The Mechanism of β -Amylase Action

1. 'MULTICHAIN' ACTION ON AMYLOSE

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Amylose, the constituent of starch which consists essentially of unbranched glucosidic chains, is hydrolysed by β -amylase in stepwise fashion starting from the non-reducing end. The sole fission products are maltose and continually shortening amylose chains.

It was concluded by Swanson (1948) that the enzyme, having started the attack on one chain, completes its hydrolysis before beginning to attack another chain. The experimental evidence included the absence of short-chain fission products in dialysates of maize amylose undergoing β -amylolysis, and the unchanging pattern of its iodine coloration spectrograms. Simultaneously, Cleveland & Kerr (1948) came to the same conclusion (see also Kerr, 1949; Kerr & Cleveland, 1951). In these cases, maize amylose was partly hydrolysed and the residual polysaccharide was recovered and examined by various methods (viscosity, iodine coloration, osmotic pressure, ferricyanide number, etc.). The results indicated that it possessed a molecular weight approximating to that of the original amylose. The fission products, examined by paper chromatography, did not contain sugars of greater chain length than the expected maltose. Since amylose must consist of chains of widely varying lengths, it was inferred that the shorter ones are hydrolysed preferentially.

This hypothesis of amylase action, which has been described as the 'single chain' mechanism, has received wide acceptance but has been criticized by Hopkins & Jelinek (1949) and by Bourne & Whelan (1950), whose observations favour the 'multichain' hypothesis, namely that the enzyme attacks chains of all lengths at random and having completed a single fission, does not necessarily continue to attack the same chain.

Meyer, Bernfeld & Press (1940) had earlier concluded that the smaller molecules in maize amylose served to protect the larger ones from retrogradation, that they were attacked first by β -amylase, and that their removal in the earlier stages of hydrolysis facilitated retrogradation or, at least, ageing of the remainder.

French, Levine, Pazur & Norberg (1950), using amyloheptaose prepared from a Schardinger dextrin (cycloheptaose), obtained evidence that

under conditions favourable to enzyme action, no pentaoose is present in partly hydrolysed reaction mixtures. This observation supports the 'single chain' theory. On the other hand, French, Knapp & Pazur (1950) found that if the enzyme was made to act under certain unfavourable conditions (pH 10, or 70°), pentaoose was formed, thus suggesting that the two mechanisms were liable to operate according to circumstances.

A review of the experimental evidence led the present authors to the opinion that no conclusion was yet justified and that the matter needed investigation by new methods. It was deemed desirable to prepare specimens of amylose and of its fission products with long and short chains, respectively, and to determine these chain lengths and the Michaelis constants of the specimens with β -amylase. These data would show whether the enzyme exhibited any marked differences in affinity for these products. As their iodine spectrograms would be characteristic and different, it should also be possible to follow their respective rates of disappearance when hydrolysed in mixtures.

EXPERIMENTAL

Materials

Amylose. This was prepared from potato starch as described by Hopkins & Jelinek (1948).

β -Amylase. This was prepared from soya beans as described by Newton, Hixon & Naylor (1943).

Amylose dextrins. Potato amylose (0.63%, w/v) was maintained at 80° in the presence of 0.1N-HCl, and after 1 and 5 hr., respectively, a quantity was neutralized. No retrogradation had occurred.

Dextrin B. This was obtained from the 1 hr. hydrolysate by heating to 85° with excess of *n*-butanol, cooling slowly at first and finally in a refrigerator at 3°. A precipitate appeared at the last stage only. This was centrifuged, dissolved and twice reprecipitated in the same way. It gave a blue iodine coloration. Yield, 24.2%.

Dextrins R. These were obtained from the 5 hr. hydrolysate which was cooled slowly with excess cyclohexanol; the small precipitate which formed was removed, and the filtrate evaporated to half volume and precipitated with 3.5 vol. 95% (v/v) ethanol. The precipitate was dissolved and reprecipitated, washed, and dried over CaCl₂ *in vacuo*. It gave a red iodine reaction. Three preparations were made. Yield, R₁ 29.7%; R₂ and R₃, about 30%. Successive

dialyses of R_1 into large volumes of still water indicated very little difference in composition between the portions found outside and inside the membrane. R_2 and R_3 were thoroughly dialysed through cellophan before use.

Reducing powers. These were determined by the method of Blom & Rosted (1947). The Somogyi (1945) method was also used but gave somewhat lower results. Unless very minute quantities had to be measured, the Blom & Rosted method was preferred. The R_m value is the reducing power as the percentage of the reducing power of the theoretically obtainable maltose.

Periodate determinations. These were made by the method of Potter & Hassid (1948), slightly modified.

Absorption values. These were determined as described by McCready & Hassid (1943), using a Spekker absorptiometer with a 1 cm. cell. For dextrans B and R_1 (Table 1) and the experiment (Table 6) in which they were used together, the readings were made on a Coleman Universal Spectrophotometer. The numerical values of the readings, calculated for a 1 cm. cell, were almost identical with the corresponding readings on the Spekker absorptiometer.

Michaelis constants (K_m). These were determined as described by Hopkins, Jelinek & Harrison (1948), except that reducing powers were measured by the methods of Blom & Rosted (1947), and of Somogyi (1945).

RESULTS

The absorption values and chain lengths of the materials used are summarized in Table 1, and the

Michaelis constants of four different preparations of amylose and of two fission products are given in Table 2. The value of K_m , expressed in molar concentration, did not vary much among the six preparations, while the maximum velocity varied even less. Data from which values of K_m were calculated are given in Table 3.

The soya bean β -amylase readily hydrolysed amylose at pH 4.8 to R_m 90 (Table 4), and must therefore have contained the Z-factor of Peat, Pirt & Whelan (1952).

In the hydrolysis of the mixture of amylose and dextrin R_3 (Table 5), absorption values lost over the period 0–30 min. are almost identical with those found for 1 mg. of dextrin R_3 /100 ml. (Table 1). Thus, in effect, attainment of R_m 48 in this reaction mixture has been accompanied by the removal of 1 mg. of dextrin R_3 /2 ml. (the volume withdrawn) or 50 mg./100 ml. of reaction mixture. Actually, rather less than this amount must have been attacked, for a small contribution to the change in absorption values would be made by the amylose when undergoing fission.

With the mixture of dextrans B and R_1 (Table 6) the loss over the period 0–45 min. is of material of similar character in its iodine coloration to dextrin R_1 .

Table 1. *Absorption values and chain lengths*

(The solutions contained 1 mg. polysaccharide, 10 mg. I_2 and 100 mg. KI in 100 ml. and were examined in a 1 cm. cell.)

	Chain length calculated from		Absorptiometer readings at (m μ .)					
	Blom & Rosted (1947)	Periodate oxidation	680	630	600	550	500	490 470
Amylose	—	Approx. 640	382	—	338	223	—	130 120
Dextrin B	Approx. 50	—	287	318	304	226	141	133 122
Dextrin R_1	12.5	—	21	42	65	112	144	141 131
Dextrin R_2	19.6	20	25	—	47	—	—	132 117
Dextrin R_3	20.9	22.5	30	—	55	—	—	154 140

Table 2. *Michaelis constants and maximum velocities of β -amylolysis of potato amylose and its short-chain fission products*

(Enzyme action carried out at pH 5.8 and 30°.)

	Mean chain length calculated from			Michaelis constant (K_m)		Maximum velocity (V) (mg. maltose/ 100 ml./ min.)
	Reducing power					
	Periodate oxidation	Blom & Rosted (1947)	Somogyi (1945)			
				(%)	$M \times 10^{-5}$	
Amylose 1	360	—	—	0.32	5.5	—
Amylose 2	300	—	—	0.32	6.7	—
Amylose 3	450	—	—	0.7	9.0	2.5
Amylose 4	660	—	—	0.85	8.0	2.5
Short-chain fission product, R_2	20	19.6	22	0.0156	4.8	3.0
Short-chain fission product, R_3	22.5	20.9	—	0.0163	4.7	2.4

Table 3. *Velocity of β -amylolysis of amylose and amylose dextrins*

(Reaction mixtures contained 0.4 mg. soya bean β -amylase, 1 ml. McIlvaine buffer, pH 5.8, for each 100 ml. substrate. Temperature, 30°.)

	Substrate concn. (mg./100 ml.)	Time (min.)	Maltose formed (mg./100 ml.)	K_m (%)	Maximum velocity (mg./100 ml./min.)
Amylose 3	44	17.8	2.8	0.70	2.5
	88	20.0	5.6	—	—
	177	22.4	11.2	—	—
	354	26.9	22.4	—	—
Amylose 4	51	22.7	3.25	0.85	2.5
	102	24.8	6.5	—	—
	205	27.1	13.0	—	—
	410	32.0	26.0	—	—
Dextrin R ₂	23.5	5.5	9.9	0.0156	3.0
	47	8.8	19.8	—	—
	94	15.2	39.6	—	—
	188	28.3	79.2	—	—
	375	54.6	158.4	—	—
Dextrin R ₃	50	11.8	21	0.0163	2.4
	100	20.5	42	—	—
	151	29.3	63	—	—
	201	38.0	84	—	—

Table 4. *Progress of action of soya bean β -amylase on amylose*

(Reaction mixture: amylose (0.5%) and soya bean β -amylase (2.0 mg./100 ml.) at pH 4.8 and 30°.)

Time (min.)	R_m
10	18
25	31.3
40	41.5
80	58.5
145	67
19 (hr.)	88

Table 5. *β -Amylolysis of a mixture of amylose and dextrin R₃*

(33.3 mg. Amylose 4, 63.8 mg. dextrin R₃, 0.5 ml. McIlvaine buffer solution, pH 5.8, and soya bean β -amylase. Total volume, 100 ml. Incubated at 30°. Samples (2 ml.) withdrawn at intervals into 10 mg. I₂ + 100 mg. KI and diluted to 100 ml.)

Time (min.)	R_m (%)	Absorption values at (m μ .)			
		680	600	490	470
0	6.4	294	300	272	250
15	36.3	293	274	148	133
30	55	266	242	101	91
Difference (0-30 min.)	48.6	28	58	171	159
60	61.6	249	222	89	84

Table 6. *β -Amylolysis of a mixture of dextrins B and R₁*

(The reaction mixture contained 208 mg. dextrin B, 182 mg. dextrin R₁, 16 ml. 0.02N acetate buffer (pH 4.6) and 1 mg. soya bean β -amylase. Total volume 220 ml. Temp. 30°. Samples (1 or 2 ml.) withdrawn at intervals into 10 mg. I₂ and 100 mg. KI, and diluted to 100 ml. A further 2 mg. of enzyme were added after 125 min. and again after 185 min.)

Time (min.)	R_m (%)	Absorption values (calculated to 1 ml. withdrawals) at (m μ .)			
		680	630	500	490
0	10	288	336	252	243
45	27.1	273	305	171	165
Difference (0-45 min.)	17.1	15	31	81	78
180	55	135	151	89	85
Difference (45-180 min.)	27.9	138	154	82	80
240	85.5	12	15	9	9

DISCUSSION

The Michaelis constant (K_m) for amylose and soya bean β -amylase has been shown to vary (Hopkins *et al.* 1948) when expressed as a percentage, but it does not follow that any such variation occurs when calculated to molar concentration. The results in Table 2 suggest that the affinities of both normal amylose and short-chain fission products, expressed on a molar basis, are all of the same order. The maximum velocities of β -amylolysis of these substrates are also approximately the same. We may therefore conclude that there is no great disparity between the rates at which the enzyme splits maltose from long and short amylose chains. In any event, both kinds of chain are liable to simultaneous attack, and it seems that the same would hold for the chains of the varying lengths actually present in amylose.

Hopkins *et al.* (1948) concluded that the longer the chain the faster the β -amylase hydrolyses the amylose chain. One would expect the reaction to be of zero order, especially when a high concentration of substrate is employed, but these authors found that the rate declined continually, and the above conclusion was reached in order to explain this observation. However, a major factor in explanation of the decline in rate reported by Hopkins *et al.* (1948) and unknown at that time, is the marked check in hydrolysis as R_m 70 is approached (Peat *et al.* 1952). It is now apparent that, at this stage, the rate of the reaction is increasingly controlled by the activity of the Z-enzyme (Peat *et al.* 1952) and, perhaps, by traces of α -amylase.

Let us consider a reaction mixture containing two dextrans derived from amylose, consisting respectively of long and short chains. This is submitted to the action of β -amylase. If the affinity constants (molar) were the same and if diffusion did not influence one constituent more than the other, the proportion of the chains of each constituent to be attacked at the outset would be the same. If the 'single chain' mechanism operated, the enzyme would complete the hydrolysis of a chain, then attack a fresh chain chosen at random from the residue of unattacked chains. As this residue would consist of the same relative proportion of the two dextrans as did the original mixture, the proportions of the components to be attacked would remain unchanged. The two dextrans would disappear from the medium at rates proportional to their original molar concentrations, their relative concentrations remaining unaltered. The absorption values at all wavelengths would decline proportionally.

If, however, the 'multichain' mechanism operates, all chains, long and short, would be shortened by an equal number of maltose units in a given time. Soon the short-chain component would have

arrived at chain length 7 and have ceased to give an iodine colour, whereas the other one would be comparatively unaffected in this respect. The polysaccharide disappearing would be iodine-reddening material, whereas the residue would give a series of absorption values corresponding to bluer material than the original. These latter changes are those which were observed (Tables 5 and 6).

This is more clearly seen when amylose itself was one constituent (Table 5), owing to the marked difference between the iodine spectrograms of the constituents, and the failure of amylose to change appreciably in this respect when the chains are slightly shortened. When amylose dextrin of chain length 50 (Table 6) was employed the result was less clear cut, but still convincing.

Even an advantage secured by short chains by virtue of diffusion rates could scarcely account for the difference between dextrans B and R_1 which had mean chain lengths 50 and 12, respectively. The reason why the short-chain constituent disappears before the other is that its chains, being shorter, are finished first.

In considering the results in Tables 5 and 6 it is not essential to postulate that the affinity of the enzyme for the long and short chains is identical. In Table 6 the whole of the dextrin R_1 originally present would be responsible for the following absorption values: 17.3 (680 $m\mu$.), 34.6 (630 $m\mu$.), 119 (500 $m\mu$.), 116 (490 $m\mu$.), these values being calculated from the data in Table 1 and the concentration of R_1 in the reaction mixture. The material removed in the first 45 min. is, from its absorption value, of the character of dextrin R_1 , and is equivalent to about 66 % of the total originally present. Since dextrin R_1 amounted to 47 % of the total substrate we thus have about 31 % of the substrate disappearing whilst only 17.1 % of the total theoretical amount of maltose has made its appearance. This could not be satisfactorily accounted for by 'single chain' amylolysis which would require that these figures agree. But on the 'multichain' mechanism the figures are easily understood. Thus, the complete β -amylolysis of dextrin R_1 would raise its R_m value from 16 to 100, or, in the reaction mixture, from 7.5 to 47, an increase of 39.5. The 17.1 increase actually observed is equivalent to shortening the mean chain length of dextrin R_1 by $(12.5 - 2) \times 17.1 / 39.5 = 4.5$, i.e. from 12.5 to 8. Since not all of the 17.1 increase in R_m is derived from dextrin R_1 , the chains of the latter would not have been shortened quite so much. This result is in much better agreement with the observed loss of absorption value than that deduced above on the 'single chain' hypothesis.

Our soya bean β -amylase exhibited slight α -amylase activity which was almost negligible at pH 4.6. Any action by α -amylase would, however,

help to change amylose and dextrin of chain length 50 into red-staining dextrans and to act in opposition to the evidence reported.

SUMMARY

1. The Michaelis constants of soya bean β -amylase with potato amylose and its short-chain fission products are of the same order. The enzyme will therefore exhibit no decided preference for either long or short chains.

2. The early disappearance of the short chains from a mixture of long- and short-chain polysaccharides is demonstrated and must be attributed to a 'multichain' mode of attack by the enzyme, the hydrolysis of short chains being soon completed because they are short.

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The Mechanism of β -Amylase Action

2. 'MULTICHAIN' ACTION ON AMYLOSE FISSION PRODUCTS

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The evidence respectively favouring the 'single chain' and 'multichain' theories of β -amylase action has been summarized in the previous paper (Hopkins & Jelinek, 1954). Particular mention must be made of the work of French, Levine, Pazur & Norberg (1950) who hydrolysed amyloheptaose with soya bean β -amylase and found only maltose and maltotriose among the fission products. Even at the stage of 55 % hydrolysis, no pentasaccharide was found, and the 'single chain' hypothesis received support. A preliminary experiment, reported briefly by Hopkins & Jelinek (1949), encouraged investigation of the action of β -amylase on amylose dextrans of chain length 10–30, the progress of which could be followed by changes in the absorption values of the iodine colorations. Such dextrans, prepared from amylose by acid or α -amylolytic fission, are colourless with iodine if shorter than chain length 8 (Swanson, 1948), and

change from red through purple to blue over the range 10–30. The absorption value at 600–680 m μ . (blue value) varies directly with chain length over this range (Bailey, Whelan & Peat, 1950). If an amylose dextrin of chain length 20 is hydrolysed by β -amylase by 'single chain' mechanism the absorption values at all wavelengths should vary inversely with increase in reducing power as percentage from R_m 10, the initial value, to approximately R_m 100 (the R_m value is the reducing power of the theoretically obtainable maltose). If, however, a 'multichain' mechanism operates, when all chains are reduced in length at equal rates, the absorption value will fall to zero by the time that the chain length has been shortened to 8 units (R_m 70) and even a little earlier when measured at 680 m μ . Moreover, the relationship would be linear over this range of chain length.

An additional method of testing for the mechanism by which amylose short-chain fission products are degraded by β -amylase employs chromato-

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