

## Photochemical inactivation of taka-amylase A

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## Photochemical inactivation of taka-amylase A

Sam Soon KIM\*

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### INTRODUCTION

The photochemical reactions, in the absence or presence of sensitizer, have been widely investigated by various workers, in various systems. Among these, the biological effects of ultraviolet and visible light have attracted the great attention of many workers from biological importance.<sup>25)</sup> The studies on living matter and body have developed into a special branch in the field of photobiology. Since the systems such as animals or unicellular organisms are too complicated to give an exact interpretation to the obtained results, it seems to be the short-cut for elucidation of light action on living things to study first the fundamental processes of physical and chemical effects of radiation on biopolymers, proteins and nucleic acids.

The present investigation deals with the photochemical inactivation of taka-amylase A by direct and indirect irradiation along this line: ultraviolet inactivation and visible one. In the latter case, the presence of suitable sensitizer is necessary. In most hitherto studied, methylene blue has been mainly used as photosensitizer,<sup>26,42-45)</sup> but this is not a biological pigment. However, riboflavin distributes commonly in living body as 5' phosphate (FMN) and as flavin adenine dinucleotide (FAD) in combination with adenosine-5'-phosphate, and is known to play important roles directly or indirectly in photo- and dark-reactions. For this reason, it is considered that riboflavin is more favorable than artificial pigments as photosensitizer.

Taka-amylase A is strongly inactivated directly by ultraviolet light<sup>21)</sup> or indirectly by visible light in the presence of riboflavin.<sup>8-12 21,34)</sup> The inactivations in both cases are ascribed to the photooxidation of con-

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stituent amino acid residues, although the detailed mechanism of photochemical processes is quite different. These were known from oxygen up-take during photoinactivation, quantitative determination of oxidized amounts of amino acid residues and action spectra for photoinactivation.

In order to clear the photophysical and photochemical processes leading to the inactivation of amylase, the effect of perturbation of paramagnetic ions on these processes was studied. The results obtained were discussed together with those from fluorescence and phosphorescence measurements. The effect of the presence of substrate on photoinactivation was also studied.

## EXPERIMENTAL

Taka-amylase A was prepared from "taka-diastrase Sankyo" and recrystallized three times from aqueous acetone solution containing 0.01 M calcium acetate by Akabori's method.<sup>1)</sup> The amylase solutions (0.143 %, pH 5.6) in the absence and presence of riboflavin ( $5.33 \times 10^{-5}$  M) were respectively irradiated by ultraviolet and visible light at 20°C in thermostat. The activity was measured at 40°C by the blue valuc method.<sup>7)</sup> The ultraviolet light was obtained from the 100 W high pressure mercury lamp (SHL-100 UV) for the measurements of photoinactivation and oxygen up-take, and from 300 W xenon lamp (XD 300 B) for the measurement of action spectrum. The visible light (320-500 m $\mu$ ) for irradiation was isolated from 500 W or 100 W projection lamp through a suitable filter system. The monochromater was used to obtain the monochromatic light, if necessary. The oxygen up-take during irradiation was measured by Warburg manometer. The bottom of main chamber (25 mm  $\times$  50 mm, fused quartz plate) was illuminated at 27.5°C in the thermostat and CO<sub>2</sub> was absorbed by 30 % KOH aqueous solution in the side chamber.

## RESULTS AND DISCUSSIONS

### 1). Photoinactivation of taka-amylase A by ultraviolet light.

The exposure of amylase solutions to ultraviolet light in the presence of air causes loss of activity, as shown in Fig. 1, (2). The activity of amylase decreases with the illumination time exponentially. From the value extrapolated to zero dose, the ultraviolet inactivation seems to be two hit event.<sup>2)</sup> The oxygen up-take occurs in the process of photoinactivation, as seen in Fig. 2, (1). This means that the photoinactivation is ascribed to the photooxidation of amino acid residues by photochemical action. Curve (2) is the oxygen up-take by the amino acid solution of which composition and concentration are the same as those of taka-amylase A. Nearly the same values of oxygen

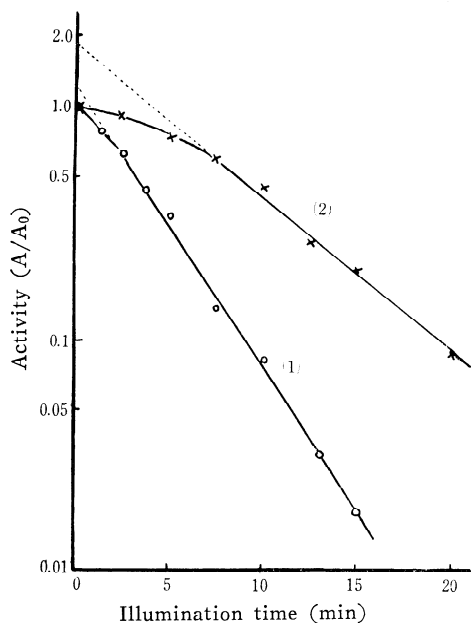


Fig. 1. Ultraviolet and visible inactivation of taka-amylase A. [amylase] =  $1.04 \times 10^{-6}$  M, [riboflavin] =  $7.8 \times 10^{-5}$  M, pH=5.6 at 20°C, (1). amylase-riboflavin system, (2). amylase only.

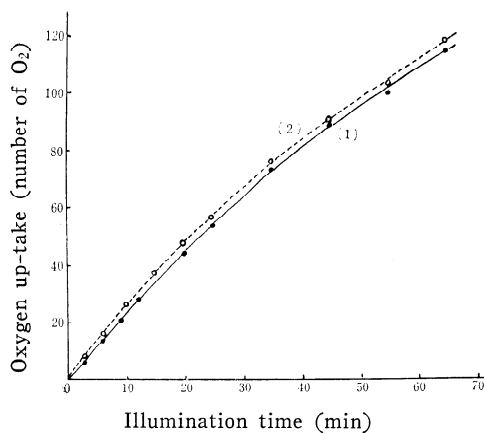


Fig. 2. Oxygen up-take with irradiation of ultraviolet light. [taka-amylase A] = 0.143 %, pH=5.6, at 27.5°C, (1). (number of O<sub>2</sub> absorbed)/(amylase molecule), (2). (number of O<sub>2</sub> absorbed)/(amino acids with equivalent constituents to amylase molecule).

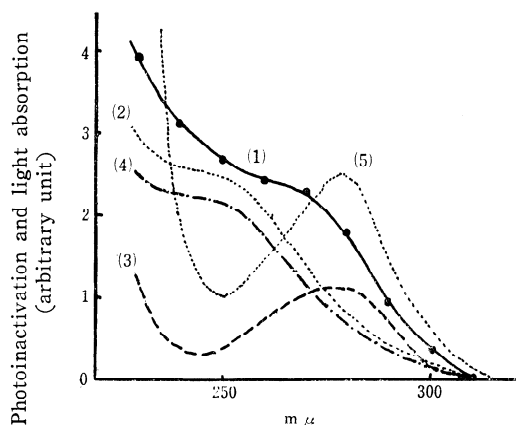


Fig. 3. Action spectrum of ultraviolet inactivation of taka-amylase A. (1). action spectrum, (2). absorption of cystine residues, (3). contribution from tryptophan residues to photoinactivation, (4). contribution from cystine residues to photoinactivation, (5). absorption of amylase.

up-take in taka-amylase A and in amino acid solutions show that the photooxidation of amino acids by ultraviolet irradiation does not strongly depend on the states of amino acids, whether they are free or bound to form the enzyme structure.

The action spectrum<sup>21)</sup> of ultraviolet inactivation is shown in Fig. 3, together with the absorption spectra of amylase and cystine. The action spectrum is quite resemble to the absorption spectrum of cystine. Taka-amylase A molecule contains 4 cystine residues which play an important role of maintaining a stereometric structure of molecule.<sup>38,39)</sup> The above-obtained results show that the light absorbed by cystine is essentially responsible for the photoinactivation of amylase on direct excitation.

The absorption band maximum of natural amylase solution is at longer wavelength by 2.5 m $\mu$  than that of "amylase" composed of the same amounts of amino acids (tryptophan, tyrosine, phenylalanine, histidine and cystine) as those in natural amylase. So, the absorption cross section of natural amylase,  $a(\lambda)$  is expressed by the following relation,

$$a(\lambda) = c \sum_i n_i a_i(\lambda - 2.5) = c a^*_i(\lambda), \quad (1)$$

where  $a_i(\lambda)$  is the absorption cross section of amino acid  $i$  in free state at the wavelength  $\lambda$ ,  $n_i$  the number of amino acid  $i$  in amylase molecule,  $a^*_i(\lambda)$  the absorption cross section of the composed "amylase" corrected for the long wavelength shift and  $c$  the normalizing factor.

If  $\phi_i$  is the quantum yield for photoinactivation by the light absorption of amino acid residue  $i$ , action spectrum  $s(\lambda)$  is expressed as follows:

$$s(\lambda) = \sum_i a^*_i(\lambda) \phi_i \quad (2)$$

Using the observed  $s(\lambda)$  (expressed as  $\cdot$  in Fig. 3) and  $a^*_i(\lambda)$ ,  $\phi_i$  is calculated from the equation (2) as shown in Table 1. The light quanta absorbed by cystine are most effective for the photoinactivation of amylase and the contributions of other amino acid residues to photo-

Table 1

Amino acid residues	Number of residues in taka-amylase A molecule	Relative quantum yield for photoinactivation (%)
Cystine	4	99.2
Tryptophan	10	0.8
Tyrosine	28	$\sim 0$
Phenylalanine	13	$\sim 0$
Histidine	7	$\sim 0$

inactivation are very small. Curve (3) and (4) show respectively the contributions from tryptophan and cystine residues to photoinactivation of amylase. Curve (1) is the addition of (3) and (4).

The prosthetic group of taka-amylase A consists of eight manoses, two hexosamines and one xylose.<sup>16)</sup> The oxidation of tryptophan, tyrosine and particularly cystine strongly modifies the stereometric structure of enzyme protein, leading to the inactivation.

When tryptophan, tyrosine or ovalbumin is illuminated with strong flash of which duration is 20–80  $\mu$ sec, the transient absorption ranging from 400  $m\mu$  to 600  $m\mu$  was observed.<sup>13–15)</sup> The band maxima for tryptophan and tyrosine are respectively 430 and 412  $m\mu$  and the duration 20 and 50  $\mu$ sec. The transient absorption of ovalbumin consists of both bands of tryptophan and tyrosine. These transient absorption are attributed to the radicals formed by the ejection of electron in the excited state of the amino acid.

When amylase is irradiated with ultraviolet light, cystine, tryptophan and tyrosine residues are excited to their excited singlet states and then go to their triplet states through the intersystem crossing of the excited singlet and triplet potentials. The electron is ejected from the triplet state to the solvent medium. This kind of photoelectron is so-called "solvated electron". The photoelectron, in the presence of oxygen, may reduce water to form  $HO_2$  radical, the reaction of which with amylase might explain the consumption of oxygen by irradiation and the oxidation of cystine, tryptophan and tyrosine residues.

For the reason mentioned-above, the fundamental process in photoinactivation of amylase by ultraviolet light seems to be the non-adiabatic transitions from excited singlet to triplet and triplet to solvent or ground state. This is shown by the perturbation effect of paramagnetic ions on singlet-triplet forbidden transition.<sup>5)</sup> The photoinactivation of amylase is enhanced by the presence of paramagnetic ions such as  $Co^{++}$  and  $Mn^{++}$ . This is shown in Fig. 4. The transition probability between singlet and triplet states is originally forbidden by spin conservation rule.<sup>32)</sup> But, if paramagnetic ions are present, this selection rule is broken by the perturbation of heterogeneous magnetic field of these ions.<sup>5)</sup> Namely, the transition probability from excited singlet to triplet state increases. This leads to the increase of the population of the photoelectrons in the solvent medium. This is the main reason why photoinactivation is enhanced by the presence of paramagnetic ions. This explanation may also be supported by the strong quenching of tryptophan phosphorescence<sup>22)</sup> (free and bound in protein) by paramagnetic ions.

The photoinactivation is strongly protected by the presence of sub-

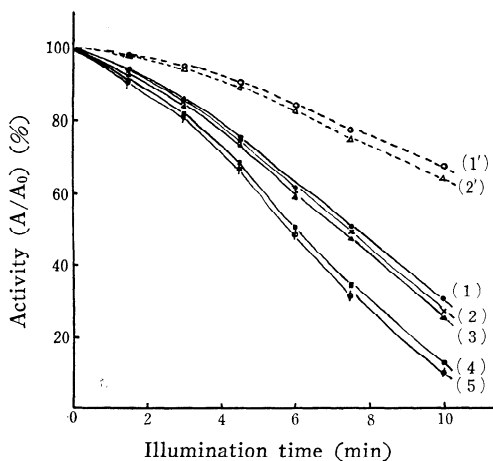


Fig. 4. Effect of the presence of paramagnetic ions and substrate on photoinactivation of taka-amylase A by ultraviolet irradiation. [taka-amylase A]=0.143% [metal ion]=0.02%, [starch]=0.6%, pH=5.6, at 27.5°C, (1). amylase only, (2). amylase+KCl, (3). amylase+CaCl<sub>2</sub>, (4). amylase + MnCl<sub>2</sub>, (5). amylase + CoCl<sub>2</sub>, (1'). amylase + starch, (2'). amylase + CoCl<sub>2</sub>+starch.

strate, starch, whether paramagnetic ions are present or not. It is known that the disordered structure of enzyme goes to the rigid, stable and ordered one upon the formation of enzyme-substrate complex and such a conformation causes the protection from various types of inactivation, for example by urea, acid and heat.<sup>27,41)</sup> The protection of photoinactivation by substrate is probably due to the protection of amino acid residues from oxidation by HO<sub>2</sub> radicals and from heat inactivation by the dissipation of excess energy of excited amino acid residues which increases the microscopic local temperature.

These are considered to be the possible explanation of the photophysical and photochemical processes of ultraviolet inactivation and the protective action of substrate from photoinactivation. However, we can not completely be neglected the effect of the trapping of photo-electrons by substrate or decomposed products on the protective action from photoinactivation.

(2). Photoinactivation of taka-amylase A by visible light in the presence of sensitizer, riboflavin.

Riboflavin is of great interest not only in its own photochemical reaction, but as a photosensitizing agent. It is known that riboflavin participates in the photo-induced production of off-flavors<sup>33)</sup> and the destruction of vitamins<sup>29)</sup> in milk and beer<sup>37)</sup> and acts as sensitizer in phototropism.<sup>40)</sup> Furthermore, it is considered to play important roles in bacterial bioluminescence,<sup>6)</sup> visual process<sup>33)</sup> and chemiluminescence accompanying photosynthesis.<sup>36)</sup> Riboflavin can sensitize the oxidation of many substrates in the presence of oxygen. Such action is known as inactivation of enzyme,<sup>8-12,21,34)</sup> inactivation of tumor,<sup>2)</sup> inactivation of microorganisms hemolysis of red cells,<sup>4)</sup> inactivation of transforming principles<sup>3)</sup> and inactivation of a fungicide.<sup>17)</sup>

The structure of riboflavin has been established by Karrer.<sup>20)</sup> The ribose is attracted *via* its number 1 carbon atom to the number 9 nitrogen in the isoalloxazine ring system. In the presence of oxygen, riboflavin is irreversibly decomposed by light to lumiflavin and lumichrome,<sup>18,23,35)</sup> as well as fragment of ribityl side chain.<sup>28)</sup> The prolonged illumination results in the production of more lumichrome at the expense of riboflavin and lumiflavin. Lumichrome and lumiflavin as well as riboflavin act as the effective sensitizer for the photoinactivation of taka-amylase A.

The effect of illumination time (min) on the photoinactivation by visible light in the presence of riboflavin is shown in Fig. 1, (1). The activity decreases exponentially with illumination time (min). This means the inactivation occurs as one hit event.<sup>21)</sup> This inactivation accompanies the oxygen up-take during the irradiation due to the oxidation of amino acid residues.\* The oxygen up-take was measured

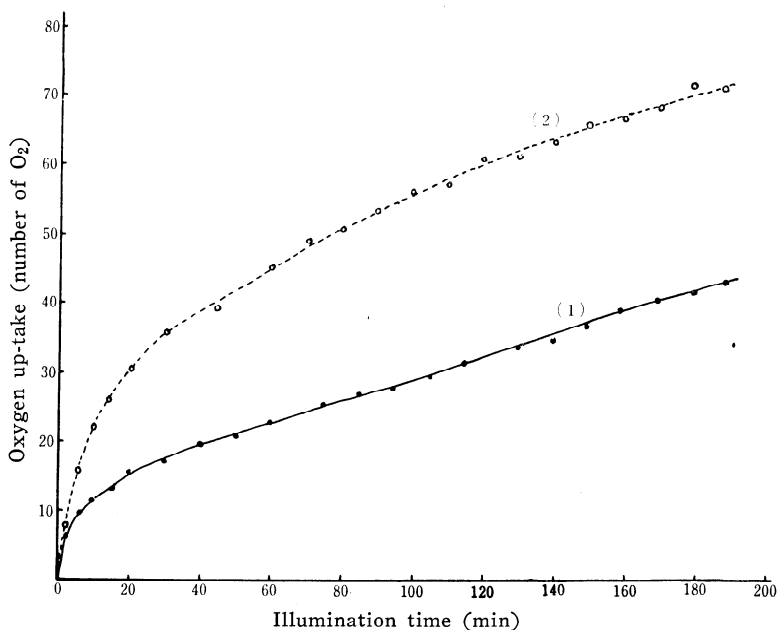


Fig. 5. Oxygen up-take with irradiation of visible light in the presence of riboflavin. [amylase]=0.143%, [riboflavin]= $5.33 \times 10^{-5}$  M, pH=5.6, at 2.75°C, (1). (number of O<sub>2</sub> absorbed)/(amylase molecule), (2). (number of O<sub>2</sub> absorbed)/(amino acids with equivalent constituents to amylase molecule).

\* The quantitative determination of oxidized amounts of amino acid residues is chemically under experiment.



by Warburg manometer. The results are shown in Fig. 5. Curve (1) is the oxygen up-take by taka-amylase A and curve (2) that by the amino acids of which composition is as same as that of natural amylase. The oxygen up-take in amylase solution is much lower than that in the amino acid solution. This seems to come from the high polymer effect

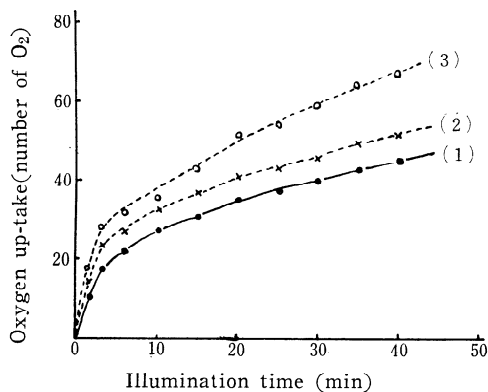


Fig. 6. Effect of denaturation on oxygen up-take of taka-amylase A. [amylase] = 0.143 %, [riboflavin] =  $5.33 \times 10^{-5}$  M, pH = 5.6, at 27.5°C, (1), native amylase, (2), amylase denatured by 0.3 M urea for 20 hours at 20°C, (3), amylase denatured by 8M urea for 20 hours at 20°C.

for the sensitized photo-oxidation, contrary to the case of ultraviolet irradiation. Such a high polymer effect is also seen in the increase of oxygen up-take when amylase was denatured by urea, as shown in Fig. 6. Namely, the amino acids are highly protected from oxidation—collision with oxidizer—by the formation of polymer structure of native amylase.

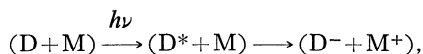
The light energy absorbed by riboflavin is dissipated as heat or lost as fluorescence by the radiative transition from excited singlet to ground state

or lost as phosphorescence by the radiative transition from triplet to ground state. The lifetime of triplet state is much longer ( $\sim 10^{-3}$  sec) than that of excited singlet state ( $\sim 10^{-8}$  sec), so that the triplet state has a much larger chance to collide with reactant than the excited singlet state. For this reason, it is considered that the triplet state is responsible for the chemical reaction in the excited state. Riboflavin or its derivatives in the triplet state may couple with oxygen molecule to form the dyeoxygen complex (triplet-triplet complex). This complex can dissociate into triplet oxygen and dye in the ground state, and singlet oxygen and dye in the ground state, according to the spin conservation law. The former is the quenching process of excited dye (phosphorescence quenching) and the latter the process of formation of active, metastable oxygen. As the ground state of oxygen is triplet, the life time of singlet oxygen is considerably long due to the spin forbiddenness. In this connection, the population of triplet state of dye molecule is essential for the oxidation of amino acid residues. The lifetime of triplet state is controlled by the mutual relation between the forbidden transition from excited singlet to triplet and from trip-

let to ground state. The selection rule for such forbidden transitions is broken by the spin-orbital coupling<sup>5)</sup> induced by the magnetic perturbation of paramagnetic ions. This results in the shortening of the lifetime of triplet state. The inhibition of photoinactivation and oxygen up-take by the presence of such ions as shown in Fig. 7 and 8 supports the above-mentioned mechanism of the photochemical processes of riboflavin-sensitized photoinactivation.

The presence of paramagnetic ions quenches the fluorescence of riboflavin only in their high concentrations ( $\sim 10^{-2}$  M), but the quenching of phosphorescence of riboflavin begins in the much lower concentrations ( $\sim 10^{-5}$  M) of paramagnetic ions.<sup>22)</sup> The life-time of the fluorescent species is the order of  $10^{-8}$  sec, so that the quencher molecule can encounter this fluorescent species only in the extremely high concentrations. On the other hand, the lifetime of triplet state of about  $10^{-3}$  sec makes quencher molecule even at the concentration of  $10^{-6}$  M possible to encounter the triplet molecule.

Riboflavin has a possibility to form a charge transfer complex with paramagnetic ions as follows:



where D and M are respectively dye molecule and metal ion, and  $D^*$  the excited dye molecule. The quenching of excited dye species by such a process can not be excluded, but the details are left in future. Riboflavin forms a charge transfer complex with tryptophan<sup>19,31)</sup> only in the extremely high concentrations ( $\sim 10^{-1}$  M) at low temperature. Therefore, though the formation of such complex can not be neglect-

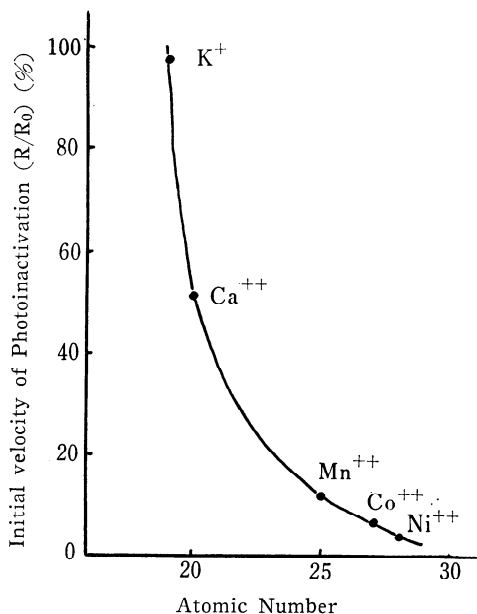


Fig. 7. Effect of paramagnetic ions on initial velocity of visible inactivation in the presence of riboflavin, [amylase] = 0.143% [metal ion] = 0.02 M, [riboflavin] =  $5.33 \times 10^{-5}$  M, at 20°C, pH = 5.6.

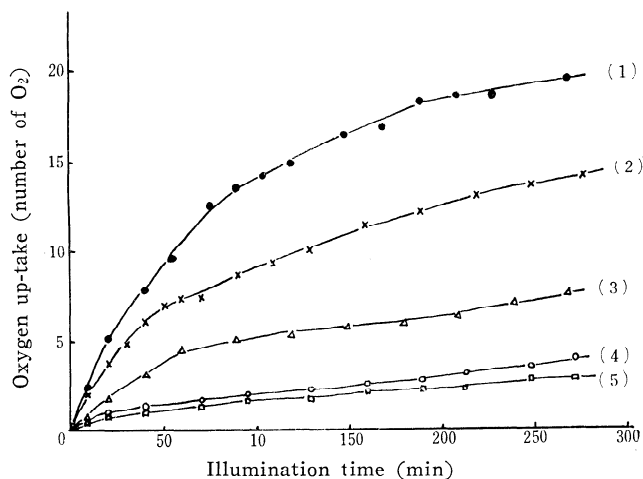


Fig. 8. Effect of paramagnetic ions on oxygen up-take of taka-amylase A irradiated by visible light. [amylase] = 0.143 %, [riboflavin] =  $5.33 \times 10^{-5}$  M, pH = 5.6, at 27.5° C, [metal ion] = 0.02 M, (1). amylase only, (2). amylase + CaCl<sub>2</sub>, (3). amylase + MnCl<sub>2</sub>, (4). amylase + NiCl<sub>2</sub>, (5). amylase + CoCl<sub>2</sub>.

ed, the low concentration employed in the present investigation may exclude the contribution of this kind of complex formation to photoinactivation of amylase.

Fig. 9 shows the changes of activity and oxygen up-take with illumination time in the absence and presence of substrate. The presence of substrate strongly protects amylase from inactivation and oxidation of amylase. The formation of enzyme-substrate complex protects enzyme from oxidation. It is considered that, as seen in Fig. 5 and 6, the photooxidation of amino acid residues depends on the structure of amylase. For this reason, the conformation change induced by enzyme-substrate complex formation may control the photochemical reaction, as stated above. Substrate is decomposed in several minutes, so that it is considered that the decomposed products also have a protective action by the formation of enzyme-product complex.

Furthermore, the oxidizers may be destroyed by the reaction with substrate or products, leading to create a favorable condition for enzyme protection.

The viscosity increase by the presence of substrate and products may decrease the collision frequency between active oxygen and amino acid residue. This also may play a part to some extent in the decrease of oxidation reaction rate.

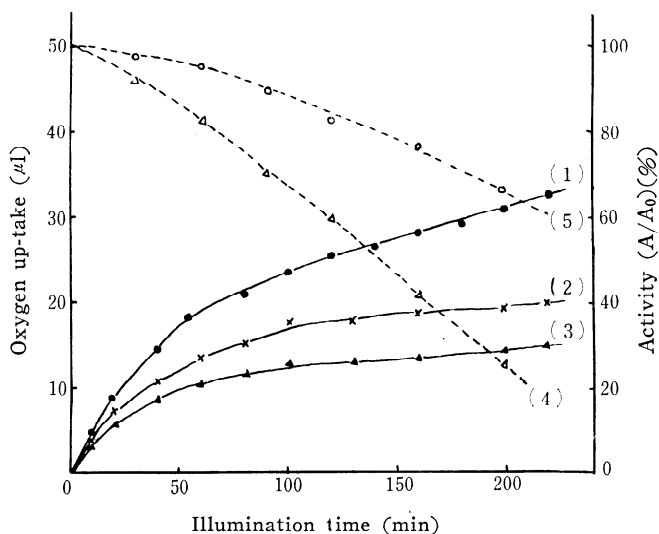


Fig. 9. Effect of substrate on oxygen up-take and visible inactivation of taka-amylase A in the presence of riboflavin. [riboflavin] =  $5.33 \times 10^{-5}$  M, pH = 5.6, at 27.5°C, (1). oxygen up-take in amylase only, (2). oxygen up-take in amylase + 0.02% starch, (3). oxygen up-take in (amylase + 0.533% starch) (4). activity in amylase only, (5). activity in (amylase + 0.533% starch).

### SUMMARY

The photoinactivation of taka-amylase A, in the absence and presence of sensitizer, riboflavin, is due to the photooxidation of amino acid residues, cystine, tryptophan, tyrosine and histidine. The process of ejection of photoelectron from triplet state *via* excited singlet state is important for the ultraviolet inactivation. The stabilization of triplet state of riboflavin is essentially important process for the riboflavin-sensitized photoinactivation of taka-amylase A. These processes can be controlled by the spin-orbital coupling effect in the presence of paramagnetic ions. The formation of enzyme-substrate and -product complexes protects enzyme from ultraviolet and visible inactivation.

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## REFERENCES

- 1) Akabori, S., Ikenaka, T. and Hagihara, B., 1954, *J. Biochem.*, **41**: 577.
- 2) Bellin, J. S., Mohos, S. C. and Oster, G., 1961, *Cancer Res.*, **21**: 1365.
- 3) Bellin, J. S. and Oster, G., 1960, *Biochim. Biophys. Acta*, **42**: 533.
- 4) Blum, H., 1941, *Photodynamic Action and Diseases Caused by Light* (Reinhold Publ. Corp., New York).
- 5) Clementi, E. and Kasha, M., 1958, *J. Mol. Spectroscopy*, **2**: 297.
- 6) Doudoroff, M., 1933, *Enzymologia*, **5**: 239.
- 7) Fuwa, H., 1954, *J. Biochem.*, **41**: 583.
- 8) Galston, A. W. and Baker, R. S., 1949, *Science*, **109**: 485.
- 9) Galston, A. W. and Hand, M. E., 1949, *Am. J. Bot.*, **36**: 85.
- 10) Galston, A. W., 1949, *Proc. Natl. Acad. Sci. U. S.*, **35**: 10.
- 11) Galston, A. W. and Baker, S. R., 1949, *Am. J. Bot.*, **36**: 773.
- 12) Galston, A. W., 1950, *Science*, **111**: 619.
- 13) Grossweiner, L. I., 1956, *J. Chem. Phys.*, **24**: 1255.
- 14) Grossweiner, L. I. and Mulac, W. A., 1959, *Rad. Res.*, **10**: 515.
- 15) Grossweiner, L. I. and Zwickel, E. F., 1959, *J. Chem. Phys.*, **31**: 1141; 1961, *ibid.*, **33**: 1411; 1963, *ibid.*, **39**: 2774.
- 16) Hanafusa, H., Ikenaka, T. and Akabori, S., 1955, *J. Biochem.*, **42**: 55.
- 17) Hendricks, B. and Berends, W., 1958, *Rec. Trav. chim. Pays-Bas*, **77**: 145.
- 18) Holmström, G. and Oster, G., 1961 *J. Am. Chem. Soc.*, **83**: 1867.
- 19) Isenberg, I. and Szent-Györgyi, A., 1958, *Proc. Natl. Acad. Sci. U. S.*, **44**: 857; 1959, *ibid.*, **45**: 1229.
- 20) Karrer, P. and Fritsche, H., 1935, *Helv. chim. Acta*, **18**: 911.
- 21) Kim, S. S., 1965, *Nippon Nogei Kagaku Kaishi*, **39**: 10.
- 22) Kim, S. S., to be published.
- 23) Kuhn, R. and Wagner-Jauregg, Th., 1933, *Ber. dtsch. chem. Ges.*, **66**: 1577, 1950.
- 24) Linschitz, H., Berry, M. G. and Schweitzer, D., 1954, *J. Am. Chem. Soc.*, **76**: 5833.
- 25) MacLaren, A. D. and Shugar, D., 1964, *Photochemistry of Proteins and Nucleic Acids* (Pergamon Press).
- 26) Nakatani, M., 1960, *J. Biochem.*, **48**: 640.
- 27) Okunuki, K., *et al.*, 1956, *Symp. Enzyme Chem.*, **11**: 99.
- 28) Oster, G. 1951, *Trans. Farad. Soc.*, **47**: 660.
- 29) Peterson, W. J., Haig, F. M. and Shaw, A. O., 1944, *J. Am. Chem. Soc.*, **66**: 662.
- 30) Pirie, A., 1960, *Nature*, **186**: 352.
- 31) Pullman, B. and Pullman, A., 1958, *Proc. Natl. Acad. Sci. U. S.*, **44**: 1197.
- 32) Reid, C., 1957, *Excited States in Chemistry and Biology* (Butterworths Scientific Publications, London), p. 83.
- 33) Samuelsson, E. G., 1959, *Milchwiss.*, **14**: 155.
- 34) Sone, K., 1959, *Nippon Nogei Kagaku Kaishi*, **33**, 1119; 1962, *ibid.*, **36**: 7.
- 35) Strauss, G. and Nickerson, W. J., 1961, *J. Am. Chem. Soc.*, **83**: 3187.
- 36) Strehler, B. L., 1951, *Arch. Biochem. Biophys.*, **34**: 239.
- 37) Stringer, W. J., 1946, *J. Inst. Brewing*, **52**: 81.
- 38) Takagi, T. and Isemura, T., 1960, *J. Biochem.*, **48**: 781.
- 39) Takagi, T. and Isemura, T., 1961, *J. Biochem.*, **49**: 43.
- 40) Thimann, K. V. and Curry, G. M., 1960, *Comparative Biology* (Florkin, M. and Mason, H. S., Ed., Acad. Press, New York) **1**: 243.

- 41) Tomita, G. and Kim, S. S., 1965, *Nature*, **205**: 46.
- 42) Weil, L., Gordon, W. G. and Buchert, A. R., 1951, *Arch. Biochem. Biophys.*, **33**: 90.
- 43) Weil, L. and Buchert, A. R., 1951, *ibid.*, **34**: 1.
- 44) Weil, L., Buchert, A. R. and Maher, J., 1952, *ibid.*, **40**: 245.
- 45) Weil, L. and Seibles, T. S., 1955, *ibid.*, **54**: 368.
- 46) Zwicker, E. F. and Grossweiner, L. I., 1963, *J. Phys. Chem.*, **76**: 549.