Enzymes of the Potato Concerned in the Synthesis of Starch

1. THE SEPARATION AND CRYSTALLIZATION OF Q-ENZYME

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Following an early recognition that plant starches are not homogeneous, extensive work in the last decade has established that most plant starches contain two main components. The isolation and characterization of the components is reviewed fully elsewhere (Kerr, 1950) and need not be described here. It is sufficient to say that these components, for which the names amylose and amylopectin are becoming commonly accepted, are readily separated from each other by fractional precipitation from aqueous solution with organic reagents, and that this difference in solubility and in other physical properties is due not to any considerable difference in chemical composition, but to a difference in molecular shape. Thus both components are polymers of glucose of high molecular weight, but amylose has a linear, or at most very slightly branched structure, while amylopectin has a very considerably branched structure.

Amylose is composed essentially of a single chain of glucose units joined by α -1:4 bonds. The exact structure of amylopectin is not known, but the available evidence (Halsall, Hirst, Hough & Jones, 1949) is consistent with the view of Haworth, Hirst & Isherwood (1937) of a main chain of glucose units linked as in amylose by α -1:4 bonds, but with an average of about every twelfth such bond replaced by an α -1:6 bond. At each of these positions a side chain of about twelve α -1:4 linked glucose units is attached at its reducing end by an α -1:4 bond. Half of the amylopectin molecule is therefore in the form of short side chains.

Along with the elucidation of the composition of starch, some insight was gained into the biological origin of its components: amylose was shown by Hanes (1940*a, b*) to be formed from glucose-1-phosphate by the action of the plant enzyme phosphorylase; amylopectin was shown by Haworth, Peat & Bourne (1944) to be formed from amylose by the plant enzyme, Q-enzyme.

The synthetic polysaccharides prepared in the laboratory with these enzymes are likely to prove of assistance in the study of natural starch, and, in addition, they can serve as model substances for the study of the effect of degree of branching on the properties of polymers. Such studies require the

provision of pure enzymes, and it was the purpose of the present work to obtain phosphorylase and Q-enzyme of a sufficient degree of purity.

Part of the method adopted was based on the technique devised at the Harvard Medical School, Boston, for the fractionation of blood plasma. In this technique precipitation is carried out at low temperature, with ethanol, from solutions of sufficiently low ionic strength for the specific properties of the proteins to exert their full effect (Cohn *et al.* 1946; Cohn *et al.* 1950). The separation of Q-enzyme by these methods, reported briefly earlier (Gilbert & Patrick, 1950), is described in this paper, and the separation of phosphorylase is described in the following paper.

METHODS

Preparation of crystalline Q-enzyme. Autumn harvested, non-sprouting King Edward potatoes were peeled carefully, washed with tap water and sliced. The slices (400 g.), after steeping for 0.5 hr. in 500 ml. of $\text{Na}_2\text{S}_2\text{O}_4$ solution (7 g./l.) were drained, washed thoroughly with tap water, and minced. The juice was pressed from the pulp through fine muslin by a hand press, and clarified by centrifuging. About 100 ml. were usually obtained. Substitution of a blender (at 0°) for a mincer improves the yield of juice to about 200 ml. From this point all operations were carried out in a cold room set at -5°, or in a refrigerated centrifuge. A 50% (v/v) solution of ethanol containing 0.01 M-disodium hydrogen citrate was prepared for use as a precipitant (50 ml. ethanol, 53.7 ml. water). Its pH was adjusted with NaOH until a pH of 6.0 at 20° was recorded for a portion diluted with 5 times its volume of 0.01 M-NaCl. The precipitant was cooled to -5°, and added at a maximum rate of 25 ml./min. with thorough but gentle stirring. A further 15 min. were allowed for equilibration before centrifuging. The temperature of the solution to which the precipitant was being added was kept about 1° above its freezing point by a refrigerator bath of ethanol.

The potato juice (100 ml., pH 5.8-6.0) was cooled to 0°, avoiding freezing, and 28.2 ml. of 50% ethanol added to bring the concentration of ethanol to 11% (at a final temperature of about -2°). After centrifuging the solution at -2° the precipitate was discarded and the ethanol concentration of the supernatant raised to 14.9% by adding 0.11 ml. of 50% ethanol/ml. of supernatant to give precipitate I. (The figure 16 instead of 14.9 was given in error by Gilbert & Patrick (1950).) The precipitate was separated by centrifugation at -5°, dissolved at 0° in 60 ml. of citrate

buffer (0.01 M-disodium hydrogen citrate, NaOH to pH 6.0), and 18 ml. of 50% ethanol added to give a concentration of 10.5% (v/v). The resulting precipitate was discarded, and the concentration of ethanol raised to 15% by the addition of 0.11 ml. of 50% ethanol/ml. of supernatant as before. Precipitate II which formed was dissolved at 0° in 40 ml. of 0.01 M-citrate solution, pH 6.0, and reprecipitated at -5° with 18.9 ml. of 50% ethanol to give precipitate III. This was dissolved at 0° in 5 ml. 0.05 M-citrate buffer pH 7.0 and the solution freeze-dried to enable the experiment to be interrupted at this point. The dried material was later reconstituted with 20 ml. water and the ionic strength of the solution increased 3.6 units by the addition of 9.3 ml. of 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution (50 g./100 ml. solution, NH_3 to pH 6.2). After standing for 2 hr. at 0° with frequent stirring, the solution was centrifuged at 0° and precipitate IV removed.

Precipitate IV was dissolved at 0° in 10 ml. of $(\text{NH}_4)_2\text{SO}_4$ solution (ionic strength 1.0); (4.4 g./100 ml., NH_3 to pH 6.2), and the solution filtered first through a Whatman no. 42 paper under gravity, and then through a sintered-glass filter (grade 3) under slight positive pressure. After raising the temperature to 10°, 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ solution pH 6.2 (filtered through a glass filter, grade 3) was added drop by drop until a faint precipitate appeared (about 5 ml. were required), after which the precipitate was just redissolved by the addition of a few drops of water. The solution was then cooled to 0°, and left overnight to crystallize in a stoppered bottle, which it almost filled to leave a minimum of air-liquid surface. The crystals, V, were centrifuged at 0°. Recrystallization was carried out by dissolving the crystals in $(\text{NH}_4)_2\text{SO}_4$ solution, as for precipitate IV, and repeating the operation above.

Analytical methods. Protein N was determined by the method of Falconer & Taylor (1946). A conversion factor of 6.25 was adopted arbitrarily to calculate protein.

The action of Q-enzyme on amylose or starch can be followed by sampling and staining with iodine solution. The stain becomes progressively less blue as the reaction proceeds (Bourne, Macey & Peat, 1945), and finally takes on the hue characteristic of amylopectin. This colour change can be used for the quantitative estimation of Q-enzyme and for a determination of activity/unit weight by applying an empirical relation described earlier (Gilbert & Swallow, 1949) for the rate of decrease of optical density at 6800 Å. It was found then that if a non-retrograding 'soluble' starch is used as substrate, the optical density D falls approximately exponentially to its final value D_∞ , thus simulating a first-order reaction. The apparent velocity constant of the reaction is proportional to the enzyme concentration under the usual conditions of testing, and therefore the activity of the enzyme may be expressed by velocity constant/enzyme concentration. D_∞ is an empirical constant which varies slightly with each preparation of starch, and is only approximately equal to the optical density of the products of reaction.

Q-enzyme activity was determined at 20°, using a test solution prepared from constituents in the proportion: 2 vol. enzyme solution, 1 vol. starch solution (10 mg. 'Analar' soluble starch/ml. of water), 2 vol. citrate buffer solution (0.2 M-disodium hydrogen citrate, NaOH such that the pH of the test solution was 7.0). 0.5 ml. samples were removed at intervals and stained with I_2 (1 mg.)-KI (10 mg.) in a solution of final volume 50 ml. The optical density of the samples was measured in 4 cm. cells with a Hilger-Spekker Absorptiometer (6800 Å., Ilford Spectrum

Filter no. 608). $\log (D - D_\infty)$ was plotted against time, t (min.), and activities calculated from the slope of the initial straight part of the line using the relation

$$\text{Activity} = - \frac{2.30 \, d \log (D - D_\infty)}{c \, dt},$$

where c is the concentration of the enzyme in the test-solution in mg./ml. The units of activity are therefore $\text{min.}^{-1} \text{mg.}^{-1} \text{ml.}$ If the concentration of the enzyme was not known or was immaterial, the activity of the solution was expressed as $-2.30 \, d \log (D - D_\infty)/dt \, \text{min.}^{-1}$.

Velocities of reaction at 10, 20 and 30° were compared using samples of solution of the crystallized enzyme prepared in 0.2 M-citrate buffer pH 7.0. The tests were carried out simultaneously and curves A, B and C in Figs. 3 and 4 obtained at these respective temperatures. Meanwhile, since Q-enzyme is thermolabile (Barker, Bourne & Peat, 1949), a measure of the rate of decay of the enzyme at 30° was obtained by keeping two samples of the enzyme solution at 30° for 88 and 176 min. respectively before adding starch. The velocities of reaction were then measured at 30° to give curves D and E in Figs. 3 and 4 (p. 184). Control solutions were kept for similar times at 10°.

Reducing power was determined by the method described by Barker *et al.* (1949). The values listed in Table 1 may be compared with one another since, under the standard conditions of testing, the volume of the test solution in each case corresponded to 1.12 times the volume of the potato juice from which the added enzyme was derived.

Phosphorylase was determined under approximately the conditions found by Green & Stumpf (1942) to lead to proportionality between enzyme concentration and phosphate liberation. A solution consisting of 'Analar' soluble starch (0.5 ml. 5% solution), 0.5 M-citrate-NaOH buffer, pH 6.0 (0.5 ml.) and enzyme solution (1.5 ml.) was brought to 35.5°, and 0.1 M-dipotassium glucose-1-phosphate (1 ml.) then added. After 12 min. 6% trichloroacetic acid (5 ml.) was added to the test solution, the precipitated protein centrifuged down, and the supernatant analysed for inorganic phosphate by the method of Allen (1940). Inorganic phosphate not due to the action of the enzyme was allowed for by running a control solution at the same time in which the enzyme had been inactivated by trichloroacetic acid before the addition of glucose-1-phosphate.

RESULTS

Electrophoretic analysis of Q-enzyme prepared by precipitation with lead acetate and ammonium sulphate. A sample of enzyme prepared by the method described by Barker *et al.* (1949) (their fraction 'Q₁') was dialysed for 48 hr. at 0° against 0.01 M-ammonium sulphate, which had been brought to an ionic strength of 0.1 with sodium chloride, and to a pH of 8.2 with ammonia. Analysis in a large sectional cell by the electrophoretic method of Tiselius showed that the constituent proteins were negatively charged and that at least six components were present (Fig. 1a). By testing small samples of solution removed from the ascending and descending boundaries, it was further shown that Q-enzyme activity was associated only with the protein moving with the highest mobility. This was a very small

fraction of the whole, and from a number of such experiments it was concluded that at the most only 5 % of the protein in the preparation was *Q*-enzyme. The very low mobility of the bulk of the protein impurity suggested that a considerable purification could be effected by electrophoresis. This was attempted, but was not pursued when it was found that the enzyme was too unstable in solution to survive the long procedure required.

Using the above figure for the activity of the crystallized enzyme (V), the concentration of *Q*-enzyme in the original potato juice was calculated to be approximately 16 mg./l.

Examination of precipitate III. Before subjecting precipitate III from the ethanol-fractionation process to further fractionation in ammonium sulphate solution, it was examined by electrophoresis, and by the solubility test of Falconer &

Fig. 1. (a) Electrophoresis of *Q*-enzyme prepared by precipitation with lead acetate and ammonium sulphate. Ascending boundary after 124 min. (b) Electrophoresis of precipitate III. Ascending boundary after 170 min. (c) Electrophoresis of precipitate IV. Ascending boundary after 76 min.

Ethanol and ammonium sulphate precipitation and crystallization of Q-enzyme. The enzyme activity/mg. protein/ml., and the yield of enzyme at different stages of the preparation of crystalline *Q*-enzyme are given in Table 1. Precipitates are numbered as under Methods. The yield is shown relative to precipitate I, since a direct determination of the *Q*-enzyme content of untreated juice is not possible because of the presence of starch precipitants and amylases. The figures are averages of the results of several experiments, each carried out with 100 ml. of potato juice. The relative yield has usually been less if considerably more juice were taken.

Taylor (1946). Philpot schlieren photographs (Fig. 1b), taken after electrophoresis in 0.1M-citrate-NaOH solution, pH 7.5, revealed a component of very low mobility representing about 60 % of the protein of precipitate III, a component of intermediate mobility (25 %), and a relatively fast component (15 %). *Q*-enzyme activity was shown to be associated only with the fast component. Precipitate III did not lose any appreciable amount of activity during either electrophoresis or the preliminary dialysis at 0° for 24 hr.

The results of the solubility-activity test carried out on precipitate III at 0° and pH 6.2 in ammo-

Table 1. Activity and yield at stages in the preparation of crystalline *Q*-enzyme

Ppt.	Activity at 20° min. ⁻¹ mg. ⁻¹ ml.	Relative yield	Reducing power of test solution after 20 hr. as apparent conversion to maltose (%)	Phosphorylase activity relative to potato juice (=1.0)
I	0.04	1.0	12.5	0.05
III	0.15	0.9	8.6	0.012
IV	0.40	0.7	4.7	< 0.007
V	2.4	0.7	1.9	< 0.0016

nium sulphate solutions of increasing ionic strength are shown in Fig. 2. Protein began to precipitate at an ionic strength of 3.0 from a solution of 0.18 mg. N/ml. (Kjeldahl). Simultaneously, the activity of the residual solution began to fall, indicating that *Q*-enzyme was present in the least soluble component. According to Fig. 2, section *AB*, the protein precipitated had a constant activity/unit weight until about 80% of the activity had been precipitated (ionic strength 3.6). A short extrapolation of *AB* to zero activity of the solution showed that if the precipitated component could be treated as homogeneous the enzyme activity was associated with a component representing 34% of the total protein.

Fig. 2. Solubility-activity curve for precipitate III.

However, in spite of the linearity of *AB*, this component evidently was not a pure protein since the electrophoretic data of Fig. 1*b* had led to a figure of 15% as a maximum. In conformity with this, electrophoretic analysis (pH 7.5, 0.1*M*-citrate-NaOH solution) of the protein precipitated at ionic strength 3.6 (Fig. 1*c*) showed the presence of two components which corresponded in mobility to the two faster components of Fig. 1*b*. There was no sign in Fig. 1*c* of the slow component in Fig. 1*b*, which therefore must remain in solution at ionic strength 3.6.

Stability of *Q*-enzyme. The crystallized enzyme does not lose more than 5% of its activity during freeze-drying from 0.05*M*-citrate-NaOH buffer pH 7.0 (cf. Barker, Bourne, Wilkinson & Peat, 1950) and is then perfectly stable at 0° in the dry state. In solution in 0.2*M*-citrate-NaOH buffer pH 7.0 the activity decays to half in 11 days at 0° or in 4 days at 17°. It will be seen in the next paragraph that the decay at 30° is extremely rapid.

Effect of temperature on the action of *Q*-enzyme on starch. The relative rates of reaction at 10, 20 and 30° were calculated from the initial slopes of the lines *A*, *B* and *C* respectively in Fig. 4 to be 0.47, 1.0,

1.0. At 30° the activity rapidly decayed during the course of the test, as shown by the progressive decrease in slope of curve *C*, and after 120 min. had fallen to 40% of its initial value. The two samples of enzyme that had been kept at 30° in the absence of

Fig. 3. The effect of temperature on the action of crystallized *Q*-enzyme on starch solutions.

Fig. 4. Activity of *Q*-enzyme. $\log (D - D_{\infty})$ as a function of time.

starch for 88 and 176 min., respectively, before being tested, had decayed even more rapidly (curves *D* and *E* respectively) and retained only 29 and 6% of their activity. The solutions kept at 10° for similar times before the addition of starch showed no loss of activity.

Fresh starch was added to the test solution corresponding to curve *C* after it had stood for 3 hr. at 30° and had cooled slowly to room temperature for 16 hr. Appreciable activity was still present, since the optical density of the iodine stain fell from 0.84 to 0.61 in 120 min. at 20°, and to 0.42 in 270 min.

Activation of Q-enzyme by ions. A suspension of crystals of the enzyme in ammonium sulphate solution was dissolved in water. The ionic strength of the resulting solution was estimated to be 0.017 by passing a sample through an exchange resin and estimating the hydrogen ion produced. The solution was diluted to an ionic strength of 0.010, and then, as a precaution against suspended enzyme which might later be dissolved at higher salt concentrations, filtered through a Whatman no. 42 paper and a G-4 sintered-glass funnel under slight positive pressure. Samples were then added to equal volumes of solutions of starch at pH 7.0 and various ionic strengths equal to or greater than 0.01 (precipitation of the enzyme by reduction in salt concentration thereby being guarded against), and the corresponding activities determined. These are given in Table 2. Further, to test that the effects were not due to suspended enzyme, a portion of the enzyme solution of ionic strength 0.01 was diluted with an equal volume of ammonium sulphate solution of ionic strength 0.01 and the resulting solution tested separately for activation, the results being indicated by the starred entries in the table.

Table 2. *Activation of Q-enzyme by ions. Activity measured in solutions of increasing ionic strength*

Ionic strength of solution	Activity $\times 10^3$ (min. ⁻¹)
0.01	1.7
0.05	2.9
0.25	4.3
1.25	6.5
0.01*	1.0
1.25*	3.0

* Enzyme solution diluted with $(\text{NH}_4)_2\text{SO}_4$ solution before testing. See text.

Dependence of the yield of enzyme on the season and on the variety of potato. Difficulty in the preparation of Q-enzyme during a certain period of the year was noted by Barker *et al.* (1949). The same trouble was also encountered in the present investigation. In the middle of March, King Edward potatoes harvested in the previous autumn were found to be almost devoid of active enzyme, although 3 weeks previously their activity had been normal. This change coincided with the beginning of sprouting in the potatoes, and at the same time activity due to an amylase of lower solubility than Q-enzyme disappeared. The amylose precipitant also increased considerably and the precipitates became oily and more difficult to work with. A standard method of fractionation could not be devised until fully grown potatoes were available in August.

With the exception of 'Ninetyfold' potatoes, all the varieties of white-skinned potatoes tested gave

very low yields of Q-enzyme in comparison with red-skinned varieties, among which there was little to choose.

DISCUSSION

The original method for preparing Q-enzyme (Bourne & Peat, 1945), after modification by Barker *et al.* (1949), consists of precipitating potato juice with lead acetate, dissolving the precipitated protein and precipitating the enzyme with ammonium sulphate. Although the method has served for the characterization of the enzyme, it is evident from the electrophoresis results above that it does not differentiate sufficiently between the enzyme and the numerous proteins which occur in potato juice. Much greater selectivity is attained by precipitation at low ionic strength with ethanol (Cohn *et al.* 1946, 1950). The number of variables available in this method make it very suitable for problems such as the present one where a minor constituent of a mixture of proteins has to be isolated.

It has to be remembered, however, that ethanol quickly inactivates Q-enzyme if certain limits of temperature and concentration are exceeded (compare the inactivation by ethanol reported by Bourne *et al.* 1945). Even under the conditions described above for the preparation, the enzyme should not be left too long in contact with ethanol, e.g. overnight. Ammonium sulphate solution is therefore a better medium than ethanol solution from which to attempt crystallization.

The influence of the season and of the variety of potato on enzyme content makes it advisable to carry out preliminary experiments before attempting a large-scale extraction of a new batch of potatoes. It is then possible to decide whether sufficient enzyme is present to be worth extraction, and whether any slight alterations in the concentrations of ethanol specified above need be made to achieve optimum results with the particular batch.

The enzymic properties of the crystallized enzyme resemble those described for the amorphous form in the references cited. The ability of the enzyme to form reducing groups when incubated with amylose is somewhat decreased but still remains, although detectable by reducing power measurements only after long incubation. It is confirmed that Q-enzyme is activated by ammonium sulphate as described by Gilbert & Swallow (1949). The experiments were conducted with particular care to eliminate any possibility that enzyme suspended, but not dissolved, at low ionic strengths was passing into solution at higher ionic strengths and simulating activation.

The lability of the enzyme is demonstrated by the results obtained for the reaction with starch at 30°

(curve *C*, Fig. 4). It is obvious that for valid comparisons of reaction rate at different temperatures the initial slope of the reaction curve must be obtained. When this is done it is still found that the velocity of reaction reaches a maximum somewhere between 20 and 30° and that an 'optimum' temperature does not result from the onset of inactivation.

The experiments in which *Q*-enzyme was kept at 30° for some time before adding starch and testing activity confirm this lability. Thus in 2 hr. only 6% of the original activity remained. It is noteworthy that the decay of activity at 30° in the solution which contained starch throughout was much less rapid, a result which suggests that starch or its reaction product tends to stabilize the enzyme.

SUMMARY

1. Potato *Q*-enzyme has been prepared in crystalline form and with high activity by a method involving precipitation with ethanol at low temperature and low ionic strength.

2. Electrophoretic analysis has been used to follow the course of the purification.

3. Activation of the enzyme by ions has been confirmed, and the effect of temperature on the activity of the enzyme has been examined.

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Enzymes of the Potato Concerned in the Synthesis of Starch

2. THE SEPARATION OF PHOSPHORYLASE

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Several accounts have been published of attempts to purify the starch phosphorylase of the potato. The original technique of Hanes (1940), in which the enzyme was precipitated from potato juice with

ammonium sulphate, has been modified by, among others, Green & Stumpf (1942), Meyer & de Traz (1944), Weibull & Tiselius (1945), Bourne & Peat (1945) and Barker, Bourne, Wilkinson & Peat (1950).