SUMMARY

1. The observations of Slein (1952) on the non-phosphorylation of galactosamine by ATP and yeast hexokinase have been confirmed.

2. A method is presented for separating galactosamine from glucosamine before assaying it.

3. The method is applied to the analysis of hydrolysates of *Aspergillus niger* cell wall.

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Studies on Carbohydrate-Metabolizing Enzymes

9. THE ACTION OF ISOAMYLASE ON AMYLOSE*

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The incomplete degradation of amylose by β-amylase was first shown by Peat, Pirt & Whelan (1952*a*, *b*), who reported β-amylolysis limits of only 68–70% for various samples of amylose. These observations have been confirmed by several workers, e.g. Hopkins & Bird (1953), Neufeld & Hassid (1955), Cowie, Fleming, Greenwood & Manners (1957). Peat *et al.* (1952*a*, *b*) also showed that impure preparations of β-amylase caused complete degradation of amylose owing to the concurrent action of β-amylase and a second carbohydrase, 'Z-enzyme'. At this time (1952–3) Z-enzyme was believed to exert a debranching action on amylose (Peat & Whelan, 1953), and, since Z-enzyme preparations showed β-glucosidase activity, it was suggested that the barriers to β-amylase were β-glucosidic linkages (Peat, Thomas & Whelan, 1952*c*). However, later work (Cunningham, Manners, Wright & Fleming, 1960; Banks, Greenwood & Jones, 1960) has shown that Z-enzyme activity is caused by a trace of α-amylase, and involves the random hydrolysis of a small number of α-(1→4)-glucosidic linkages. The barriers to β-amylase in amylose are therefore bypassed by Z-enzyme, and not selectively removed as was originally suggested.

An additional complication in this work was the finding by Gilbert (1958), later confirmed by others (Banks, Greenwood & Thomson, 1959; Liddle, Manners & Wright, 1961), that oxygen treatment of amylose introduces barriers to enzymic degradation. It is therefore necessary to prepare amylose under anaerobic conditions to avoid inadvertent modification of the substrate, as almost certainly occurred in the earlier studies.

The nature of the barriers to β-amylase has been the subject of several investigations. Possibilities include (*a*) an anomalous linkage [i.e. other than the α-(1→4)-glucosidic type] in the middle of an amylose chain, (*b*) a branch point, or (*c*) the presence of ester phosphate groups, although treatment of amylose with phosphatase preparations does not increase the β-amylolysis limit (Peat *et al.* 1952*c*; Banks & Greenwood, 1961). We have therefore re-examined the possibility that the anomalous linkages represent α-(1→6)-glucosidic inter-chain linkages of the type present to the extent of 4–5% in amylopectin.

The presence of anomalous linkages in amylose is inferred from the specificity requirements of

* Part 8: Cunningham, Manners & Wright (1962).

Table 1. *Distribution of enzymes in the fractions obtained by precipitation with acetone*

Isoamylase activity is given in the units defined in the Methods and Materials section; maltase and isomaltase activities represent increases in reducing-sugar content, expressed as mg. of apparent glucose (Somogyi estimation); α-amylase activity is given as mg. of apparent maltose; branching activity represents the percentage decrease in iodine-staining power of soluble starch, part of which is due to α-amylase.

Fraction no.	Isoamylase	Maltase	Isomaltase	α-Amylase	Branching enzyme
1	22	0·01	0·00	0·00	9
2	131	0·16	0·00	0·00	1
3	56	0·49	0·08	0·15	29
4	—	0·28	0·23	0·25	25
5	—	0·00	0·15	0·05	23

β-amylase and phosphorylase and their proportion (probably 0·1 %) is too low for analysis by conventional chemical methods. However, the outer α-(1→6) inter-chain linkages in amylopectin are hydrolysed by the specific debranching enzymes R-enzyme (Hobson, Whelan & Peat, 1951) and isoamylase (Gunja, Manners & Khin Maung, 1961). The former has not been completely separated from α-amylase, so that isoamylase was chosen for detailed study.

Isoamylase was previously prepared from brewer's yeast, but, although the preparation was free from α-amylase, the removal of maltase impurity was difficult. (During the degradation of a starch polysaccharide by the combined action of β-amylase and isoamylase, maltase impurity would cause erroneously high apparent percentage conversions into maltose.) Isoamylase is also present in baker's yeast and its purification from this source has therefore been investigated.

We now report that isoamylase hydrolyses both the anomalous linkages in amylose and the inter-chain linkages in amylopectin, thus establishing their probable identity. A preliminary account of these results has appeared (Kjølberg & Manners, 1962).

METHODS AND MATERIALS

Analytical methods. The methods used have been described by Gunja *et al.* (1961), except that, for β-amylolysis, crystalline sweet-potato β-amylase (purchased from the Worthington Biochemical Corp.) dissolved in 0·01 % serum albumin (see Walker & Whelan, 1960) was also used. This sample of β-amylase was free from Z-enzyme (cf. Cunningham, Manners & Wright, 1962).

Unless otherwise stated, 0·1–0·2 M-sodium citrate–sodium phosphate buffer, pH 6·0 or pH 8·0, was used.

Measurement of enzymic activity. (*a*) Isoamylase. This activity was assayed by the increase in iodine-staining power of glycogen. Digests containing oyster glycogen (2 mg./ml.; 5 ml.) and enzyme preparation in buffer, pH 6·0 (0·75 mg./ml.; 5 ml.), were prepared and samples (2 ml.) removed at intervals for iodine-staining. Under these conditions, 1 unit of isoamylase activity causes an increase in $E_{435\,m\mu}$ of 0·01 as measured by a Unicam SP. 600 spectrophotometer in 6 hr. Specific activities are expressed per mg. of protein nitrogen.

(*b*) Maltase, isomaltase, yeast branching enzyme and α-amylase. Maltase and isomaltase activities were estimated by incubation of 0·2 % substrate with an equal volume of enzyme solution (0·75 mg. of isoamylase preparation/ml.) in buffer, pH 6·0, for 24 hr. at 20°. Samples were removed for paper chromatography and determination of reducing power.

Yeast branching enzyme was assayed from the decrease in iodine-staining power of soluble starch (cf. Gunja, Manners & Khin Maung, 1960). Digests containing equal volumes of soluble starch (0·1 % solution) and enzyme preparation (0·075 %) in buffer, pH 6·0, were prepared and the percentage decrease in iodine-staining power at 600 mμ was measured at intervals. Samples (5 ml.) were also removed for measurements of reducing power and the results, expressed as mg. of apparent maltose, provide a measure of the α-amylase activity.

Substrates. Amylose was prepared from the following varieties of potato: Great Scot (sample I), Kerr's Pink (sample II), Duke of Kent (sample III), and from oats (amylose V), by fractionation of the whole starch with thymol and butan-1-ol, under nitrogen, as described by Cowie *et al.* (1957). Amylose VI was the sample from potato starch (var. King Edward) described previously (Cowie *et al.* 1957). Incubation of all samples with barley β-amylase containing Z-enzyme gave 98–100 % conversion into maltose, showing the absence of amylopectin; this was also shown by the iodine-binding powers, as measured by potentiometric titration, which were in the range 18·5–19·5 mg. of iodine bound/100 mg. of polysaccharide. The degrees of polymerization of the amylose samples, determined viscometrically (see Cowie *et al.* 1957), were of the order of 2000.

Amylose β-dextrin was prepared by incubation of oat amylose (0·23 g.) with crystalline β-amylase (5600 units) for 21 hr. at pH 4·7 in a total volume of 120 ml. The conversion into maltose was 78 %. Maltose was removed by dialysis and the polysaccharide isolated under nitrogen as the butan-1-ol complex.

Glycogen α-dextrin was the sample L.D. 2 described by Bell & Manners (1951), and contained a small amount of maltose impurity.

Preparation of isoamylase. Dried baker's yeast (300 g.) was extracted as described by Gunja *et al.* (1961) and protein fractions precipitated at −5° to −10° in acetone concentrations of 0–16, 16–28, 28–35, 35–42 and 42–50 % (v/v) were collected, dissolved in water, dialysed at 0° for 18 hr. and freeze-dried in buffer, pH 6·0 (Table 1). The yields of the fractions were: 1, 0·5 g.; 2, 4·5 g.; 3, 0·95 g.; 4, 1·4 g.; 5, 1·05 g. The specific activities of fractions 1, 2 and 3 were

22, 131 and 56 respectively; fractions 4 and 5 were apparently contaminated with α-amylase, since the iodine-staining power of glycogen was decreased.

Fraction 2 (750 mg.) was dissolved in buffer, pH 6·0 (110 ml.), cooled to −5° and protein fractions were precipitated by the addition of cold acetone (A.R.) at −15°, at concentrations of 0–16, 16–25, 25–33 and 33–45% (v/v). After solution in water and dialysis the fractions were freeze-dried in buffer, pH 6·0. The yields were: 2a, 0·2 g.; 2b, 0·25 g.; 2c, 0·2 g.; 2d, 0·3 g. Specific activities of the first three fractions were 17, 159 and 59 respectively.

RESULTS

Properties of baker's-yeast isoamylase

Effect of pH. In sodium citrate–sodium phosphate buffer, isoamylase action was optimum at pH 6·0 but the pH–activity curve was so broad that, at pH 8·0, 70% of the maximum activity was observed. By contrast, the activity of branching enzyme at pH 8·0 was less than 10% of that at pH 6·0, and at pH 8·0 the maltase activity was negligible.

Action on β-limit dextrins. Amylopectin or glycogen β-limit dextrin (0·2%) was incubated with an equal volume of fraction 2 (0·1% in buffer, pH 6·0, equivalent to 1·5 units/mg. of polysaccharide). The iodine-staining power of glycogen β-dextrin increased by 18% within 24 hr., but with amylopectin β-dextrin the increase was negligible (cf. Gunja *et al.* 1961). After 24 hr. the enzyme was inactivated by heating and the pH adjusted to 4·8. The β-amylolysis limits of the amylopectin and glycogen β-dextrins were 25 and 25% respectively, whereas without isoamylase treatment the β-amylolysis limits were 2 and 0%.

Action on panose. Solutions (1 ml.) of panose (0·2%) and isoamylase (fraction 2, 0·1%) were incubated at pH 6·0 and 8·0 for 24 hr. Paper-chromatographic analysis of both digests showed that no hydrolysis had occurred.

Action on α-dextrin. Dextrin (1·85 mg./ml.; 10 ml.) was incubated with an equal volume of isoamylase (1·1 mg./ml. in buffer, pH 6·0 or pH 8·0) at 20°. Samples were removed after 24 and 40 hr. for measurement of reducing power. The results (Table 2) show that the purified isoamylase has no action on glycogen α-dextrin at either pH 6·0 or 8·0. This was confirmed by paper chromatography, which showed the absence of glucose.

Table 2. *Action of isoamylase on α-dextrin*

For composition of digests, see text. Results are expressed as mg. of apparent maltose.

Time of incubation (hr.)	24	40
Substrate control	0·53	0·54
α-Dextrin and fraction 2, pH 6·0	0·70	0·74
α-Dextrin and fraction 2, pH 8·0	0·56	0·55
α-Dextrin and fraction 2b, pH 6·0	0·56	0·56
α-Dextrin and fraction 2b, pH 8·0	0·56	0·55

Tests for enzymic impurities. Fractions 2a and 2b contained a trace of branching enzyme and maltase. At pH 8·0 the branching enzyme was inactivated (there was no change in the iodine-staining power of amylose), and on incubation with maltose the increase in reducing power during 48 hr. was only 2%. Fraction 2c contained a small amount of branching enzyme, but no maltase. Fraction 2b was completely free from α-amylase, since on incubation with linear amylose there was no decrease in specific viscosity. The action of this fraction at pH 8·0 on amylose was therefore examined in detail.

Amylose or its β-dextrin (about 0·15%; 20 ml.) was incubated with 10 ml. of isoamylase fraction 2b (0·25% in 0·1 M-sodium citrate–sodium phosphate buffer, pH 8·0; enzyme concentration, 1·5 units/mg. of polysaccharide). The digests were divided; part (equivalent to about 15 mg. of substrate) was filtered (G.3 sintered-glass filter) into a viscometer, and the specific viscosity at 25° measured after 0·5, 6 and 21 hr. The pH was then adjusted to 5·5 (with acetic acid), sweet-potato β-amylase (800 units) was added and the β-amylolysis limit determined. The second portion of the digests was kept at 20° for 21 hr., heated (5 min. at 98°) to inactivate the enzyme, cooled, the pH was adjusted to 4·8, and the digest filtered. The β-amylolysis limits were measured; the results were constant within 24 hr., but analysis was continued for 40 hr.

Action of purified isoamylase on amylose and its β-dextrin

Action on amylose. Incubation of various samples of amylose with isoamylase (1·5 units/mg. of polysaccharide) resulted in a small but significant increase in β-amylolysis limit and a limited fall in specific viscosity (Table 3). The sizes of the changes were similar with a particular sample of amylose, and indicate that 'debranching' had occurred.

With amylose III and VI, the successive action of isoamylase and β-amylase gave 90% conversion into maltose, a value about 10% less than theoretical. Previous work on isoamylase (Gunja *et al.* 1961) and the related plant R-enzyme (Hobson *et al.* 1951) has shown that the (1→6)-linkages attaching long side chains to the molecule are apparently hydrolysed only with difficulty, since the debranching action on a starch-type polysaccharide is appreciably less than that of the corresponding β-dextrin, which has side chains of only two or three glucose residues. (The natural substrates for these enzymes are phosphorylase limit dextrins with side chains of only four to six glucose

Table 3. *Action of isoamylase on amylose*

For composition of digests, see text. Results are shown before (*b*) and after (*a*) isoamylolysis.

Amylose sample	Specific viscosity (*b*)	Specific viscosity (*a*)	Decrease (%)	β-Amylolysis limit (*b*)	β-Amylolysis limit (*a*)	Increase (%)
I	0·359	0·350	3	97	100	3
II	0·205	0·180	12	90	96	6
III	0·211	0·182	14	85	90*	5
VI	0·199	0·160	20	76	90*	14
V	0·206	0·166	19	76	88*	12
β-Dextrin	0·172	0·170	1	6	77	71

* The combined action of β- and iso-amylase gave β-amylolysis limits of 95% with amylose III, 97% with amylose VI and 98% with amylose V.

residues.) The combined action of iso- and β-amylase on the three samples of amylose was therefore examined; as expected, the β-amylolysis limit was increased further (Table 3). Moreover, the addition of a further 1·5 units of isoamylase/mg. of amylose after 24 hr., followed by heat-inactivation, increased the β-amylolysis limit of amylose V from 88 to 94%.

Action on amylose β-dextrin. Isoamylase caused a large increase in β-amylolysis limit (71%) of this polysaccharide, but without affecting the specific viscosity (Table 3). This result, which represents about 95% degradation of the original amylose, is in full agreement with the proposed action pattern of isoamylase, and provides confirmation of the absence of α-amylase from the enzyme preparation.

DISCUSSION

The isoamylase of baker's yeast shows a similar action to that of the brewer's-yeast enzyme in causing extensive 'debranching' of amylopectin and glycogen β-dextrins by the hydrolysis of outer α-(1→6)-glucosidic inter-chain linkages. The size and structure of the substrate is important since α-(1→6)-linkages in the disaccharide isomaltose, the trisaccharide panose or in oligosaccharide α-dextrins are not hydrolysed.

The results in Table 3 show clearly that isoamylase causes a significant increase in the β-amylolysis limit and a comparable decrease in the specific viscosity of amylose. This therefore indicates that isoamylase has a 'debranching' action on amylose similar to that on amylopectin; moreover, the magnitude of the changes in viscosity is greater than that expected from the removal of small side chains containing perhaps one to three glucose residues. Some of the amylose molecules therefore have a low degree of branching. In view of our demonstration of the heterogeneity of amylose (Cowie *et al.* 1957), branching will be confined to the largest molecules, and probably amounts to only one or two branch points per molecule.

The possibility that amylose is slightly branched has been the subject of some controversy (cf. Meyer, 1952). Potter & Hassid (1951) determined the degree of polymerization and average chain length of several samples of potato and maize amylose and found that in some the average number of non-reducing terminal glucose residues per molecule was considerably greater than 1. In agreement with this conclusion, Kerr & Cleveland (1952) found that the rate of sugar production from potato and tapioca amylose by amyloglucosidase and by β-amylase indicated the presence of one to two and two to three branches per molecule respectively. More recently, studies of the hydrodynamic behaviour of amylose by viscosity, light-scattering and sedimentation methods suggest that limited long-chain branching occurs in certain subfractions of amylose (Greenwood, 1960).

The present results are not caused by gross contamination of the substrates with amylopectin. A certain mixture of linear amylose and amylopectin might have a β-amylolysis limit of 75%, which on isoamylolysis would be increased by 10–15%, but on incubation with β-amylase and Z-enzyme (Cunningham *et al.* 1960) the conversion into maltose would be only 75% whereas the experimental values were 98–100%.

The presence of linkages other than the α-(1→4)-glucosidic type in amylose was first established by Peat *et al.* (1952*a*, *b*), but their suggestion that the anomalous linkages were β-glucosidic, attaching single glucose units to a main chain, has not been proved. Hopkins & Bird (1953) examined the action of almond emulsin (β-glucosidase) on amylose β-dextrin and found that glucose was not produced. More recently, Baba & Kojima (1955) showed that purified β-glucosidase from apricot emulsin had no action on the barriers to β-amylolysis. The presence of β-glucosidic linkages in

amylose is therefore unlikely, particularly since their synthesis would require an enzyme system capable of polymerizing both α- and β-glucose residues. The alternative suggestion that ester phosphate groups are the barriers to β-amylase action is also not supported by experimental evidence.

It is doubtful if the nature of the anomalous linkages (probably only 0·1 %) can be rigorously proved, but their identity as α-(1→6)-glucosidic inter-chain linkages may be inferred as the most probable, in view of the known specificity requirements of isoamylase. The presence of α-(1→6)-glucosidic linkages in amylose would be in accord with, and provide some simplification of, current views on the biosynthesis of starch (for review, see Manners, 1962). These linkages could arise from the action of Q-enzyme on completely linear chains of α-(1→4)-linked glucose residues. It is not therefore necessary to postulate either that additional and as yet unknown starch-metabolizing enzymes are present, or that those already known can, by a chance error, synthesize an anomalous linkage. Further, Whistler & Doane (1961) have shown that starches from high-amylose corn can be fractionated to give polysaccharides intermediate in properties between 'amylose' and 'amylopectin'.

The present results are not in accord with the theory of starch biosynthesis proposed by Erlander (1958), in which a glycogen-type polysaccharide is formed first, and this then undergoes partial debranching to yield amylopectin and short linear chains of α-(1→4)-linked glucose residues, which combine to form amylose.

SUMMARY

1. Incubation of various samples of amylose with isoamylase causes a significant increase in β-amylolysis limit and a comparable decrease in specific viscosity.

2. The results are not due to α-amylase impurity in the enzyme preparation, or to contamination of the amylose with amylopectin.

3. It is concluded that isoamylase exerts a 'debranching' action on amylose, and that the polysaccharide is slightly branched.

4. Since isoamylase appears to be specific for α-(1→6)-glucosidic linkages, the anomalous linkages in amylose are most probably α-(1→6)-glucosidic inter-chain linkages similar to those present in amylopectin.

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