waxy-maize amylopectin.* The amylose precipitate from 200 ml. of potato juice was extracted, after elution of D-enzyme, with 0·1% (w/v) waxy-maize amylopectin in 0·05M-citrate buffer (pH 7·0; 20 ml.), and the extracts were centrifuged and filtered to remove amylose particles. Qualitative tests on a portion (0·5 ml.) of the extract showed considerable Q-enzyme activity. The bulk of the extract was then added to a 0·25% (w/v) solution (30 ml.) of the same specimen of amylopectin and incubated at 20°. Portions (2 ml. each) were removed at intervals and stained with iodine (2 mg.) and potassium iodide (20 mg.) in a final volume of 100 ml. and the absorption values measured at 680 mμ. The absorption value remained constant at 0·210 during 48 hr.

(ii) *Potato amylopectin.* The Q-enzyme was extracted from an amylose precipitate with 0·1% (w/v) potato amylopectin in citrate buffer (25 ml.) as described above. Qualitative tests having indicated Q-enzyme activity, the extract (20 ml.) was added to a digest containing potato amylopectin (505 mg.; 100 ml.) and 0·05M-citrate buffer (pH 7·0; 25 ml.) and incubated under toluene at 20°. Portions (0·5 ml. each) were removed at intervals and stained with iodine as described and the absorption values were measured in the E.E.L. colorimeter with 1·3 cm. cells and Ilford filter No. 608 (peak transmission at 680 mμ). Portions (2 ml. each) of the digest were also removed at 60 hr. and 108 hr. for qualitative tests of Q-enzyme activity. In both cases the enzyme was still highly active. Further portions (30 ml. each) were removed at intervals, boiled for 5 min., cooled, and dialysed at 2° for 4 days against running distilled water. The impermeate solutions were freeze-dried, and the recovered polysaccharides dried to constant weight at 60°. Portions (50 mg. each) of the polysaccharides were subjected to periodate oxidation (Table 2).

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