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 β -Amylolysis: Union of Enzyme and Substrate

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It is well known that β -amylase liberates maltose from starch and its components in the β form (Kuhn, 1925; Freeman & Hopkins, 1936). This was established by observing the mutarotation of the fission products and establishing that it was the maltose itself which was undergoing this change. In one type of experiment the optical rotation of the β -amylase/starch reaction mixture is read continuously during the reaction and samples are withdrawn from time to time and treated with alkali to stop enzyme action and hasten mutarotation. The rotations of the former series are lower than the corresponding ones of the latter (corrected for dilution) because β -maltose ($[\alpha]_D + 118^\circ$) has yet to change into α -maltose ($[\alpha]_D + 137^\circ$).

In repeating some experiments of this kind and taking direct readings of optical rotation of the reaction mixture very soon after mixing the enzyme and substrate it was observed that the rotation was higher, not lower, than that of the corresponding mutarotated solution. After the reaction had proceeded further this position was reversed and subsequent readings conformed to those previously reported by the above authors. The phenomenon was confirmed with starch, amylose and a short-chain amylose dextrin and was considered to be well outside the range of experimental error. The simplest explanation is that an enzyme-substrate compound is formed, the optical rotation of which (dextrorotatory) is greater than that of the substrate.

MATERIALS AND METHODS

Substrates. Soluble starch was prepared from potato starch by the method of Hanes & Cattle (1938), $[\alpha]_D^{20} + 198^\circ$ in water; amylose from potato starch by precipitation with thymol followed by repeated recrystallizations with butanol

as described by Hopkins & Jelinek (1948). Achroic amylose dextrin was prepared by hydrolysing amylose with bacterial α -amylase (prepared from *B. subtilis*) to the achroic stage and its separation from the lower-molecular hydrolytic products in a charcoal-Celite column as described by Bailey, Whelan & Peat (1950). The lower fractions eluted by ethanol up to 25% (v/v) were separated and the remainder eluted with ethanol (30%, v/v, and higher) but not further fractionated, $[\alpha]_D^{20} + 186^\circ$ in water.

Enzymes. We are indebted to the Wallerstein Laboratories, New York, for a preparation of β -amylase of high purity from ungerminated barley, and to Dr A. K. Balls for crystalline sweet potato β -amylase (4 times recrystallized).

Reactions were performed as follows. The substrate and enzyme were prepared in solution, and after standing for a period of 1 hr. at the temperature at which the reaction was to be carried out, the optical rotation of each was measured in the polarimeter at that temperature. Our soluble starch fell in $[\alpha]_D$ by about 0.6 angular degree for each degree ($^\circ$ C) rise in temperature. In the case of amylose special precautions were necessary, such as reading immediately after filling the polarimeter tube (see Results). The reaction mixtures contained no added buffers, the pH being about 6.

On mixing suitable volumes of amylase (5 ml.) and substrate (100 ml.), after a short vigorous shaking a portion was transferred to a jacketed polarimeter tube and the first reading obtained as quickly as possible, and later readings at intervals. These are represented graphically in Figs. 1-3 and are described there as 'continuous' curves. The remainder of the reaction mixture was kept in a flask in a water bath at the same temperature. Samples (10 ml.) were withdrawn at intervals and rapidly run into 1 ml. of 0.2N-NaOH. After about 30 min. the optical rotation was measured at the reaction temperature. The results (corrected for dilution) are shown in the figures as 'withdrawals' curves. Such small fall in α_D of starch or other substrate or maltose as was caused by the alkali (after correcting for dilution) was allowed for, control experiments having been performed. Portions of the final reaction mixture, after amylolysis had ceased, treated with equal volumes of NaOH and

water in parallel withdrawals, gave with the alkali a small fall in α_D , about 1–2% of its value. Similar results were obtained if the reaction was checked with 0.2N ammonia instead of NaOH.

In the cases of amylose and the achroic dextrin, the progress of the reaction was also followed by determining reducing power by the method of Blom & Rosted (1947). From these data the values of α_D for the mutarotated products were calculated on the assumption of a linear relationship between increase of reducing power and fall in α_D over the range of the experiment. This has received subsequent justification by the observations reported by Whelan, Bailey & Roberts (1953) that the Freudenberg relationship holds for these unbranched short-chain dextrans from maltose upwards.

The curves in Figs. 1–3 described there as ' β -maltose' curves were obtained in the following way by calculation from the data of the 'withdrawals' curves. Our starch and amylose had $[\alpha]_D^{20} + 198^\circ$. Taking that of $\alpha\beta$ -maltose as $+137^\circ$ and of β -maltose as $+118^\circ$ and allowing for the increase in weight resulting from hydrolysis, it was calculated that the fall in α_D in producing β -maltose would be 1.37 times as great as that for the formation of an equal quantity of $\alpha\beta$ -maltose. (Actually the fall in α_D during β -amylolysis of starch is partly due to changes in $[\alpha]_D$ of the residue, but the correction arising from this is small and does not affect the conclusion.)

Both the 'withdrawals' and the ' β -maltose' curves start from the initial value of α_D calculated from the values of α_D of substrate and amylase (the amylase contributing less than 1% of this initial rotation). The 'continuous' curve was extrapolated to the zero axis.

RESULTS

Soluble starch, alone or previously partly hydrolysed by β -amylase, when treated with NaOH, as described, fell in value of α_D by about 1.5% of its

Fig. 1. Change in optical rotation of starch during β -amylolysis. Soluble starch (2.18 g. in 100 ml. solution) was mixed with Wallerstein β -amylase (2.5 mg. in 5 ml. solution) at 16° . α_D was read in a 1 dm. tube. $\odot$ — $\odot$, 'Continuous' curve, readings of reaction mixture; $\triangle$ — $\triangle$, 'withdrawals' curve, readings after addition of alkali (corrected for dilution, see text); $\odot$ — $\odot$, calculated ' β -maltose' curve (see text). After 130 min. 2.5 mg. more β -amylase were added.

original value. Enzyme added to starch so pre-treated was inactivated, since the value of α_D of the mixture was and remained that which was calculated from those of the enzyme and alkali-treated starch.

Soluble starch undergoing hydrolysis by β -amylase exhibited values of α_D as shown in Fig. 1, amylose and its achroic dextrin as in Figs. 2 and 3 respectively. When the reading could be made soon enough, the value exceeded that calculated for the initial mixture. In all cases the early readings were

Fig. 2. Change in optical rotation of amylose during β -amylolysis. Amylose solution (0.425 g; 100 ml.) was mixed with Wallerstein β -amylase (100 mg. in 10 ml. solution) at 16° . α_D was read in a 2 dm. tube. Symbols as in Fig. 1. The reading at 6 min. on the 'withdrawals' curve was calculated from a reducing-power determination.

Fig. 3. Change in optical rotation of achroic dextrin during β -amylolysis. Achroic dextrin (0.765 g. in 100 ml. solution) was mixed with Wallerstein β -amylase (25 mg. in 5 ml. solution) at 19° . α_D was read in a 2 dm. tube. Symbols as in Fig. 1; α_D in 'withdrawals' curve calculated from reducing power.

Table 1. *Optical rotations of reaction mixtures at the start and end of β -amylolysis*

Readings after addition of NaOH were corrected for dilution and for the small effect of the NaOH on the carbohydrate.

		Optical rotation, α_D , in 2 dm. tube ($^{\circ}$)					
		Temp. ($^{\circ}$)	At start		At end (or after slowing down)		
Substrate	β -Amylase		Without NaOH (extrapolated from 'continuous' curve)	Calculated from α_D of constituents	Without NaOH	With NaOH	R_m^*
Starch	Wallerstein	16	Approx. 4.5	4.32	3.62	3.61	61
	Wallerstein	8	7.40	7.08	—	—	—
	Crystalline	19	3.68	3.55	3.05	3.02	56
	Crystalline	16	4.82	4.64	{ 4.45† 4.17‡	{ 4.27† 4.06‡	{ 30 47
Amylose	Crystalline	16	—	2.04	1.65	1.64	74
	Crystalline	16	—	1.72	1.37	1.39	72
	Crystalline	16	Approx. 1.55	1.50	1.16	1.17	82
	Crystalline	17	Approx. 1.70	1.63	1.28	1.28	80

* Reducing power as percentage of theoretically obtainable maltose.

† Readings taken after the initially fast reaction had slowed down markedly.

‡ Readings taken when the reaction had ceased.

Table 2. *Changes in optical rotation of starch solution during α -amylolysis*The digest contained Lintner soluble starch (2 g.) and malt α -amylase ($\equiv$ 100 g. malt) in 0.015N acetate (125 ml.; pH 4.6) at 20 $^{\circ}$.

Time (sec.)	α_D in 1 dm. tube ($^{\circ}$)	R_m^*
0	2.94	0
60	3.04	—
120	3.09	—
130	3.06	25.8
24 hr.	2.18	83.6

* For definition, see Table 1.

higher than those of corresponding withdrawals corrected for dilution and for the small effect of the alkali. The curve of the former, however, ultimately intersected that of the alkali-treated withdrawals. The latter, redrawn to represent the corresponding rotation if the maltose present had been solely in the β -form, remained more or less uniformly below the curve of the untreated reaction mixture.

The general patterns of the curves were similar in all the experiments, i.e. for different substrates and for reactions at different rates. Of course the rate must be fast enough to show the phenomenon. A relatively slow reaction gave time to take the necessary readings and to show the rise brought about by mixing enzyme and substrate, before amylolysis had caused sufficient fall to mask this rise (Fig. 1). The phenomenon was obtained at three temperatures (Table 1), with the Wallerstein enzyme, the Balls crystalline β -amylase and with barley and soya amylases prepared in these laboratories (results not given).

When amylose was used, certain difficulties ensued which were attributed to birefringence and to the rapidity with which this surface-active

colloid concentrated on the walls of the polarimeter tube. This was most marked when the crystalline enzyme was used. Fig. 2 shows one successful experiment with amylose. Less surface activity was shown by amylose prepared by leaching potato starch at 63 $^{\circ}$ (described by Hopkins & Jha (1950) and termed by them 'Baldwin' amylose). The difficulty was avoided by the use of the achroic amylose dextrin (Fig. 3).

With all substrates, the abnormal rotatory power had disappeared by the end of amylolysis (see Table 1). It was not possible to demonstrate the stage at which this had happened (presumably a gradual process) because mutarotation masked it.

In one or two cases in which crystalline β -amylase was used the amylolysis became quite slow at a stage appreciably before the usual limit, and the accompanying mutarotation became negligible. In such cases the readings of α_D taken before still exceeded those taken after the addition of alkali (Table 1).

In one experiment (Fig. 1) a further addition of enzyme was made after the original reaction had become very slow. No rise in α_D was observed.

No rise or change in α_D was observed in the following control experiments: (i) when active amylase was replaced by its equivalent of inactivated β -amylase preparation, (ii) when a very active preparation of yeast saccharase was mixed with soluble starch, (iii) when β -amylase was added to amylopectin β -limit dextrin (α -amylodextrin).

A similar rise with malt α -amylase is clearly seen in the results reported by Freeman & Hopkins (1936), the essential figures of which are reproduced in Table 2. A flocculum formed in the reaction mixture very soon after the observed rise in rotation and prevented further direct readings.

DISCUSSION

When β -amylase is mixed with starch or the other substrates used, including the dialysable achroic dextrin, there is an immediate rise in optical rotation, and the raised value can be observed before the fall due to amylolysis masks it. Subsequently to this, comparison of α_D of the reaction mixture with that observed in portions in which the reaction is arrested and mutarotation completed, shows that the former continues to be the higher. Later still, even after this has ceased to be the case, the value of α_D exceeds that which is to be expected on the basis that the maltose which has been liberated is in the β form. The 'continuous' curve remains above and approximately parallel to the ' β -maltose' curve until the reaction stops or mutarotation becomes appreciable. Under the conditions of experiment (pH 6, no added salts) the effect of mutarotation of β -maltose would be negligible for several minutes after the reactions were commenced. (For velocity constants of mutarotation see Freeman & Hopkins, 1936; Isbell & Pigman, 1937.)

This abnormality in optical rotation ceases when the amylolysis ceases and is not exhibited when β -amylase is added to the reaction mixture at the end of the reaction (Fig. 1), or to amylopectin β -limit dextrin, on which β -amylase does not act. Nor was it observed when starch was mixed with heat-inactivated β -amylase or with an active enzyme such as saccharase which does not function with starch. The colloidal character of starch and amylose is not responsible since the dialysable achroic amylose dextrin exhibits the phenomenon.

One is led to the conclusion that the rise in optical rotation is associated with the enzymic reaction and is due to the formation of an enzyme-substrate compound. The rise is appreciable and is equivalent to about 3-4 % of the optical rotation of the substrate. It is about equal to the rotatory power that would be lost on the fission of 6-8 % of the α -1:4-glucosidic linkages followed by change of the reducing groups so formed to the $\alpha\beta$ equilibrium. This suggests that the enzyme unites specifically with more glucosidic groups than the two concerned in the fission, and indeed it is now considered that this must be the case. β -Amylase does not hydrolyse α - or β -maltosides with which it has been tested (Helferich & Petersen, 1935), phosphohexaose (Posternak, 1951), maltotriose only with difficulty (Bailey *et al.* 1950; Whelan *et al.* 1953) and is apparently only able to unite with and hydrolyse efficiently maltotetraose and longer chains. In

passing it may be added that malt α -amylase exhibits this phenomenon of increase in rotation in such degree as to suggest union with an even greater length of chain, but this merits proper investigation with crystalline enzymes.

The conception of a multipoint contact between enzyme and substrate (Gottschalk, 1950) is probably nowhere more appropriate than in considerations of the amylases, and it is to such a union that the optical rotatory changes are mainly to be ascribed. However, there is another factor. In the cases of both α - and β -amylase the change in optical rotation is in the positive sense. As the α -glucosidic linkage which is split by these enzymes itself contributes appreciably in the positive sense to the rotatory power of the polysaccharide, the strain imposed on this linkage by the enzyme might well increase this contribution.

SUMMARY

1. On mixing β -amylase with a solution of starch, amylose or an achroic dextrin, an initial increase in optical rotation takes place.

2. During the β -amylolysis reaction, the optical rotation of the mixture remains above that calculated from the values of the enzyme, substrate and fission products.

3. The rise in optical rotation is ascribed to the formation of an enzyme-substrate compound, and is regarded as experimental evidence of such.

4. A similar rise in optical rotation has been reported when α -malt amylase is mixed with Lintner soluble starch.

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