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The Selective Hydrolysis of Amylopectin and Amylose by β -Amylase

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(Received 11 July 1949)

The common starches are now generally recognized as consisting of a branched component (amylopectin) and a linear one (amylose), hydrolysable by β -amylase which attacks the α -glucosidic chains at their non-reducing ends to yield about 50 and 100 % maltose respectively. Potato amylopectin has one such chain end for every 24–26 glucosidic groups present, whereas potato amylose has one to every 200–500 or more.

An investigation has been undertaken of the relative rates of disappearance of these two components of starch during hydrolysis by β -amylase. The proportion of amylose and amylopectin remaining in a partly hydrolysed starch paste can be calculated from the absorption spectra of its iodine colour under standard conditions. If amylopectin is preferentially hydrolysed amylose will accumulate and the iodine colour will be relatively bluer.

MATERIALS AND METHODS

Substrates. Three commercial samples of potato starch, one each of wheat, rice, a soluble starch (A.R.) and a Lintner soluble starch prepared in our laboratory from potato starch were used. Amylose was prepared from potato starch by the thymol precipitation method of Bourne, Donnison, Haworth & Peat (1948), and amylopectin by cyclohexanol and thymol precipitation as described by Hopkins & Jelinek (1948). In some cases the amylopectin was preserved in solution, sterile.

Starch and amylopectin were made into a cream with cold water, poured into boiling water and boiled with continuous mechanical stirring for 15 min. With native starches, 0.05 g. of NaCl/100 ml. was added 10 min. before the end of boiling. Amylose was preserved under butanol and dissolved in hot water freshly for use, the butanol being driven off at about 80–90°.

Preparation of amylopectin. The purest preparation of amylopectin made in these laboratories was obtained by precipitation of potato starch paste with cyclohexanol followed 3 days later by thymol (Hopkins & Jelinek, 1948). It had been stored for 2 years and was difficult to get into solution unless finely ground, and all the precautions required in the preparation of starch paste were employed. A white flocculum was thrown out, apparently retrograded amylose, which was separated and the solution centrifuged clear. There were indications that unless it was well dispersed it hydrolysed only half as rapidly as other specimens of amylopectin. The data on which Fig. 1 was constructed were obtained with this sample.

Preparation of amylose and amylopectin for determination of Michaelis constants. Potato starch was stirred with water at 63° for 15 min., the clear portion separated and the amylose prepared from it by thymol precipitation (amylose I, yield 4.6–6.2 %). The starch residue was dispersed by boiling for 30 min., centrifuged to remove small amounts of residues, and the residual amylose (II) precipitated by thymol, leaving amylopectin II. The amylose preparations were purified and stored as described by Hopkins & Jelinek (1948). The amylopectins were kept in sterile solution, amylopectin II for 3 months and amylopectin III (prepared in the same way as II) for 6 days. (Amylopectin I derived from the 63° infusion (yield 0.8–1.1 %), will be referred to later.)

Amylose I corresponds to the β -amylose of Baldwin (1930) and the amylose of Meyer, Bernfeld & Wolff (1940), but, having been precipitated with thymol, it should be free from amylopectin. In Fig. 2 it is described as 'Baldwin amylose'.

Enzyme preparations. Both β -soya and barley amylase were prepared as described by Hopkins, Jelinek & Harrison (1948).

Reaction mixtures. Conditions of pH, temperature, etc., are given in the appropriate tables or in the text. In view of the effects of salts on spectrometric measurements of iodine coloration, the concentration of buffer solutions was kept down to a 1 % addition of 0.1 M-acetate (for pH 4.6) and citrate-phosphate for pH 5.8 and 7.

Progress of hydrolysis was followed and Michaelis constants were determined as described by Hopkins *et al.* (1948).

Iodine absorption spectra were determined with appropriate solutions in a 1 cm. cell in a Hilger Spekker absorptiometer at 20° using, in combination with the heat filter (H 503), the Ilford filters 608 (6800–7000 Å. transmission), 607 (6000 Å.), 603 (4900 Å.) and 602 (4700 Å.). These solutions contained 10 mg. of I_2 + 100 mg. of KI/100 ml. together with 5 mg. of starch or amylopectin or its equivalent from a reaction mixture undergoing hydrolysis, but in the case of amylose only 1 mg. was present. In all cases the solution was kept at 20° for 2 hr. to enable the full colour to develop, and allowance was made for the colour due to I_2 alone by making the reading against a corresponding I_2 solution.

RESULTS

Calibration of Spekker absorptiometer for the measurement of amylose, amylopectin and α -amylo-dextrin. Amylose (0–1.2 mg./100 ml.), amylopectin (0–6 mg.) and α -amylo-dextrin (0–8 mg.) were found to give linear relationships between carbohydrate concentration and absorptiometer reading for each colour filter. One mg. of amylose/100 ml. gave readings 0.390, 0.350, 0.145, 0.124 for the 608, 607, 603 and 602 filters respectively; those for amylopectin can be read from Fig. 1.

Fig. 1. Iodine coloration of amylopectin during hydrolysis by β -amylase. Ordinates. Extinctions (Spekker absorptiometer readings) 5 mg. of amylopectin (or its equivalent during hydrolysis) per 100 ml.; $\times - \times - \times$, Ilford filter no. 607; $\circ - \circ - \circ$, Ilford filter no. 608.

The readings for amylose and amylopectin bore an additive relationship over all ranges up to and including 0.8 mg. of amylose and 5 mg. of amylopectin per 100 ml. It was thus possible to calculate from such readings the quantities of amylose and amylopectin in mixtures of the two. It remained to determine how their respective iodine colour values change during the progress of hydrolysis by β -amylase. Such volume of reaction mixture as con-

tained 5 mg. of amylopectin at the start was withdrawn at intervals for readings in the absorptiometer. The latter, plotted against the percentage hydrolysis, gave straight lines to 51 % (Fig. 1). From these data, the corresponding figures for 5 mg. of the residual polysaccharide per 100 ml. were calculated, thus bringing all the values to the same basis. These results were approximately constant for any one filter. The same is true of amylose, readings, calculated to the basis of 1 mg. of residual polysaccharide per 100 ml., remaining constant until the hydrolysis is well advanced.

From all these observations it was concluded that the relative rates of disappearance of amylose and amylopectin could be calculated from the absorptiometer readings and percentage hydrolysis, provided the process was confined to the hydrolysis of about 50 % of the amylopectin. The 608 and 607 filters should be used, as by far the greatest difference between amylose and amylopectin is observed with them.

Certain precautionary tests were made. It was desirable to dilute amylose as far as possible before adding the iodine, otherwise the amylose-iodine complex tended to flocculate.

No influence outside the range of experimental error was exerted on the absorptiometer readings by variation in pH of the reaction mixtures tested between 4.6 and 7.0, or by the use of acetate or citrate-phosphate buffers (0.0001N) in the liquid as used in the absorptiometer or by such concentrations of maltose and sodium chloride as could be present under the experimental conditions.

Hydrolysis of mixtures of amylose and amylopectin by β -amylase. Table 1 shows absorptiometer readings taken during the reaction calculated to the basis of 5 mg. of residual polysaccharide per 100 ml. The rise in values for the 608 and 607 filters indicates increase in blueness and also that the unhydrolysed residue is becoming richer in amylose and poorer in amylopectin. Decrease in redness, i.e. in the 603 and 602 filter values, due to the loss of amylopectin is obscured by a greater increase due to the relative increase in amylose. The amylose-iodine complex, although characterized by its high blue values, nevertheless yields higher values with the 603 and 602 filters than its equivalent of amylopectin. The most significant figure is the ratio 608/602, an independent indication in that it takes into account the 602 filter readings and at the same time is independent of errors arising from the determination of percentage hydrolysis. The 607 and 603 filter values were throughout this work confirmatory of those of 608 and 602 respectively, but are not quite so distinctive. This ratio rises, but after 33 % hydrolysis it falls again, indicating that the enzyme is no longer liberating maltose from amylopectin faster than from amylose.

Table 1. *Extinction coefficients of unhydrolysed residue during hydrolysis of amylose-amylopectin mixture by β -amylase*

(Extinction coefficients are Spekker absorptiometer readings calculated to a basis of 5 mg. of residue + 10 mg. of iodine in 100 ml. Reaction mixture: in 100 ml., 0.25 g. of amylose, 0.75 g. of amylopectin, 0.8 mg. of soya β -amylase, pH 7, temp., 30°.)

Time (hr.)	Hydrolysis (%)	Ilford filters				Ratio 608 : 602
		608	607	603	602	
0	0	0.720	0.770	0.530	0.460	1.57
1	22.7	0.800	0.845	0.537	0.470	1.70
2	33.0	0.850	0.900	0.545	0.448	1.90
4	36.7	0.815	0.870	0.520	0.423	1.89
21	43.8	0.764	0.826	0.533	0.426	1.79
74	46.7	0.704	0.770	0.525	0.413	1.71

Table 2. *Progress of hydrolysis of amylose-amylopectin mixture and of potato starch paste*

(Conditions of reaction as for Table 1.)

(1) Time (hr.)	(2) Residual polysaccharide (%)	(3) Fraction of amylose in residue (x)	(4) Residual amylose (product of columns (2) and (3))*	(5) Residual amylopectin*	(6) Amylose lost by hydrolysis*	(7) Amylopectin lost by hydrolysis*
Amylose-amylopectin mixture						
0	100	0.256	25.6	74.4	—	—
1	77.3	0.308	23.8	53.5	1.8	20.9
2	67.0	0.344	23.0	44.0	2.6	30.4
4	63.3	0.322	20.4	42.9	5.2	31.5
21	56.2	0.290	16.3	39.9	9.3	34.5
74	53.3	0.250	13.3	40.0	12.3	34.4
Potato starch paste (1%)						
0	100	0.211	21.1	78.9	—	—
0.25	88.4	0.229	20.3	68.1	0.8	10.8
1.0	64.8	0.275	17.8	47.0	2.3	31.9
1.5	59.3	0.298	17.7	41.6	3.4	37.3
2.0	57.9	0.279	16.1	41.8	5.0	37.1
3.0	56.6	0.264	14.9	41.7	6.2	37.2
6.0	52.8	0.232	12.2	40.6	8.9	38.1
25.0	47.6	0.132	6.3	41.3	14.8	37.6

* As percentage of polysaccharide in original mixture.

The absorptiometer value using the 608 filter for 5 mg. of starch recorded in Table 1 is 0.720, or 0.144 for 1 mg. From the calibration data 1 mg. of amylose would give 0.390 and 1 mg. of amylopectin 0.060. If we take x and $(1-x)$ for the weights of amylose and amylopectin respectively in 1 g. of starch, then $0.390x + 0.060(1-x) = 0.144$, whence $x = 0.255$. The corresponding figure calculated from the 607 filter values (0.350, 0.086, 0.154) is 0.257, and the mean of the two is 0.256. In this way the figures recorded in Table 2 (upper half), column 3, were calculated.

It will be seen from the last two columns that in the early stages amylopectin was disappearing over ten times as fast as amylose, but later the process was put into reverse. Hydrolysis of potato starch paste (Table 2, lower half) was similar, the small difference being easily accounted for. The check in amylopectin hydrolysis occurred earlier in the arti-

ficial mixture because there was less amylopectin in it. When 34.5% of amylopectin (column, 7 upper half) or 37.3% (lower half) had gone, hydrolysis of that constituent almost ceased and the rate of maltose formation as a whole declined sharply.

The behaviour of potato and other starch pastes, including Lintner soluble starches, under a number of conditions was investigated and three examples are given in Table 3, which shows the quantities of amylose and amylopectin disappearing, calculated as above. In the case of the soluble starch the blue colour values for amylose needed for this calculation were taken from readings on amylose made from the same starch.

It was observed that the ratio of rates of maltose formation from amylopectin/amylose is (a) about 20 for potato starch at pH 4.6 and 30°, (b) reduced by more alkaline pH, (c) varyingly reduced when soluble starch is substituted, (d) unaffected by concen-

Table 3. *Course of hydrolysis of amylose and amylopectin*

(Results show the amounts of amylose (AML) and amylopectin (AMP) which have disappeared at various times, expressed as percentage of original starch. 1.0% of starch in medium. 608/602 refers to ratio of absorptiometer readings with 608 and 602 Ilford filters, cf. Table 1.)

Nature of starch ...	...	Potato		Potato		Soluble (A.R.)	
pH of medium ...	...	4.6		7.0		4.6	
Temp. of medium ...	...	30°		40°		30°	
Enzyme, mg./100 ml. ...	...	Barley, 20		Soya, 0.8		Soya, 1.6	
Time (hr.)		AML	AMP	608/602	AML	AMP	608/602
0	—	—	—	1.64	—	—	1.59
0.25	0.7	11	—	1.72	—	—	1.45
0.5	—	—	—	—	—	—	1.41
1.0	1.5	32	—	1.84	3.4	26.5	1.88
1.5	—	—	—	—	—	—	1.42
2.0	2.3	37	—	1.80	5.2	33	1.77
3.0	3.3	37	—	1.72	7.0	34	1.67
5.0	—	—	—	—	9.4	36	1.65
6.0	4.9	39	—	1.73	—	—	—
24	12	39	—	1.39	18	37	0.93
48	—	—	—	—	21	37	—
144	18	40	—	0.80	—	—	—

tration and source (barley, soya, yeast) of β -amylase, (e) unaffected by alkaline dispersion of potato starch instead of boiling.

Fig. 2. Initial velocity-substrate concentration relationship plotted according to the procedure of Lineweaver & Burk (1934) for the system soya β -amylase-amylose and amylopectin. Units: v , mg. of maltose formed per min. in 100 ml. of reaction mixture; c , percentage amylose or amylopectin in original reaction mixture. For sources of amylose, see p. 319.

Determination of Michaelis constants of amylose and amylopectin. The procedure described by Hopkins *et al.* (1948) was used with these preparations, but the

numerical values obtained may not safely be compared with those reported by these authors. In the present work soya β -amylase acting at pH 5.8 and 30° was employed. In Fig. 2 the graphs of the reciprocals of reaction velocity are plotted against reciprocals of substrate concentration and yield straight lines, thus showing that both kinds of amylose and also amylopectin conform in their hydrolysis by soya β -amylase to the Michaelis-Menten theory. The graph for amylopectin III almost coincided with that of amylopectin II which is shown in Fig. 2. The values calculated from these results were:

	'Baldwin amylose'	Residual amylose	Amylopectin II	Amylopectin III
Soya β -amylase, pH 5.8, 30°	—	—	—	—
Michaelis constant, K_m (%)	0.22	0.32	0.15	0.18
Maximum velocity, V	1.26	1.0	2.67	2.94

The relative rates of decomposition of two substrates A and B , present in equal concentrations and competing for the same enzyme, are given by the formula (Haldane, 1930)

$$\frac{\text{Rate for } A}{\text{Rate for } B} = \frac{V_A}{V_B} \times \frac{K_B}{K_A} = K \text{ (selectivity constant),}$$

where V_A and V_B are the respective maximum velocities, and K_A and K_B the respective Michaelis constants. Taking the mean values for amylopectin and those of the residual amylose (the main constituent), then, in equimixtures of the two,

$$\frac{\text{Rate of hydrolysis of amylopectin}}{\text{Rate of hydrolysis of amylose}} = \frac{2.80}{1.0} \times \frac{0.32}{0.165} = 5.4.$$

In starch, with nearly 4 parts of amylopectin to 1 part of amylose, the ratio of rates of hydrolysis is much higher.

Relative rates of hydrolysis of different preparations of amylopectin. The preparations I, II and III, described earlier, were submitted to treatment and hydrolysis as summarized in Table 4. The newly prepared sample hydrolysed 1.4 times as fast as the corresponding sample which had been stored for 4 months, but precipitation, etc., of the latter made no difference.

fast as from the amylose. This ratio is maintained until about 20–25 % of the theoretically equivalent maltose has been split off. The Michaelis constants also indicate that the soya amylase has only about twice the affinity for amylopectin, notwithstanding its possession of some 8–20 times as many terminal points per unit of weight as amylose. This could be due to the close proximity to one another of the end groups in the amylopectin molecule which could not all be attached simultaneously to the large enzyme molecule. Alternatively, the shortness of the

Table 4. *Hydrolysis of potato amylopectin by β -amylase*

(Results as percentage of theoretically equivalent maltose. Reaction mixture: amylopectin, 0.2 %; soya β -amylase, 0.4 mg./100 ml.; pH 5.8; temp., 30°.)

Method of preparation ...	From 63° infusion (see p. 319)	Thymol precipitation. Stored 4 months in sterile solution with thymol	As II, but stored only 8 days	As II, but subsequently precipitated with ethanol, dried, stored 1 day	As IV, but stored in butanol 1 day
	I	II	III	IV	V
10 min.	—	6.7	8.7	6.8	6.6
20 min.	15.4	13.8	17.4	13.9	13.4
30 min.	18.4	18.1	23.0	17.8	17.8
24 hr.	51.0	46.4	49.6	46.3	46.4
120 hr.	52.0	50.2	50.1	50.2	48.5

DISCUSSION

The chain length of potato amylose reported in the literature ranges from 200 upwards. The figures are far from certain owing to the considerable effect of any experimental error in the end-group assay on the result of the calculation (Brown, Halsall, Hirst & Jones, 1948). Meyer, Bernfeld, Boissonnas, Gürtler & Noelting (1949), using other methods, report 300 for potato amylose, and Potter & Hassid (1948) 930 and 980 by methods based on viscosity and periodate oxidation respectively.

If it be assumed that β -amylase can attack a non-reducing end group of amylopectin or of amylose with equal facility and hydrolyse either chain to produce maltose at the same rate, then we should expect amylopectin to yield maltose $300/25 = 12$ times (say) as fast as amylose under identical conditions. In starch, which contains approximately 4 parts of amylopectin to 1 part of amylose, about 48 parts of maltose would be derived from amylopectin for 1 from amylose. If the relationship remained unaffected by the diminishing chain length, the amylopectin would be transformed into its limit dextrin (α -amylodextrin), corresponding to about 50 % hydrolysis, by the time that only 0.8 part of amylose had disappeared.

However the experiments with potato starches at pH 4.6 and a consideration of the Michaelis constants of amylose and amylopectin led alike to the conclusion that β -amylase splits maltose from the amylopectin component of starch paste about 20 times as

branches and complexity of the amylopectin molecule may impede the attachment.

Both in starch paste and in separate solution amylopectin hydrolysis is almost linear with time, whereas that of amylose soon declines in rate (Tables 2 and 3; Hopkins *et al.* 1948). The consistent behaviour of a constituent in paste, alone or in mixtures, supports the view that starch is composite, with which not all workers agree (Le Corvaisier, 1948).

The complete removal of amylose from starch was only achieved at pH 7, under which conditions the rate of hydrolysis of amylose was faster. These conditions, pH 7 and 40°, would be those most suitable for the preparation of α -amylodextrin.

The amylose of Lintner starches was hydrolysed as much as one-third as fast as the amylopectin; this was found also with this particular amylose in separate solution. Presumably a certain amount of hydrolytic fission of the amylose had occurred during the acid treatment in the production of the starch, resulting in a great increase in the molarity and rate of hydrolysis of amylose in the corresponding reaction mixtures.

A similar case is that of potato starch macerated with water at 63° which yields an infusion containing about 5 % of the original starch, of which about 4.5 % is amylose and 0.5 % amylopectin. This amylose, presumably composed of relatively short chains which have made their exit from slightly swollen granules, hydrolyses much faster than normal amylose in solutions of 0.2 % concentration,

owing to its higher molarity. The amylopectin which accompanies it into the infusion, amylopectin I, gave much lower blue values with iodine than the other preparations, and indeed resembled in this respect the best preparation otherwise obtained. It can scarcely be representative of the amylopectin of potato starch, and presumably consists of the smaller or less complex molecules.

It is worthy of note that infusion of potato starch for 15 min. at 63°, followed by a further 15 min. at 45–50° before centrifuging, led to the separation of fractions of both amylopectin and amylose, which differ from the main portions of these substances.

SUMMARY

1. β -Amylase splits maltose from the amylopectin constituent of potato starch about 20 times as rapidly as from the amylose.

2. Its reactions with both amylose and amylopectin conform to the Michaelis and Menten formulations, the values of K_m (expressed as percentage)

being about twice as great for amylose as for amylopectin.

3. The hydrolysis of an artificial mixture of 4 parts of amylopectin and 1 part of amylose follows the same course as the hydrolysis of starch.

4. The results support the accepted view that starch paste contains these ingredients in unmodified form.

5. From structural considerations it might be expected that amylopectin would be hydrolysed even more rapidly than was found to be the case.

6. The rate of hydrolysis of amylopectin is to some extent influenced by its mode of preparation.

7. The amylose which diffuses from slightly swollen potato starch granules at 63° is hydrolysed much faster than normal potato amylose, while the amylopectin which accompanies it gives an unusually low blue value with iodine.

8. The amylose of Lintner starches is hydrolysed much faster than normal amylose, it having presumably been split in the acid treatment and its molarity in solutions correspondingly raised.

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On the Mechanism of Secretion of Ions by Gastric Mucosa and by Other Tissues

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(Received 25 July 1949)

In this paper the theory of gastric secretion, discussed by Crane, Davies & Longmuir (1948*a*), is extended and certain new evidence is presented.

All the explanations of ionic secretion so far proposed have been more or less incomplete. Franck & Mayer (1947) have examined the general mechanisms required for an 'osmotic diffusion pump'; Rosenberg (1948) has discussed the thermodynamic relationships which must hold for secretory processes; Stiehler & Flexner (1938) related the secretion of chloride into the cerebrospinal fluid to maintained

differences of electrical potential and of redox potential across the choroid plexus; Hollander (1943) proposed that the separation of gastric hydrochloric acid is due to differential permeability to ions. None of these authors have related secretion to the activity of particular physico-chemical systems within the cell.

More specific proposals have been made by Crane *et al.* (1948*a*) and Crane & Davies (1948), who have also reviewed a number of other suggestions of the chemical mechanism involved. They distinguished