*Biochimica et Biophysica Acta*, 341 (1974) 497–504


BBA 67165

# EFFECT OF PHOTOOXIDATION OF BACTERIAL LIQUEFYING α-AMYLASE DEPENDENT ON THE DEGREE OF POLYMERIZATION OF LINEAR SUBSTRATES

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(Received September 7th, 1973)
(Revised manuscript received December 5th, 1973)

## SUMMARY

The effect of pH on the rate of inactivation of liquefying α-amylase from *Bacillus subtilis* (α-1,4-glucan 4-glucanohydrolase (liquefying), EC 3.2.1.1) by photosensitized oxidation in the presence of methylene blue indicated that the modification of a histidyl residue causes loss of the enzyme activity for the hydrolysis of soluble starch catalyzed by this enzyme.

The activity of the enzyme photooxidized at 25 °C and pH 7.0 was measured by using maltooligosaccharides with the degree of polymerization *n* ranging from 3 to 12.6 as substrates. A remarkable difference in the effect of the modification was observed depending on the *n* value of the substrate; the rate of hydrolysis of maltooligosaccharides with *n* larger than 5 was crucially decreased, whereas that of smaller substrates was not appreciably affected. This result indicates that the affinity of a subsite which is quite distant from the catalytic site is decreased in consequence of the modification. The location of the damaged subsite was estimated by considering the predominant productive binding modes which can be predicted from the knowledge already obtained concerning the arrangement of subsite affinities and the action patterns of this enzyme.

## INTRODUCTION

Recently, arrangements of subsite affinities of several exo- and endo-amylases, including the number of subsites, values of subsite affinities and location of the catalytic site, were determined from the measurement of the rate parameters of a series of linear substrates [1–6] and also from quantitative analysis of products with end-labeled maltooligosaccharides [7, 8]. A theory employed in these analyses predicts that an action pattern of the amylase could be drastically altered by changing one (or

Abbreviation used; $G_n$: maltooligosaccharide with degree of polymerization *n*.

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more) of the subsite affinities caused by the chemical modification of the amino acid residue(s) involved in one (or more) of the subsites [9].

From the results of the modification of liquefying α-amylase from *Bacillus subtilis* with acrylnitrile and with 2-methoxy-5-nitrotropone, it was suggested that a histidyl residue may be important for the hydrolysis of soluble starch catalyzed by this enzyme [10].

Studies of the pH effects on the inactivation of an enzyme by photooxidation have been known to be useful to distinguish a histidyl residue from tryptophanyl, tyrosyl and methionyl residues which could also be destroyed by photooxidation*. In this paper, we aimed to modify the histidyl residue of bacterial liquefying α-amylase by photooxidation in the presence of methylene blue at various pH values, and to investigate the effect of the photooxidation on the rate of enzyme reaction towards a series of maltooligosaccharides as substrates. The most important result obtained is that the effect of the photooxidation on the enzyme activity is strongly dependent on the degree of polymerization of the linear substrates. The result was analyzed in the light of the subsite theory [1–3, 11] to estimate the location of the subsite whose affinity to the glucose residue was decreased by the modification.

## MATERIALS AND METHODS

### *Materials*

Crystalline liquefying α-amylase from *B. subtilis* was purchased from Daiwa Kasei Co., Osaka. The concentrations of the enzyme solutions were estimated from the absorption at 280 nm assuming that $A_{1cm}^{1\%} = 25.6$ [12]. Homologous maltooligosaccharides were prepared from an acid hydrolyzate of cycloheptaamylose or amylose by partition chromatography on cellulose powder according to the procedure of Thoma et al. [13].

### *Methods*

For the photooxidation of the enzyme, an incubation mixture was prepared by adding 1 ml of methylene blue solution (0.1 mg/ml) and 2 ml of the enzyme solution (6 mg/ml) to 2 ml of buffer solution (0.05 M acetate buffer from pH 4.0 to pH 6.0 and 0.05 M Tris–HCl buffer from pH 6.5 to pH 9.0). Illumination was achieved by a 100 W tungsten lamp placed at a distance of 25 cm from the reaction vessel (diameter 2.5 cm) which was thermostatted at 25 °C. Infrared rays were cut off through a glass dish filled with water and placed between the lamp and the vessel.

Amino acid analyses were performed by the method of Moore et al. [14] using a Hitachi KLA-3 amino acid analyzer. The enzyme was photooxidized at pH 4.5 and 7.0 for 20 and 60 min, desalted through a column (2.0 cm × 30 cm) of Sephadex G-25, and hydrolyzed with 6 M HCl at 110 °C for 22 h. Methionine sulfoxide was analyzed after hydrolyzing the enzyme in 2.5 M NaOH at 110 °C for 19 h [15]. Tryptophan contents were determined by the method of Spies and Chambers [16].

The hydrolytic reactions of various substrates catalyzed by the intact and the modified enzyme were carried out at 25 °C in 0.05 M acetate buffer (pH 5.85). The

* The cysteinyl residue may also be photooxidized. However, this enzyme has no cysteinyl residue.

time course of the reaction was followed by the determination of the reducing end according to the method described elsewhere [17].

## RESULTS AND DISCUSSION

The rate of the photosensitized inactivation of bacterial liquefying α-amylase measured with soluble starch as a substrate followed first-order kinetics at every pH studied. Typical examples of the first-order plots are shown in Fig. 1. The pH depend-

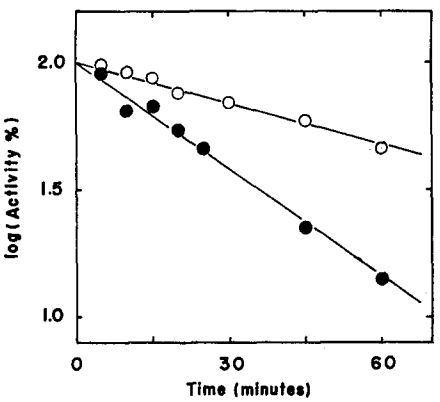


Fig. 1. A typical example of the time-course of inactivation of bacterial liquefying α-amylase photooxidized by methylene blue. Soluble starch (0.5%) was used as a substrate for measurement of the enzyme activity at pH 5.85. The photooxidation was conducted at pH 5.85 (○) and pH 7.45 (●) at 25 °C.

ence of the relative magnitude of the rate constant of inactivation is shown in Fig. 2. This plot closely resembles the titration curve of a histidyl residue with p$K$ about 5.8. In accordance with this, the results of the amino acid analyses listed in Table I show that about one histidyl residue is destroyed by the photooxidation at pH 7.0. Table I also shows that slightly more (less than one) tryptophanyl residue appears to be oxidized at pH 7.0 than at pH 4.5, which prevents us from definitely concluding that only a histidyl residue is exclusively modified. However, the clear pH dependence of the loss of enzyme activity shown in Fig. 2 may be taken as good evidence for believing that the modification of a histidyl residue is the cause of the activity loss of this enzyme towards soluble starch as a substrate.

The effect of the photooxidation of the enzyme at pH 7.0 on the initial rate of enzyme reaction towards various maltooligosaccharides ($G_3 \sim G_7$ and $G_{12.6}$) as substrates was studied at a substrate concentration $s$ sufficiently lower than the $K_m$ value of each substrate. The results are shown in Fig. 3, where the remaining enzyme activity towards various substrates is plotted against the time of the modification. It is interesting to note that the effect of photooxidation on the enzyme activity is distinctly different depending on the degree of polymerization, $n$, of substrate; the rates of inactivation for $G_3$, $G_4$ and $G_5$ are much lower than those for the longer substrates, $G_7$ and $G_{12.6}$. If the modified group(s) were directly involved in the catalytic hydrolysis of the substrate linkage, the effect of modification would be the same for all substrates,

TABLE I

AMINO ACID COMPOSITION OF PHOTOOXIDIZED BACTERIAL LIQUEFYING α-AMYLASE

Each enzyme sample was analyzed by the method of Moore et al. [14] after 22 h of acid hydrolysis with 6 M HCl at 110 °C. The values are expressed as the number of amino acid residues per 1 mole of bacterial liquefying α-amylase (BLA).

| Amino acids | Intact BLA | BLA modified at pH 4.5 | | BLA modified at pH 7.0 | |
|---|---|---|---|---|---|
| | | For 20 min | For 60 min | For 20 min | For 60 min |
| Asp | 52.5 | 53.3 | 53.9 | 53.6 | 52.4 |
| Thr | 21.5 | 22.6 | 23.1 | 23.2 | 22.4 |
| Ser | 24.0 | 24.8 | 24.6 | 24.3 | 24.5 |
| Glu | 41.0 | 42.9 | 42.9 | 40.3 | 42.1 |
| Pro | 16.0 | 15.2 | 15.6 | 15.5 | 15.0 |
| Gly | 38.0 | 38.4 | 37.8 | 37.2 | 37.3 |
| Ala | 29.0 | 29.0 | 29.0 | 29.0 | 29.0 |
| Val | 23.0 | 24.1 | 24.3 | 24.2 | 23.7 |
| Met* | 5.0 | 4.9 | 5.1 | 4.3 | 4.9 |
| Ile | 16.0 | 17.6 | 16.5 | 16.7 | 16.7 |
| Leu | 21.5 | 22.2 | 21.8 | 22.0 | 21.8 |
| Tyr | 23.5 | 24.1 | 24.1 | 24.2 | 23.6 |
| Phe | 16.0 | 16.6 | 17.1 | 16.9 | 16.7 |
| Trp** | 14.0 | 13.4 | 13.0 | 12.7 | 12.3 |
| Lys | 25.0 | 24.9 | 25.2 | 25.2 | 25.5 |
| His | 11.5 | 11.3 | 11.0 | 10.3 | 10.0 |
| Arg | 17.0 | 15.9 | 16.0 | 15.8 | 16.8 |

* Methionine sulfoxide was not detected in the alkaline hydrolyzate of BLA photooxidized for 20 min at either pH employed [15].

** Determined on desalted samples by the method of Spies and Chambers [16].

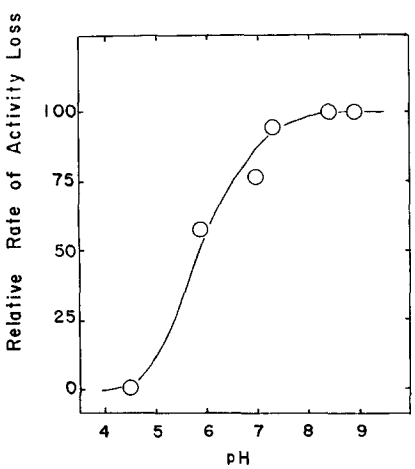


Fig. 2. The pH profile of the rate of inactivation of bacterial liquefying α-amylase photooxidized by methylene blue. The solid line represents the titration curve of a histidyl residue with p$K$ 5.8.

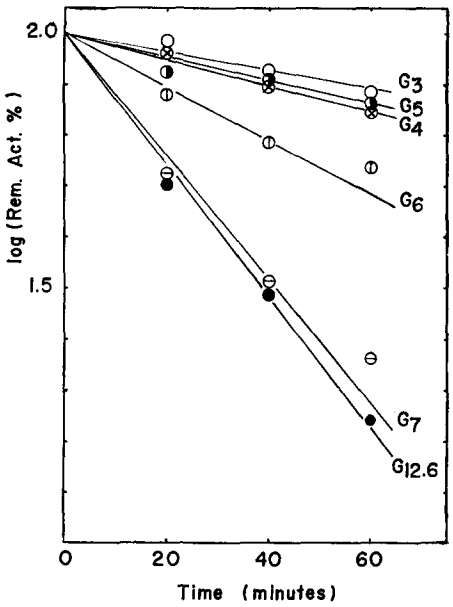


Fig. 3. The remaining activities of bacterial liquefying $\alpha$-amylase which was photooxidized for 20, 40 and 60 min at pH 7.0 and 25 °C measured by using a series of maltooligosaccharides ($G_n$) as substrates. The enzyme reactions were conducted at 25 °C and pH 5.85 with the substrate concentration $s$ and the enzyme concentration $e_0$ described below: $G_3$: $s$ = 5.56 mM, $e_0$ = 21.6 $\mu$M; $G_4$: $s$ = 2.85 mM, $e_0$ = 14.4 $\mu$M; $G_5$: $s$ = 2.66 mM, $e_0$ = 4.80 $\mu$M; $G_6$: $s$ = 2.02 mM, $e_0$ = 719 nM; $G_7$: $s$ = 2.60 mM, $e_0$ = 240 nM; $G_{12.6}$: $s$ = 48.6 $\mu$M, $e_0$ = 13.5 nM.

probably leading to a nearly complete loss of the enzyme activity. Therefore, the results can be interpreted to indicate that the modified group is not essential for the catalytic cleavage of substrate linkage but is important for the formation of a productive complex especially for longer substrates.

According to the subsite theory [1–3, 11], the rate parameter $k_0/K_m$, where $k_0$ and $K_m$ are the molecular activity and the Michaelis constant, respectively, involves only the constants for productive complexes and is given by the following equation;

$$k_0/K_m = k_{int} \sum_p K_{n,p} = 0.018\, k_{int} \sum_p \exp\Big(\sum_i^{cov.} A_i/RT\Big)_{n,p} \quad (1)$$

where $R$ and $T$ are the gas constant and the absolute temperature, respectively. $k_{int}$ is the intrinsic rate constant of hydrolysis of substrate linkage in a productive complex, which was assumed to be constant irrespective of the degree of polymerization, $n$, of substrate and of binding modes. $K_{n,p}$ is the association constant of $n$-mer substrate in a productive binding mode specified by the subscript $p$*, $A_i$ is the subsite affinity of the $i$-th subsite, and $\big(\sum_i^{cov.} A_i\big)_{n,p}$ is the sum of the affinities of the subsites covered by the $n$-mer substrate in the relevant binding mode $p$. Eqn 1 predicts that the change in $A_i$

* The number which specifies a productive binding mode $p$ is conveniently taken to be the number of subsite at which the reducing end glucose residue of the substrate is situated (cf. Fig. 5).

caused by the chemical modification would affect the $k_o/K_m$ of an $n$-mer substrate which does occupy the $i$-th subsite in its productive binding mode(s). Therefore, the subsite modified by photooxidation can possibly be identified by investigating the magnitude of the modification effect on $k_o/K_m$ as a function of the degree of polymerization $n$ of maltooligosaccharides.

The value of $k_o/K_m$ of a substrate can be obtained from the initial rate $v$ at a substrate concentration $s$ sufficiently lower than its $K_m$ value, according to the following equation;

$$v = k_o e_o s/(K_m + s) \simeq (k_o/K_m)e_o s \tag{2}$$

where $e_o$ is the molar concentration of the enzyme. At an appropriate substrate concentration for each substrate which satisfies $s \ll K_m$, the initial rate was determined to obtain $k_o/K_m$ for both the intact and the modified enzymes. The effect of the photooxidation on $k_o/K_m$ is shown in Fig. 4, where the ratio $(k_o/K_m)_{\text{modified}}/(k_o/K_m)_{\text{intact}}$ is

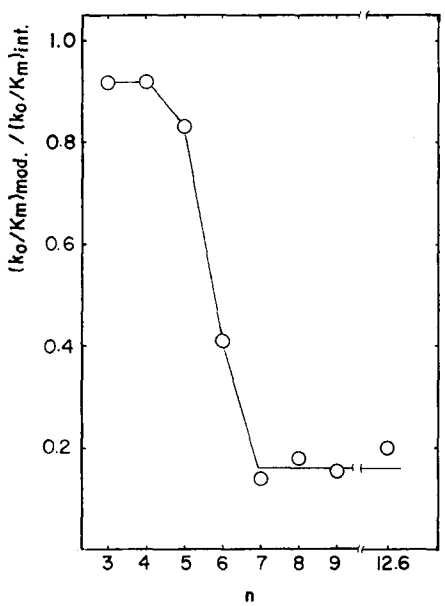


Fig. 4. The relationship between the ratios of $(k_o/K_m)_{\text{modified}}$ to $(k_o/K_m)_{\text{intact}}$ and the degree of polymerization $n$ of maltooligosaccharides. The modification was performed at 25 °C for 1 h at pH 7.0.

plotted against $n$. A distinct decrease in the ratio is observed when $n$ exceeds 5. In other words, the effect of the modification is significant for $n > 5$, but insignificant for $n < 6$.

From the results of the product analyses by thin-layer chromatography and from the arrangement of the subsite affinities already evaluated [5, 6], the predominant productive complexes of maltooligosaccharides are considered as those depicted in Fig. 5. The sum $\sum_p K_{n,p}$ in Eqn 1 is determined approximately by one (or more) of the

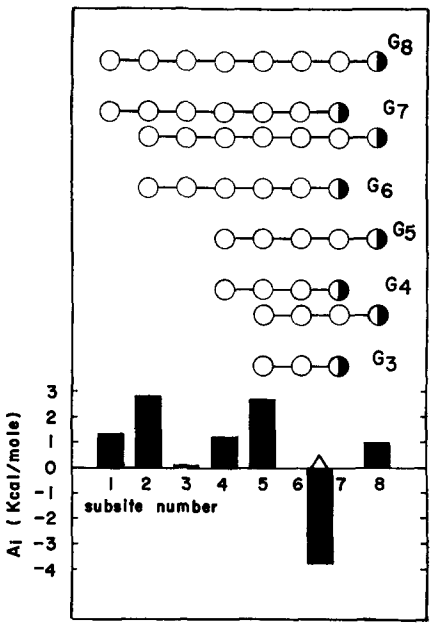


Fig. 5. Histogram showing the subsite affinities of bacterial liquefying $\alpha$-amylase and predominant productive binding mode(s) of maltooligosaccharides. The wedge represents the catalytic site. The open and the half-black circles represent a glucose residue and the one at the reducing end, respectively. The values of the subsite affinities are those already evaluated [5, 6]. The predominant productive binding mode(s) were estimated from the histogram and the product distribution was observed by thin-layer chromatography [5, 6].

largest association constant(s) of the predominant productive complex(es). Thus, referring to the predominant productive binding mode(s) (Fig. 5), we have;

$$(k_o/K_m)_5 = k_{int}\, K_{5,\,8} = 0.018\, k_{int} \exp\,[(A_4 + \,.\,.\, + A_8)/RT] \tag{3}$$

$$(k_o/K_m)_6 = k_{int}\, K_{6,\,7} = 0.018\, k_{int} \exp\,[(A_2 + \,.\,.\, + A_7)/RT] \tag{4}$$

$$(k_o/K_m)_7 = k_{int}\,(K_{7,\,8} + K_{7,\,7}) = 0.018\, k_{int}\,\{\exp[(A_1 + \,.\,.\, + A_7)/RT] + \exp\,[A_2 + \,.\,.\, + A_8)/RT]\} \tag{5}$$

and so on, where $(k_o/K_m)_n$ is the value of $k_o/K_m$ for an $n$-mer substrate. A negligible effect of the photooxidation on $k_o/K_m$ for substrates with $n < 6$ (Fig. 4) indicates that the subsite affinities $A_4$, $A_5$, $A_6$, $A_7$ and $A_8$ are not affected by the modification (see Fig. 5). On the other hand, the sudden decrease in the $k_o/K_m$ ratio for $G_6$ and $G_7$ is consistent with the supposition that one (or more) of the subsite affinities, $A_1 \sim A_3$ (presumably $A_1$ or $A_2$), has been decreased by the photooxidation of the enzyme. An ingenious interpretation of this effect would be that the modified (presumably a histidyl) residue is directly involved in the interaction between the modified subsite and a glucose residue of the substrate in its productive binding mode. However, the possibility that the subsite affinity is altered indirectly, as a consequence of the modification of an amino acid residue which is not located at that subsite, might not be excluded.

Finally we would like to emphasize that a study of this kind will be useful for

locating the subsite whose affinity towards a substrate residue is affected by the chemical modification of some amino acid residue(s) of the enzyme. It would provide us with a new means to research the "structure–function relationship" of enzymes at the level of "subsite structure–action pattern relationships".

ACKNOWLEDGEMENT

The authors wish to thank Miss F. Murakami for the performance of amino acid analyses.

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