Studies on Carbohydrate-Metabolizing Enzymes

3. YEAST BRANCHING ENZYME*

By ZEENAT H. GUNJA, D. J. MANNERS AND KHIN MAUNG

Department of Chemistry, University of Edinburgh

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The classical researches of Hanes, Peat and their co-workers (for reviews, see Barker & Bourne, 1953; Whelan, 1958) have established that two distinct enzymes are involved in the synthesis of amylopectin from α -D-glucosyl phosphate. Thus P-enzyme (potato phosphorylase) action results in the formation of linear amylose-type molecules which are converted into amylopectin by Q-enzyme. Alternatively, the combined action of P- and Q-enzymes on α -D-glucosyl phosphate also yields a branched polysaccharide, although Q-enzyme alone has no action on the glucoside.

In the animal kingdom a related enzyme system may be partly responsible for glycogen synthesis. Muscle phosphorylase synthesizes amylose-type chains from α -D-glucosyl phosphate and the concurrent action of this enzyme and an enzyme preparation (branching factor or supplementary enzyme) from heart or liver extracts gave a glycogen-type polysaccharide, since the latter gave a red-brown coloration with iodine and, unlike amylose, could function as a primer for muscle phosphorylase (Cori & Cori, 1943). In general, however, the branching enzymes from animal tissues have not been highly purified and the products of enzyme action have not been rigidly characterized.

Glycogen is one of the reserve polysaccharides of brewer's yeast (Manners & Khin Maung, 1955a); hence, extracts of this yeast should provide a source of branching enzyme. This enzyme was first encountered as an impurity in yeast-phosphorylase preparations (unpublished work), which, acting on α -D-glucosyl phosphate, synthesized a branched polysaccharide which had a β -amylolysis limit of 44% and gave a red-brown stain with iodine.

This paper describes some of the properties of partly purified yeast branching enzyme and its action on amylose. Furthermore, since yeast glycogen contains 7-8% of 1:6-glucosidic linkages, it was of interest to examine the action of yeast branching enzyme on an amylopectin containing only 4-5% of branch points. Preliminary accounts of some of this work have been published (Manners & Khin Maung, 1956; Gunja & Manners, 1959).

METHODS AND MATERIALS

Analytical methods. Reducing sugars and protein N were determined by the methods of Somogyi (1952) and Robinson & Hogden (1940) respectively. Enzyme digests containing isoamylase were deproteinized by Nelson's (1944) method. Paper chromatography was carried out as described by Manners & Khin Maung (1955a) and Archibald & Manners (1959).

Iodine staining. Polysaccharide sample (0.5-1.0 ml.) was stained with iodine solution [0.2% of I_2 in 2% (w/v) KI; 0.5-1.0 ml.] in a total volume of 50-100 ml. The solution also contained 1 drop of 5N-HCl. The absorption value of the polysaccharide-iodine complex [for definition see Bourne, Haworth, Macey & Peat (1948)] was measured (a) by a Hilger Spekker photoelectric absorptiometer in 1 or 4 cm. cells with an Ilford no. 608 (approx. 680 m μ) filter (amylose or starch) or no. 603 (approx. 490 m μ) filter (waxy-maize starch) against an iodine-water blank, or (b) with a Unicam SP. 500 spectrophotometer with 1 cm. cells against an iodine-water blank. Amylose, starch and amylopectin solutions were measured at 630, 600 and 540 m μ respectively.

Phosphorylase activity. The conditions of Green & Stumpf (1942) were used; inorganic phosphate was measured by the method of Fiske & Subbarow (1925).

Analysis of polysaccharides

Glucose content. The polysaccharides (0.1%, w/v) were hydrolysed with 2N- H_2SO_4 at 100° for 2 hr. and the reducing-sugar content (as glucose) was determined with the Somogyi reagent. Analytical figures are based on the observed glucose contents.

β -Amylolytic limit. Polysaccharide (25 mg.), 0.2M-sodium acetate buffer (pH 4.6; 4 ml.) and β -amylase (approx. 1250 units) were incubated in a total volume of 25 ml. at 35° for 24 hr. The maltose content of a portion (3 or 5 ml.) was then determined. Under these conditions enzyme action was 93% complete in 4 hr. and the maltose content after 24 and 48 hr. was identical (Liddle, 1956).

β -Amylolytic limit after incubation with isoamylase. Polysaccharide (25 mg.), 0.2M-sodium acetate buffer (pH 6.0; 4 ml.), isoamylase (50 mg.) and water (7 ml.) were incubated at 20° for 24 hr. The digest was heated (3 min. at 98°), cooled and β -amylase solution (approx. 1250 units) and water (to 25 ml.) were added. After 24 hr. at 35°, the maltose content of the digest was determined after deproteinization of a sample.

Simultaneous action of β -amylase and isoamylase. Polysaccharide (20-25 mg.), 0.2M-sodium acetate buffer (pH 6.0; 4 ml.), isoamylase (40 mg.) and β -amylase (1000 units) were incubated for 24 hr. at 20° in a total volume of

* Part 2: Archibald & Manners (1959).

25 ml. After deproteinization of a sample (3 ml.) the maltose content was measured.

α -Amylolytic limit. Polysaccharide (30 mg.), 0.1 M-NaCl solution (10 ml.), freeze-dried α -amylase (2.5 mg.) and water (to 50 ml.) were incubated at 35° for 24 hr. Portions (5 ml.) were examined for reducing sugar (expressed as apparent percentage conversion into maltose, P_M).

Limiting viscosity number. By means of a modified Ubbelohde viscometer, the specific viscosity, η_{sp} , at $25 \pm 0.02^\circ$ of four concentrations of the polysaccharide in 0.1 M-NaCl or M-KOH was determined. The limiting viscosity number, $[\eta]$, was obtained by extrapolation of η_{sp}/c against c to zero concentration (c as g./ml.).

Enzyme preparations

Pressed brewer's yeast (*Saccharomyces cerevisiae*) was kindly supplied by Wm. Younger and Co. Ltd. It was washed with water, dried at 35° in a current of air and stored in air-tight bottles at 0°. Isoamylase was prepared by the method of Manners & Khin Maung (1955b) and Gunja, Manners & Khin Maung (in preparation). α - and β -Amylase were as described by Liddle & Manners (1957); soya-bean β -amylase was also used (Manners & Khin Maung, 1955a).

Substrates

Amylose. Various samples, made by the fractionation of potato starch with butanol (H. Baum & G. A. Gilbert, cited by Whelan, 1955), aluminium hydroxide (Bourne, Donnison Peat & Whelan, 1949) or thymol and butanol (Greenwood & Robertson, 1954), were used.

Amylopectin. Two samples of amylopectin were prepared by the thymol fractionation of potato starch (Greenwood & Robertson, 1954). Malted-barley amylopectin was kindly provided by Dr G. O. Aspinall (Aspinall, Hirst & McArthur, 1955). Waxy-maize starch was a commercial sample.

Glycogen. Glycogens isolated from brewer's yeast (Manners & Khin Maung, 1955a), rabbit muscle (Liddle & Manners, 1957) and horse-diaphragm muscle (Lawrie, Manners & Wright, 1959) were used. Floridean starch was the sample II described by Fleming, Hirst & Manners (1956).

β -Dextrins. Amylopectin β -dextrin and glycogen β -dextrin were prepared by the prolonged action of β -amylase on waxy-maize starch and *Mytilus edulis* VI glycogen (Liddle & Manners, 1957), followed by dialysis (to remove maltose and inorganic material) and isolation by precipitation with ethanol or freeze-drying.

Preparation of yeast branching enzyme

The starting material was air-dried brewer's yeast. The following procedure has given preparations of yeast branching enzyme of satisfactory activity, although with different batches of yeast the proportion of contaminating enzymes (phosphorylase, isoamylase, isomaltase and maltase) has varied. Dried yeast (100 g.) was extracted with 0.1 M-NaHCO₃ solution (500 ml.) at 35° for 2 hr., and the extract centrifuged at 0°. To the solution (about 350 ml.) an equal volume of cold saturated (NH₄)₂SO₄ solution was added. The precipitate was collected by centrifuging at 1° and dissolved in 0.1 M-sodium citrate buffer (pH 6.6; 100 ml.). Aq. 50% (v/v) ethanol-0.01 M-sodium citrate buffer, pH 6.8, was added at -5° to give an ethanol concentration of 25%.

The precipitate was removed and the ethanol concentration of the supernatant solution was increased to 30%. This precipitate was collected and triturated with 0.1 M-sodium citrate buffer (pH 6.8; 40 ml.) for 30-60 min. Insoluble material was removed by centrifuging and the solution treated with an equal volume of cold saturated (NH₄)₂SO₄ solution. The precipitate was collected, dissolved in 0.1 M-sodium citrate buffer (pH 6.8; 20 ml.) and freeze-dried. Yield 2.0-2.6 g.; N content 1.6-2.3%. The material was free from iodophilic polysaccharide.

A digest containing amylose (2.5 mg.), 0.2 M-sodium citrate buffer (pH 6.8; 1 ml.), yeast branching enzyme (45 mg.) and water (4 ml.) was prepared and incubated at 20°. The absorption values (Spekker units) after 0, 0.5, 1.5 and 20.0 hr. were 0.44, 0.21, 0.15 and 0.04 respectively. The digest did not contain reducing sugars (Somogyi reagent and paper chromatography).

Tests for contaminating enzymes. (a) Maltase and iso-maltase. Yeast branching enzyme (20 mg.) was incubated with maltose, maltotriose and isomaltose (10 mg.) in 0.2 M-sodium citrate buffer (pH 6.8; 0.5 ml.). Paper-chromatographic analysis after incubation at 20° for 24 hr. showed the presence of both glucose and unchanged oligosaccharide. The enzyme preparation thus contains traces of maltase and isomaltase, although the presence of these enzymes will not interfere with measurements of activity of yeast branching enzyme.

(b) α -Amylase. The test is based upon that of Hobson, Whelan & Peat (1950) except that glycogen β -dextrin was used. Amylose (5 mg.), amylopectin β -dextrin or glycogen β -dextrin (10 mg.) was incubated with yeast branching enzyme (60 mg.), 0.2 M-sodium citrate buffer (pH 6.8; 2 ml.) and water (8 ml.). Portions (1 ml.) were removed after 0, 20, 60 and 1080 min. The results are shown in Fig. 1. Since the iodine-staining power of glycogen β -dextrin

Fig. 1. Detection of α -amylase in preparation of yeast branching enzyme. Relationship between the changes in the absorption value of O, amylose and glycogen β -dextrin or ●, amylopectin β -dextrin during incubation with yeast branching enzyme.

remained constant it is concluded that yeast branching enzyme is essentially free from α -amylase. The action on amylopectin β -dextrin will be considered later.

(c) Isoamylase. Incubation of yeast branching enzyme (1958 preparation) with glycogen gave an insignificant increase in iodine-staining power and β -amylolysis limit. By contrast, preparations made in 1955-56 contained a trace of isoamylase since the β -amylolysis limit of yeast glycogen increased by 4-5% after incubation for 4-14 hr.

(d) Phosphorylase. In preliminary experiments the protein precipitated at ethanol concentrations of 17-23 and 23-30% was found to be free from phosphorylase.

RESULTS

Properties of yeast branching enzyme

Effect of enzyme concentration. Although amylose is the normal substrate for branching enzymes, Gilbert & Patrick (1952) have shown that soluble starch may also be used. With this substrate, preliminary experiments showed that with digests containing starch (2%, w/v; 0.5 ml.), 0.2M-sodium citrate buffer (pH 6.8; 1 ml.), yeast branching enzyme (10-25 mg.) and water to 5 ml., and incubated at 20° for 10 min., activity of yeast branching enzyme as followed by iodine staining was proportional to enzyme concentration. Under these conditions a unit of activity may be defined as the amount of enzyme causing a decrease of 0.1 Spekker absorptiometer unit in the starch-iodine complex. A typical preparation of yeast branching enzyme has an activity of 66 units/g.

Effect of temperature and pH. Figs. 2 and 3 show that action of yeast branching enzyme is optimum at about 20° and pH 7.0.

Effect of possible activators. To determine the effect of maltose, maltotriose, cellobiose and glyco-

gen on the action of yeast branching enzyme on amylose, the following digests were prepared: amylose (2.9 mg.), yeast branching enzyme (15 mg.), 0.1M-sodium citrate buffer (pH 6.8; 0.5 ml.), activator (5 mg.) and water (3.5 ml.). The reaction was followed by iodine staining. The rate of decrease of absorption value during incubation for 40 min. in a control digest was the same as observed above. The maltosaccharides and cellobiose thus have no effect.

Effect of inhibitors. (a) Mercuric chloride. Digests containing amylose (2 mg.), yeast branching enzyme (10 mg. in 0.2M-sodium citrate buffer, pH 6.8, 0.5 ml.) and mercuric chloride solution (to 2 ml.) were incubated at 20° for 30 min. The results (Table 1) show that yeast branching enzyme

Fig. 3. Effect of pH on activity of yeast branching enzyme. Yeast branching enzyme (100 mg. in 10 ml. of water) was dialysed at 20° against distilled water for 12 hr. Digests contained amylose (2.9 mg.), dialysed yeast branching enzyme solution (1.5 ml.) and 0.2M-sodium phosphate buffer (1.5 ml.). Absorption values (Spekker absorptiometer readings) were taken after incubation at 20° for 15 min.

Fig. 2. Effect of temperature on activity of yeast branching enzyme. Amylose (1 mg.) and yeast branching enzyme (20 mg. in 0.1M-sodium citrate buffer, pH 6.8; 1 ml.) were incubated at various temperatures for 30 min. The reaction was followed by measurements of absorption values with a Spekker absorptiometer.

Table 1. *Effect of mercuric chloride on the activity of yeast branching enzyme on amylose*

See text for composition of digests. Absorption values are given in Spekker absorptiometer units.

Concn. of mercuric chloride (mm)	Decrease in absorption value	Inhibition (%)
0	0.65	0
0.5	0.00	100
0.2	0.39	40
0.1	0.54	17
0.05	0.60	8

is completely inhibited by 0.5 mM-mercuric chloride.

(b) Ammonium molybdate. Amylose (4.5 mg.), 0.2M-sodium citrate buffer (pH 6.8; 1 ml.), yeast branching enzyme (25 mg.) and ammonium molybdate (12%, w/v) solution (final vol. 5 ml.) were incubated at 20°. The absorption values of the amylose-iodine complexes were measured at intervals. The results, summarized in Table 2, show that yeast branching enzyme is only partly inhibited by 2% (w/v) ammonium molybdate.

Action on amylose

Amylose (40 mg.), 0.2M-sodium citrate buffer (pH 6.8; 5 ml.), yeast branching enzyme (110 mg.) and water (to 25 ml.) were incubated at 20°. After 1, 2 and 4 hr. enzyme action resulted in a 66, 74 and 81% decrease in absorption value at 680 m μ , and the β -amylolysis limit fell from 84.1% to 50.4, 49.3 and 48.6% respectively. These results differentiate yeast branching enzyme from an α -amylase.

The change in absorption spectrum of the amylose-iodine complex during action of yeast branching enzyme is shown in Fig. 4. After prolonged incubation the residual polysaccharide showed maximum absorption at 520 m μ , the solution giving a brown stain. No increase in reducing power was detected.

The above-described changes indicate the formation of branch points in the amylose, the nature of which was examined enzymically. Amylose (32 mg.), 0.2M-sodium citrate buffer (pH 6.8; 4 ml.) and yeast branching enzyme (80 mg. in 4 ml. of water) were incubated at 20° for 4.5 hr. The enzyme was inactivated by heating (3 min. at 98°) and the digest was cooled and divided into three parts. To part A, water (1 ml.) and β -amylase solution (2 ml.; 800 units) were added; to part B, water (1 ml.) and α -amylase solution (2 ml.) were added. After incubation at 35° for 24 hr., the apparent percentage conversions into maltose were A, 45.4; B, 91.9. To part C, isoamylase solution (25 mg. in 1 ml. of water) was added. After incubation at 20° for 18 hr., the isoamylase was deactivated by heating (3 min. at 98°), cooled and β -amylase solution (2 ml.; 800 units) added. The β -amylolysis limit was 65.3%. The results show that the branched polysaccharide synthesized from amylose still contains a high proportion of α -1:4-glucosidic linkages, and that many of the newly formed inter-chain linkages are susceptible to isoamylase. Since isoamylase is specific for α -1:6-glucosidic linkages (Manners & Khin Maung, 1955b; Gunja, Manners & Khin Maung, in preparation), this characterizes many of the new inter-chain linkages.

The above evidence is consistent with the conversion of amylose by yeast branching enzyme into an amylopectin-type polysaccharide. The properties of the latter were then studied in detail.

Preparation of synthetic amylopectin

Amylose-butanol complex (about 0.5 g.) dissolved in water (250 ml.) was incubated at 20° with yeast branching enzyme (900 mg. in 10 ml. of

Table 2. Effect of ammonium molybdate on activity of yeast branching enzyme on amylose

See text for composition of digests. Absorption values are Unicam spectrophotometer readings at 630 m μ .

Time of incubation (hr.)	...	...	Decrease in absorption value (%)		
			1.0	2.5	4.5
Control			26	41	58
Ammonium molybdate (1%, w/v)			21	32	50
Ammonium molybdate (2%, w/v)			16	22	29

Fig. 4. Light-absorption curves of iodine-stained products during action of yeast branching enzyme on potato amylose. Age of digest (min.): (a) 0; (b) 10; (c) 30; (d) 60; (e) 120.

Table 3. Conversion of amylose into an amylopectin-type polysaccharide by yeast branching enzyme

For composition of digest see text. Absorption values are Unicam spectrophotometer readings at 630 m μ .

Time of incubation (hr.)	Decrease in absorption value (%)	$\lambda_{\max}$ (m μ)	β -Amylolysis limit (%)
0	0	630	97.1
2	20.0	—	78.8
6	29.8	—	73.8
10	38.5	585	70.2
20	61.6	570	60.8
25	63.1	570	59.0
30	67.7	570	58.3
48	77.7	550-555	55.1

Table 4. *Effect of yeast branching enzyme on the absorption value of various amylopectin-type polysaccharides*

Polysaccharide (30–40 mg.), 0.2M-sodium citrate buffer (pH 6.8; 5 ml.), yeast branching enzyme (20–100 mg.) and water in a final vol. of 25 ml. were incubated at 20°. Portions (0.5 or 1.0 ml.) were stained with iodine, at intervals. A Spekker absorptiometer (filter no. 603) was used for waxy-maize starch and a Unicam SP. 500 spectrophotometer at 540 m μ for malted-barley amylopectin and β -dextrin, and at 555 m μ for synthetic amylopectin, to measure the absorption values.

Time of incubation (hr.)	Absorption value			
	Waxy-maize starch	β -Dextrin	Malted-barley amylopectin	Synthetic amylopectin
0	0.160	0.429	0.295	0.525
2	0.085	—	—	—
3	—	0.392	0.270	0.392
4	0.075	—	—	—
24	0.050	0.375	0.230	0.265
48	—	—	—	0.220

Table 5. *Effect of yeast branching enzyme on the β -amylolysis limit of potato amylopectin*

The digest contained potato amylopectin I (42 mg.), 0.2M-sodium citrate buffer (pH 6.8; 5 ml.), yeast branching enzyme (70 mg.) and water to 25 ml. Portions (5 ml.) were removed at intervals, heated (98° for 3 min.), cooled and β -amylase solution (3 ml.; 1200 units) was added. After incubation at 35° for 24 hr. the maltose content was determined.

Time of incubation (hr.)	0	1	2	3
β -Amylolytic limit (%)	59.6	53.0	52.2	49.5

water) and 0.2M-sodium citrate buffer (pH 6.8; 40 ml.). Portions were withdrawn at intervals for measurement of absorption value (0.5 ml.) and β -amylolysis limit (4.5 ml.). The latter solution was heated (3 min. at 98°) to inactivate yeast branching enzyme, cooled and β -amylase solution (500 units in 1 ml. of 0.2M-sodium acetate buffer, pH 4.6) was added, with water to 50 ml. After incubation at 35° for 24 hr., the maltose content was determined. The results are summarized in Table 3, and indicate the absence of isoamylase from the preparation of yeast branching enzyme.

After 48 hr. the digest was heated (15 min. at 98°), cooled and dialysed against successive changes of distilled water for 48 hr. at room temperature. The polysaccharide-protein mixture was then concentrated *in vacuo* at 35° to one-fifth of the original volume. Since the polysaccharide was only partly soluble, coagulated protein could not be removed by filtration. Deproteinization was therefore effected by the toluene-sodium chloride method (Anderson & Greenwood, 1955). Sodium chloride to 0.1M-concentration was added and the mixture shaken with freshly distilled toluene ($\frac{1}{3}$ vol.) for 18 hr. The denatured protein was removed from the toluene-water interface and the aqueous solution collected. The deproteinization was repeated three times. The final aqueous solution

was dialysed (24 hr., against distilled water) and the polysaccharide isolated by freeze-drying.

The product had the following properties: glucose content, 81%; α -amylolysis limit, 89%; β -amylolysis limit, 57% (before) and 82% (after) treatment with isoamylase; $[\eta]$ in M-potassium hydroxide, 55.0; the iodine complex showed $\lambda_{\max}$ 550–555 m μ and absorption value $\lambda_{\max}$ 1.30. These properties are typical of amylopectin-type polysaccharides (see Table 9). On incubation with yeast branching enzyme a further decrease in iodine-staining power was observed (Table 4).

Action on amylopectin-type polysaccharides

Iodine-staining measurements. Incubation with amylase-free yeast branching enzyme resulted in a marked decrease in iodine-staining power (Table 4), although the rate was much greater with waxy-maize starch and the synthetic amylopectin, both of which have a lower degree of branching than the malted-barley amylopectin and amylopectin β -dextrin. This behaviour distinguishes yeast branching enzyme from the plant Q-enzymes (Peat, Turvey & Jones, 1959).

β -Amylolytic. Under similar conditions an appreciable (10%) decrease in the β -amylolysis limit of potato amylopectin was observed (Table 5).

In view of these results, the product of action of yeast branching enzyme on potato amylopectin was investigated in detail.

Preparation of synthetic glycogen

Amylopectin (1.3 g.; sample II, prepared by thymol fractionation of potato starch var. Kerr's Pink) was dissolved in water (100 ml.). To the solution after filtration through sintered glass (G. 4) was added 0.2M-sodium citrate buffer (pH 6.8; 30 ml.) and an aqueous solution of yeast branching enzyme (900 mg.; 20 ml.), and the digest was incubated at 20°. Samples (0.5 or 1.0 ml.) were withdrawn at intervals for determination of absorption

value (Unicam spectrophotometer at 540 m μ) and β -amylolysis limit. The results are shown in Table 6.

After 24 hr. the enzyme was inactivated by heating at 98° for 15 min. Coagulated protein was removed by filtration (G. 3 and G. 4 filters) and the filtrate dialysed against running distilled water at room temperature for 48 hr. The solution was concentrated *in vacuo* and the polysaccharide isolated by freeze-drying.

The product had the following properties: glucose content, 69 %; α -amylolysis limit, 82 %; β -amylolysis limit, 46 %; β -amylolysis limit after pretreatment with isoamylase, 66 %.

The polysaccharide was purified by treatment with ice-cold 4 % trichloroacetic acid. The freeze-dried material was dissolved in water (90 ml.), cooled to 0° and 40 % trichloroacetic acid (10 ml.) was added slowly with stirring. After 18 hr. a small precipitate was removed by centrifuging at 0°, and 2 vol. of cold absolute ethanol was added to the solution. The polysaccharide was dissolved in water, reprecipitated with ethanol, washed with ethanol and dried over P₂O₅. Yield, approx. 0.9 g.

The purified polysaccharide was freely soluble in cold water and the solution gave a red-brown stain with iodine. It had the following properties: $[\alpha]_D + 198^\circ$ (c, 0.2 in water); glucose content, 95 %; α -amylolysis limit, 79 %; β -amylolysis limit, 47 %; β -amylolysis limit after pretreatment with isoamylase, 75 %; the iodine complex had $\lambda_{\max}$ 480 m μ and absorption value $\max$ 0.56; $[\eta]$ in 0.1 M-sodium chloride, 11.7. The combined action of isoamylase

and β -amylase gave 90 % conversion into maltose; under similar conditions glycogen and amylopectin gave 95 and 84 %.

The polysaccharide (241 mg.) was oxidized with a saturated solution of potassium metaperiodate (120 ml.) at room temperature. Samples (10 ml.) were analysed for formic acid at intervals; after 10, 12, 15 and 16 days, 0.68, 0.82, 0.86 and 0.86 ml. of 0.01013 N-sodium hydroxide were required for neutralization. The final titre corresponds to an average chain length of 13.5 glucose residues.

The remaining solution of periodate-oxidized glycogen (70 ml.) was filtered and neutralized with ethylene glycol (5 ml.). After dialysis against running tap water at room temperature for 3 days the solution was concentrated (5 ml.). A part (1 ml.) of the solution was hydrolysed with 2 N-sulphuric acid at 100° for 2 hr., neutralized with barium carbonate, concentrated and deionized with Amberlite IR-4B and IR-120 resins. No glucose could be detected by paper chromatography. The remaining solution was treated with potassium borohydride (20 mg.) at room temperature for 3 days. The polyalcohol was precipitated with ethanol, washed with ethanol and then hydrolysed with 2 N-sulphuric acid (2 ml.). The neutralized deionized hydrolysate did not contain glucose (tested by paper chromatography).

Action on glycogen-type polysaccharides

Yeast branching enzyme had no significant effect on the iodine-staining power or β -amylolysis limit of glycogen or Floridean starch [the latter is an algal polysaccharide closely resembling glycogen in many properties (Fleming *et al.* 1956)], as shown in Table 7. The results also confirm the absence of isoamylase from the preparation of yeast branching enzyme.

DISCUSSION

This investigation shows that extracts of brewer's yeast contain an active branching enzyme (systematic name amylo-1:4 $\rightarrow$ 1:6-transglucosidase) in addition to the previously reported phosphorylase and isoamylase. This finding has been confirmed

Table 6. *Conversion of amylopectin into a glycogen-type polysaccharide by yeast branching enzyme*

See text for composition of digest.

Time of incubation (hr.)	Decrease in absorption value (%)	β -Amylolysis limit (%)
0	0	53.2
2	29.6	52.0
5	39.8	51.5
6.5	47.0	50.5
8	56.0	49.5
18	92.0	47.2

Table 7. *Effect of yeast branching enzyme on the absorption value and β -amylolysis limit of glycogen-type polysaccharides*

For composition of digests see Table 4. (a) Absorption value; (b) β -amylolysis limit.

Time of incubation (hr.)	Rabbit-muscle glycogen		Horse-muscle glycogen		Floridean starch	
	(a) (480 m μ)	(b) (%)	(a) (480 m μ)	(b) (%)	(a) (540 m μ)	(b) (%)
0	0.105	40	0.119	55	0.227	35
3	0.098	41	—	—	0.230	34
24	0.101	41	0.117	56	0.226	35
48	0.108	—	0.118	—	—	—

independently by Hopkins (1955). In the course of purification of yeast phosphorylase (D. J. Manners & Khin Maung, unpublished work), protein fractions precipitated with ethanol showed the least phosphorylase activity and the greatest activity of yeast branching enzyme. Fractionation of yeast proteins with ethanol-sodium citrate at low temperature, as used by Gilbert & Patrick (1952) for the purification of potato Q-enzyme, was therefore carried out. Although all the fractions showed some activity, that obtained at 25-30 % ethanol concentration was most active; the partly purified enzyme was finally isolated by freeze-drying in the presence of sodium citrate buffer, pH 6.8. This method has been applied to the related potato enzymes (Barker, Bourne, Wilkinson & Peat, 1950). The majority of the preparations of yeast branching enzyme were substantially free from phosphorylase and isomylase but contained traces of maltase and isomaltase, the presence of which does not affect the interaction between yeast branching enzyme and starch-type polysaccharides. The detection of traces of α -amylase is extremely difficult; for example, some preparations of β -amylase contain an α -amylase (Z-enzyme) which appears to attack amylopectin β -dextrin but not glycogen β -dextrin (W. L. Cunningham, D. J. Manners, A. Wright & I. D. Fleming, unpublished work). With this proviso, the results in Fig. 1 and Table 7 suggest that a normal α -amylase is not a significant contaminant of the preparation of yeast branching enzyme. Hopkins (1955) has also concluded that α -amylase is absent from brewery yeasts.

The action of yeast branching enzyme on both amylose and amylopectin results in a decrease in both iodine-staining power and β -amylolysis limit (Tables 3-6) without the concomitant production of reducing groups. The introduction of branch points into the substrate rather than random hydrolysis is therefore indicated. Enzyme action on amylose is optimum at pH 7.0 and 20°, these conditions being similar to those of other branching enzymes (Table 8). Yeast branching enzyme, like potato Q-enzyme, is completely inhibited by mercuric chloride, but only partially by ammonium molybdate and does not require a carbohydrate activator or co-substrate. In this respect, *Polytomella coeca* Q-enzyme, the action of which is markedly stimulated by certain carbohydrates (Barker, Bebbington

& Bourne, 1953), would appear to be unique (see also Lerner & Uwah, 1956).

Action on amylose

The action of yeast branching enzyme results in a marked change in iodine-staining properties, shown by: (1) a large and rapid decrease in absorption value at all wavelengths, and especially at 680 m μ ; (2) a displacement in λ_{max} from 630 to about 520-550 m μ . These changes are accompanied by a 50 % decrease in β -amylolysis limit. Since some of the newly formed inter-chain linkages can be hydrolysed by isomylase, this indicates that they are α -1:6-glucosidic in nature. These results may be compared with the similar action of potato Q-enzyme, and the apparent inability of the animal branching enzymes to attack this polysaccharide (Barker & Bourne, 1953; Cori & Cori, 1943).

Under our experimental conditions amylose is converted into an amylopectin-type polysaccharide. This was difficult to purify, and analysis was confined to iodine-staining and enzymic degradation, since protein impurities interfere with periodate oxidation. Nevertheless, the results obtained (Fig. 5, and Table 9) clearly show the close structural relationship between the synthetic and a natural (potato) amylopectin, particularly with regard to P_M values on α -amylolysis, which are directly related to the degree of branching.

The synthetic amylopectin represents an intermediate in the conversion of amylose into glycogen since on reincubation with yeast branching enzyme further branching takes place. The relative proportion of branching enzyme is therefore a major factor in controlling the degree of branching, in accord with the findings of Barker, Bourne, Peat & Wilkinson (1950).

Action on amylopectin

In contrast with plant Q-enzymes, yeast branching enzyme has a marked action on amylopectin-type polysaccharides. On incubation with waxy-maize starch (or its β -dextrin), potato amylopectin or malted-barley amylopectin, the iodine-staining power or β -amylolysis limit of the substrate or both are appreciably reduced (Tables 4-6). The end-product of action of yeast branching enzyme on potato amylopectin appears to be a glycogen-type polysaccharide since the extent of

Table 8. Properties of branching enzymes

Source	Optimum pH	Optimum temperature	Reference
Potato Q-enzyme	7.0	21 $\pm$ 1°	Barker, Bourne & Peat (1949)
Broad-bean Q-enzyme	7.25-7.5	20	Hobson, Whelan & Peat (1950)
Green-gram Q-enzyme	7.0	24	Ram & Giri (1952)
<i>Polytomella coeca</i> Q-enzyme	7.3	25-33	Bebbington, Bourne, Stacey & Wilkinson (1952)
Yeast branching enzyme	7.0	20	—

Table 9. *Properties of amylopectin-glycogen-type polysaccharides*

Property	Potato amylopectin	Synthetic amylopectin	Rabbit-liver glycogen	Synthetic glycogen	Brewer's-yeast glycogen
Average chain length (glucose residues)	22.0	—	12.6	13.5	13.2
Proportion of 1:6-linkages (%)	4.5	—	8.0	7.4	7.6
Iodine complex					
$\lambda_{\max.}$ (m μ)	545	550–555	460	480	430
Absorption value $\max.$	1.30	1.30	0.34	0.56	0.34
α -Amylolysis (P_M value)	89	89	78	79	79
β -Amylolysis limit (%)*					
Before pretreatment	53	57	46	47	44
After pretreatment	80	82	78	75	68
Limiting viscosity number					
In 1 M-potassium hydroxide	205	55	—	—	—
In 0.1 M-sodium chloride	—	—	10	12	7

* 'Before' and 'after' refer to pretreatment with yeast isoamylase.

Fig. 5. Light-absorption curves of iodine-stained polysaccharides. Solutions contained 0.01% of polysaccharide (A, synthetic amylopectin; B, potato amylopectin; C, synthetic glycogen) and 0.02% of iodine in 2.0% potassium iodide, and were read against an iodine-iodide reference solution in a Unicam SP. 500 spectrophotometer. The curve for rabbit-liver glycogen (D) is taken from Liddle (1956).

enzymic degradation, end-group content and physical properties (especially limiting viscosity number) are very similar to those of brewer's-yeast glycogen and a normal sample of rabbit-liver glycogen (Table 9). It follows that two classes of 'branching enzyme' may be distinguished: (a) those that have no action on amylopectin, e.g. potato Q-enzyme (Peat *et al.* 1959); (b) those acting on amylopectin, e.g. liver branching enzyme (Larner, 1955). For this reason it is suggested that the term 'Q-enzyme' be confined to the former class of enzymes (cf. Hopkins, 1955).

The present results suggest that the enzymic conversion of amylose into glycogen occurs through an intermediary amylopectin-type polysaccharide, in agreement with generally accepted views, as reviewed by Barker & Bourne (1953). However, Erlander (1958) has proposed that the starch components are produced by the action of a debranching enzyme (not yet detected or isolated) on glycogen, and that amylopectin is a partly debranched glycogen. This theoretical reaction is virtually the reverse of that now demonstrated *in vitro*. It is clear that the detailed mechanism of glycogen and amylopectin synthesis *in vivo* requires further investigation, and that the pathways for synthesis and degradation may, in fact, involve separate enzyme systems [cf. Villar-Palsai & Larner (1958) Robbins, Trant & Lipmann (1959)].

On the basis of partial acid-hydrolysis (Wolfrom & Thompson, 1956, 1957) and periodate-oxidation studies (Abdel-Akher, Hamilton, Montgomery & Smith, 1952; Hamilton & Smith, 1956) it has been suggested that both amylopectin and glycogen contain a small proportion of α -1:3-glucosidic linkages. In view of this, the nature of the inter-chain linkages introduced by yeast branching enzyme into amylopectin was examined, as follows: (a) periodate-oxidized synthetic glycogen was converted into the corresponding polyalcohol and hydrolysed with acid: glucose could not be detected; (b) the outermost inter-chain linkages of the synthetic glycogen are, like those of the parent amylopectin, susceptible to isoamylase, which has no action on α -1:3-glucosidic linkages; (c) the combined action of β -amylase and isoamylase causes 90% degradation. In view of the relatively small amount of synthetic glycogen available for analysis (the chemical studies by the workers cited above

required 10–100 g. quantities), our results are considered to provide qualitative evidence against the synthesis of a significant proportion of α -1:3-glucosidic linkages.

Action on other polysaccharides

The results in Table 7 show that yeast branching enzyme has no significant action on the β -amylolysis limit and iodine-staining properties of normal glycogen (muscle glycogen was used since it is more iodophilic than yeast glycogen). A polysaccharide containing 8% of α -1:6-glucosidic linkages probably represents the highest degree of branching obtainable under these conditions *in vitro*. However, the failure of yeast branching enzyme to act on horse-muscle glycogen (average chain length 17) and ability to react slowly with malted-barley amylopectin (average chain length 18) suggests that the specificity of yeast branching enzyme is not entirely controlled by the degree of branching in the substrate; further, 'glycogen' and 'amylopectin' may differ structurally in an as yet unknown way.

The appreciable action of yeast branching enzyme on amylopectin β -dextrin shows that chains carrying 'stubs' of two to three glucose residues can be transferred intact. This provides further evidence for the view that yeast branching enzyme has a rather wider specificity than its plant and animal counterparts.

SUMMARY

1. Extracts of brewer's yeast contain a branching enzyme (amylo-1:4 $\rightarrow$ 1:6-transglucosidase) which may be isolated by ethanol-sodium citrate fractionation at -5° .

2. Enzyme action on amylose is optimum at pH 7.0 and 20° , and is inhibited by mercuric chloride (0.5 mM).

3. The reaction is characterized by a marked decrease in iodine-staining power and β -amylolysis limit of the substrate without the concomitant production of free reducing groups.

4. Enzyme action on amylose yields initially an amylopectin-type polysaccharide; on incubation with further enzyme, amylopectin is converted into a glycogen-type polysaccharide.

5. The mode of synthesis of glycogen and the properties of related animal and plant branching enzymes are discussed.

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REFERENCES

- Abdel-Akher, M., Hamilton, J. K., Montgomery, R. & Smith, F. (1952). *J. Amer. chem. Soc.* **74**, 4970.
 Anderson, D. M. W. & Greenwood, C. T. (1955). *J. Sci. Fd Agric.* **6**, 587.
 Archibald, A. R. & Manners, D. J. (1959). *Biochem. J.* **73**, 292.
 Aspinall, G. O., Hirst, E. L. & McArthur, W. (1955). *J. chem. Soc.* p. 3075.
 Barker, S. A., Bebbington, A. & Bourne, E. J. (1953). *J. chem. Soc.* p. 4051.
 Barker, S. A. & Bourne, E. J. (1953). *Quart. Rev. Chem. Soc., Lond.*, **7**, 56.
 Barker, S. A., Bourne, E. J. & Peat, S. (1949). *J. chem. Soc.* p. 1705.
 Barker, S. A., Bourne, E. J., Peat, S. & Wilkinson, I. A. (1950). *J. chem. Soc.* p. 3022.
 Barker, S. A., Bourne, E. J., Wilkinson, I. A. & Peat, S. (1950). *J. chem. Soc.* p. 84.
 Bebbington, A., Bourne, E. J., Stacey, M. & Wilkinson, I. A. (1952). *J. chem. Soc.* p. 240.
 Bourne, E. J., Donnison, G. H., Peat, S. & Whelan, W. J. (1949). *J. chem. Soc.* p. 1.
 Bourne, E. J., Haworth, W. N., Macey, A. & Peat, S. (1948). *J. chem. Soc.* p. 924.
 Cori, G. T. & Cori, C. F. (1943). *J. biol. Chem.* **151**, 57.
 Erlander, S. R. (1958). *Enzymologia*, **19**, 18.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Fleming, I. D., Hirst, E. L. & Manners, D. J. (1956). *J. chem. Soc.* p. 2831.
 Gilbert, G. A. & Patrick, A. D. (1952). *Biochem. J.* **51**, 181.
 Green, D. E. & Stumpf, P. K. (1942). *J. biol. Chem.* **142**, 355.
 Greenwood, C. T. & Robertson, J. S. M. (1954). *J. chem. Soc.* p. 3769.
 Gunja, Z. H. & Manners, D. J. (1959). *Chem. & Ind.* p. 1017.
 Hamilton, J. K. & Smith, F. (1956). *J. Amer. chem. Soc.* **78**, 5910.
 Hobson, P. N., Whelan, W. J. & Peat, S. (1950). *J. chem. Soc.* p. 3566.
 Hopkins, R. H. (1955). *European Brewery Convention Congress*, p. 52.
 Larner, J. (1955). In *Methods in Enzymology*, vol. 1, p. 222. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Larner, J. & Uwah, D. N. (1956). *J. Amer. chem. Soc.* **78**, 3647.
 Lawrie, R. A., Manners, D. J. & Wright, A. (1959). *Biochem. J.* **73**, 485.
 Liddle, A. M. (1956). Ph.D. Thesis: University of Edinburgh.
 Liddle, A. M. & Manners, D. J. (1957). *J. chem. Soc.* p. 3432.
 Manners, D. J. & Khin Maung (1955a). *J. chem. Soc.* p. 867.
 Manners, D. J. & Khin Maung (1955b). *Chem. & Ind.* p. 950.

- Manners, D. J. & Khin Maung (1956). *Biochem. J.* **63**, 16 p.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Peat, S., Turvey, J. R. & Jones, G. (1959). *J. chem. Soc.* p. 1540.
- Ram, J. S. & Giri, K. V. (1952). *Arch. Biochem. Biophys.* **38**, 231.
- Robbins, P. W., Trant, R. R. & Lipmann, F. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 6.
- Robinson, H. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 727.
- Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
- Villar-Palsai, C. & Larnier, J. (1958). *Biochim. biophys. Acta*, **30**, 449.
- Whelan, W. J. (1955). In *Methods in Enzymology*, vol. 1, p. 195. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Whelan, W. J. (1958). In *Encyclopedia of Plant Physiology*, vol. 6, p. 154. Ed. by Ruhland, W. Berlin: Springer-Verlag.
- Wolfson, M. L. & Thompson, A. (1956). *J. Amer. chem. Soc.* **78**, 4116.
- Wolfson, M. L. & Thompson, A. (1957). *J. Amer. chem. Soc.* **79**, 4212.

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Studies on Vitamin E

1. THE DETERMINATION OF TOCOPHEROLS IN ANIMAL TISSUES

By E. E. EDWIN, A. T. DIPLOCK, J. BUNYAN AND J. GREEN
Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

(Received 13 July 1959)

In connexion with a programme designed to study the vitamin E status of animals under different nutritional conditions, a rapid and accurate method of determining tocopherols in animal tissues was required. Two problems exist: the extraction, without loss, of the material sensitive to oxidation and its separation from large quantities of other reducing substances before determination.

The extraction problem was of most immediate interest and has received considerable attention from previous workers in the field. Apart from blood and milk, which cause no special extraction difficulties, some kind of comminution or homogenization of the tissue is usually necessary, and a number of different methods have been used by previous workers. Karrer, Jaeger & Keller (1940) chopped organs as finely as possible and extracted them with ethanol-benzene (1:1). Devlin & Matthill (1942) homogenized rat muscle in the Waring Blendor with the foaming solvent mixture (Skellysolve B-ethanol; 8.5:10.0) of Moore & Ely (1941), three separate extractions being used to ensure complete extraction. Hines & Matthill (1943), in the first systematic study of the vitamin E content of animal tissues, used the same procedure. Emmerie & Engel (1943) first introduced the use of boiling ethanol as solvent. Kaunitz & Beaver (1946) adopted a lengthy procedure, emulsifying muscle first with large quantities of acetone in the Waring Blendor and then with several mixtures of acetone and Skellysolve B. Even after this procedure, reducing substances could still be extracted from the tissue. Kringstad

& Folkvord (1949), studying cod roes, homogenized the tissue and extracted with ether. Wanntorp & Nordlung (1947) and more recently Benze (1954) treated the problem by directly saponifying the whole tissue, but recoveries from this procedure are not given. Quaife & Dju (1949), in one of the best studies on vitamin E analysis, criticized both saponification techniques and the use of the Waring Blendor, and they adopted a dry homogenization technique, which they considered essential for the analysis of animal tissues. The tissues were first diced and then frozen in liquid nitrogen, ground in an iron mortar at -70° , sieved and finally extracted with hot ethanol vapour. A 24 hr. extraction period was necessary to remove reducing substances completely. Bratzler, Loosli, Krukovsky & Maynard (1950), studying tocopherol levels in the pig, freeze-dried the tissues and then ground the organs in a Wiley mill and the muscle in a Waring Blendor with solid carbon dioxide, finally extracting with ethanol vapour for 24 hr. Swick & Baumann (1952) used a similar technique, but homogenized smaller samples in ethanol before extraction in a Soxhlet apparatus for 18 hr. Draper & Csallany (1958) comminuted samples of rat and rabbit tissue by grinding with acid-washed sand.

The problems connected with the removal of the extensive reducing impurities from the tocopherol extract were attacked by earlier workers in many different ways and have led to extremely variable estimates for the vitamin E contents of animal tissues. The various analytical modifications that