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The Chemical Modification of Biopolymers. III. The Introduction of SH Groups into Taka-amylase A

Shuichi SUZUKI, Yutaka HACHIMORI and Ryuichi MATOBA

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Meguro-ku, Tokyo

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The enzymic activity of Taka-amylase A on amylose, as measured by the blue-value method, increased to 180% of the original when one mole of mercaptosuccinyl group was introduced into one mole of the enzyme. Similar results were obtained for the activity measured by the Semogyi-Nelson method; the introduction of one or two mercaptosuccinyl groups raised the activity to 130—140% of that of the native enzyme. Even after the attachment of six mercaptosuccinyl groups per mole, the activity was 120% of the original. However, the enzymic activity was not elevated, but was lowered, by succinylation. When the sulfhydryl groups of the mercaptosuccinyl groups were blocked with *p*-chloromercurybenzoate or iodoacetamide, the enzyme was inhibited. These results suggest that the sulfhydryl groups of mercaptosuccinyl residues play an important role in increasing the enzymic activity.

Recently, Hartman and Wold[1] reported that the activity of bovine pancreatic Ribonuclease A (EC 2.7.7.16) treated with dimethyladipimidate was elevate to 160% of that of the natural material.

1) F. C. Hartman and F. Wold, *Biochemistry*, **6**, 2439 (1967).

Ikenaka[2] reported that phenylazobenzoyl-Taka-amylase had an activity on α-phenylmaltoside 2.5 times higher than that of the natural material, while its activity on amylose was diminished.

The introduction of sulfhydryl groups into proteins has been attempted by many investigators. Klotz[3] found that *S*-acetylmercaptosuccinic anhydride reacted selectively with free amino groups under mild conditions.

This paper will report on the preparation of mercaptosuccinyl Taka-amylase A (EC 3.2.1.1.) and their properties.

Experimental

Materials. The Taka-amylase A was prepared from Taka-diastase of the Sankyo Co., Ltd., by the method of Akabori *et al.*[4] The protein concentration was estimated by spectrophotometry, with an extinction coefficient, $E^{1\%}_{1cm}$, of 22.1 at 278.5 mμ,[5] or by the method of Lowry.[6] The *S*-acetylmercaptosuccinic anhydride was synthesized by adding thioacetic acid to maleic anhydride according to the method of Holmberg and Schjånberg,[7] it was recrystallized from benzene (melting point 76—77°C). The amylose was purchased from the Nagase Sangyo Co.

Preparation of the Modified Taka-amylase A. Mercaptosuccinyl and succinyl Taka-amylase were prepared as follows. One m*l* of a protein solution (3.6×10^{-4}M) was added to 6 m*l* of a 0.2M phosphate buffer, pH 8.0. One m*l* of *S*-acetylmercaptosuccinic anhydride or succinic anhydride (3.6×10^{-3}M to 1.8×10^{-1}M) in dioxane was then added to the buffered protein solution. After 30 min, 3 m*l* of a 0.33M hydroxylamine solution in a 0.2M phosphate buffer (pH 7.5) was added in order to remove the acetyl groups which blocked the sulfhydryl groups. The mixture was left to stand for 10 min at 20°C, and then dialyzed against distilled water for 24 hr at 20°C. The extent of mercaptosuccinylation was estimated by determining the sulfhydryl groups spectrophotometrically at 255 mμ by mercaptide formation using *p*-CMB, according to the method of Boyer.[8] The extent of succinylation was estimated by the decrease in the amino groups, using the method of Matsushima *et al.*[9]

Activity Measurement. The hydrolytic activity of the natural and modified Taka-amylase A was measured by the decrease in the absorbance at 700 mμ due to iodine-amylose complex formation (the blue-value method[10]) and by the increase in reducing groups (the Somogyi-Nelson method[11,12]). The digestion was conducted at 37°C for 30 min at pH 5.5.

Results and Discussion

Determination of mercaptosuccinyl Groups. *p*-Chloromercuribenzoate reacted with mercaptosuccinic acid stoichiometrically, the molar extinction coefficient of the product at 255 mμ was 5.8×10^3 at pH 4.6 in a 0.2M acetate buffer. The mercaptosuccinyl groups in protein were estimated by means of this coefficient after the *p*-chloromercuribenzoate had been reacted at 25°C for 24 hr.

The number of mercaptosuccinyl groups introduced per mole of the enzyme reached a constant value after 20 to 60 minutes' incubation (Fig. 1).

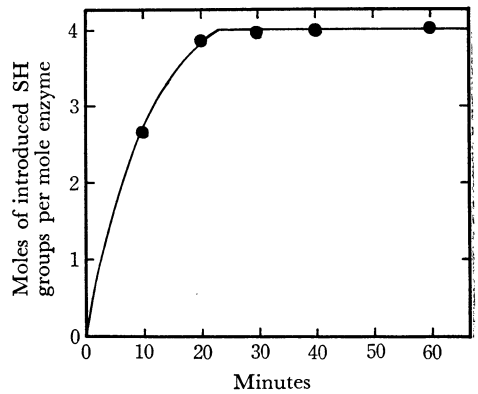

Fig. 1. The time course of mercaptosuccinylation reaction of Taka-amylase A at 2°C, in 0.15M phosphate buffer at pH 8.0.

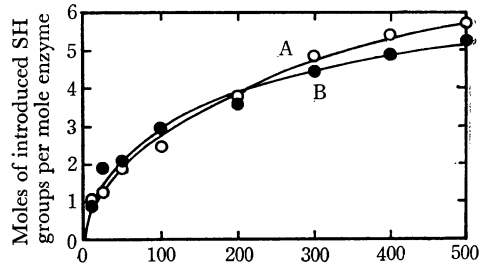

Fig. 2. The effect of concentration of *S*-acetylmercaptosuccinic anhydride on the extent of mercaptosuccinylation at 2°C (Curve B, closed circle) and 25°C (Curve A, open circle).

Figure 2 shows the effect of the concentration of *S*-acetylmercaptosuccinic anhydride on mercaptosuccinylation at 25°C and 2°C. The number of mercaptosuccinyl groups introduced into the enzyme increased in accordance with the increase in the

2) T. Ikenaka, *J. Biochem.*, **46**, 297 (1959).
3) I. M. Klotz and R. E. Heiney, *Arch. Biochem. Biophys.*, **96**, 605 (1962).
4) S. Akabori, R. Ikenaka and B. Hagihara, *J. Biochem.*, **41**, 577 (1954).
5) T. Takagi and H. Toda, *ibid.*, **52**, 16 (1962).
6) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7) B. Holmberg and E. Schjånberg, *Arkiv. Kemi. Mineral. Geol.*, **14A**, No. 7, 1 (1940).
8) P. D. Boyer, *J. Amer. Chem. Soc.*, **76**, 4331 (1954).
9) A. Matsushima, Y. Hachimori, Y. Inada and K. Shibata, *J. Biochem.*, **61**, 328 (1967).
10) H. Fuwa, *ibid.*, **41**, 583 (1954).
11) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
12) N. Nelson, *ibid.*, **153**, 375 (1944).

Table 1. Folin test for *O*-succinylation of protein tyrosine[13]

The results are given in the ratio of optical densities (O.D.) at 750 mμ measured at pH 8.0 and at pH 11.0.

Moles introduced SH per mole enzyme	O.D. pH 8.0/O.D. pH 11.0		
	After 3h	24h	48h
8	0.98	0.95	0.95
4	0.98	0.96	0.99

concentration of *S*-acetylmercaptosuccinic anhydride, it was six when a 500-fold excess mole was applied.

O-Mercaptosuccinylation of Tyrosyl Residues. The extent of the acylation of tyrosyl residues in this enzyme was estimated by the Folin phenol reagent test.[13]

The buffered solutions of the modified enzyme with four and eight moles of mercaptosuccinyl groups per mole were adjusted to pH 8 and 11 respectively. After 3, 24, and 48 hrs incubation at 25°C, the Folin phenol reagent was added to the solutions and they measured for absorbancy at 750 mμ. The results are summarized in Table 1. It may be concluded that the *O*-esterification of the tyrosyl residues occurred to only a slight extent, if at all, during the mercaptosuccinylation reaction.

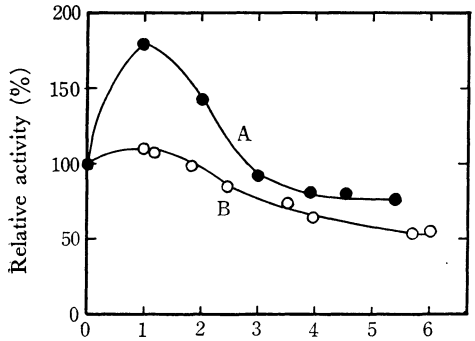

Fig. 3. The activities of native and modified Taka-amylase A on amylose measured by the blue value method. The modified enzymes mercaptosuccinylated at 2°C (Curve A, closed circle), and at 25°C (Curve B, open circle) were used.

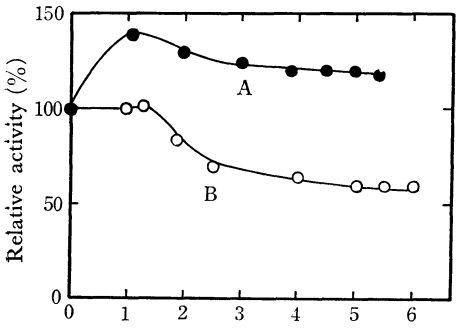

Fig. 4. The activities of native and modified Taka-amylase A on amylose measured by the Somogyi-Nelson method. The modified enzymes mercaptosuccinylated at 2°C (Curve A, closed circle) and at 25°C (Curve B, open circle) were used.

13) G. L. Miller, *ibid.*, **146**, 339 (1942).

Enzymic Activities of Mercaptosuccinyl Takaamylase A. The pH optimum of either the natural or the modified enzyme was around pH 5.5. The following experiments were, therefore, performed at this pH value. The results are shown in Figs. 3 and 4. When one mole of the mercaptosuccinyl group was introduced per mole of the enzyme at 2°C, the activity on amylose, measured by the blue-value method (Curve A in Fig. 3), increased to 180% of that of the natural material. The activities of the modified enzymes with more than three mercaptosuccinyl groups were, however, lower than that of the natural material. Similar results were obtained for the activity measured by the Somogyi-Nelson method (Curve A in Fig. 4); when one mercaptosuccinyl group was attached to the enzyme at 2°C, the activity was 140% of that of the natural

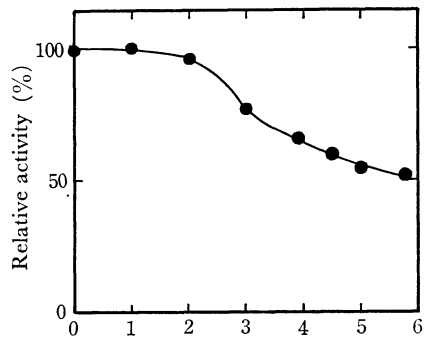

Fig. 5. The activities of native and succinyl Taka-amylase A on amylose measured by the Somogyi-Nelson method.

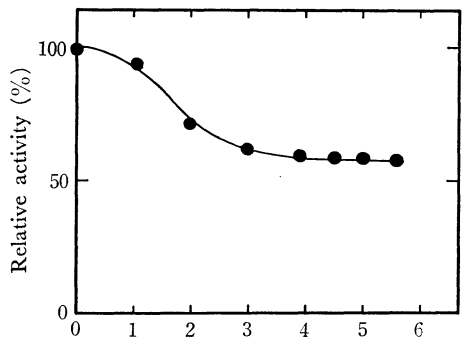

Fig. 6. The activities of native and succinyl Taka-amylase A on amylose measured by the blue value method.

material and 120% of the original activity was retained even after the attachement of six mercaptosuccinyl groups per mole of the enzyme. However, when the modification was made at 25°C, both activities of the modified enzymes decreased in proportion to the number of the mercaptosuccinyl residues introduced into the enzyme (Curves B in Figs. 3 and 4).

The difference between the activities of the modified enzymes prepared at 2°C and 25°C may be due to the difference in the mercaptosuccinylated sites.

Role of the Sulfhydyl Introduced into the Enzyme. The Activity of Succinyl Taka-amylase A. Succinyl Taka-amylase A was prepared has been described in the "Experimental" section, and its activity was compared with that of mercaptosuccinyl Taka-amylase A.

The optimum pH of the succinyl Taka-amylase A was the same as that of the natural enzyme. The activities of the modified enzymes prepared at 2°C were measured by the Somogyi-Nelson method and the blue-value method, and were then plotted against the numbers of succinyl groups introduced into the enzyme. These results are shown in Figs. 5 and 6. No enhancement of the activity was observed on succinylation by either method, in contrast to the case of mercaptosuccinylation.

Effect of the SH Reagents on the Activity of the Mercaptosuccinyl Taka-amylase A. One m*l* of iodoacetamide or *p*-CMB (1.44×10^{-7}M to 3.6×10^{-6}M) solution in a 0.2M phosphate buffer at pH 8.0 was added to 1m*l* of a natural or mercaptosuccinilated enzyme solution (7.2×10^{-9}M). After 4 hr, treatment at 25°C, the mixture was diluted and tested for the enzyme activity by the blue-value method at pH 5.5. The results are shown in Figs. 7 and 8.

The activity of the native enzyme on amylose was not affected by the addition of iodoacetamide (Curve B in Fig. 7), but that of the modified enzyme, which was elevated 1.8 times as compared with

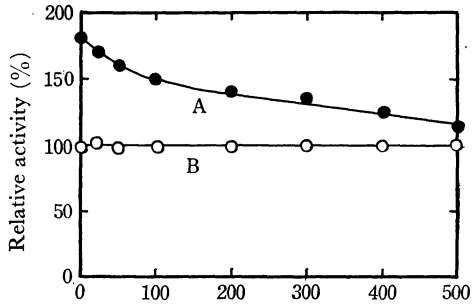

Fig. 7. The inhibition of native (Curve B, open circle) and modified Taka-amylase A (Curve A, closed circle) by iodoacetamide.

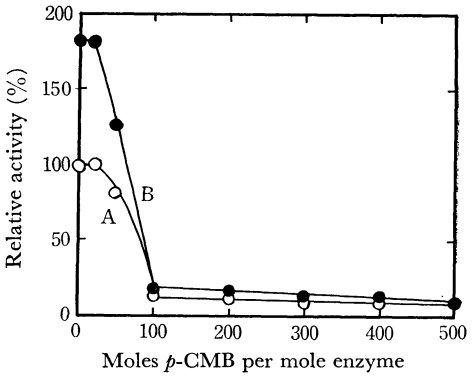

Fig. 8. The inhibition of native (Curve B, open circle) and modified Taka-amylaseA (Curve A, closed circle) by *p*-CMB.

that of the natural enzyme, was appreciably inhibited and approached that of the natural enzyme A in Fig. 7). On the contrary, *p*-CMB inhibited both modified and natural enzymes, as is shown in Fig. 8.

These results suggested that the sulfhydryl group of the mercaptosuccinyl residue plays an important role in the enhancement of the enzymic activity.