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THE INTERACTION OF L-ASCORBIC ACID WITH THE ACTIVE CENTER OF MYROSINASE

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Summary

Only L-ascorbic acid activated plant myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), whereas ascorbic acid analogs did not.

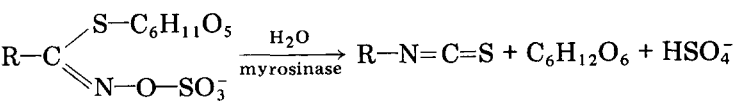
The enzyme protein was conformationally changed by the addition of L-ascorbic acid to the spectrophotometric analysis, approx. 1.5 amino residues appeared on the surface of the enzyme and about 2.3 tryptophan residues were buried in the molecule when 1 mM L-ascorbic acid was added.

Optimum temperature for the myrosinase activity was approx. 55°C without L-ascorbic acid, but with L-ascorbic acid it was about 35°C; that for β-glucosidase activity was the same (55°C) with or without L-ascorbic acid.

The effect of chemical modification of the functional groups of myrosinase on the interaction of L-ascorbic acid was investigated and the interaction of L-ascorbic acid with the active center of the enzyme is proposed.

Introduction

Plant myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), contained mainly in the Cruciferae family, is β-thioglucosidase which catalyzes the hydrolysis of mustard oil glucosides to goitrogenic isothiocyanates, glucose and bisulfate. It is specifically activated by L-ascorbic acid.



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In previous papers, we described the purification, physicochemical properties [1] and functional groups [2] of myrosinase from yellow mustard powder (*Brassica juncea*). We also found that the activation mechanism of myrosinase by L-ascorbic acid depends on a slight conformational change in the protein due to the presence of L-ascorbic acid, not to the dissociation and association mechanism of myrosinase [3]. Furthermore, we reported that the hydrolysis of *p*-nitrophenyl β-glucoside by myrosinase was not accelerated by L-ascorbic acid, but was inhibited competitively at a high concentration of L-ascorbic acid [4].

In this study, we spectrometrically investigated the interaction of L-ascorbic acid with the enzyme due to its inhibitory effect or to chemical modification. Reagents for discriminating the states of amino acids were used on both myrosinase and β-glucosidase activities in connection with the activation of the enzyme. The relationship between the active center and the activation mechanism of the enzyme by L-ascorbic acid is discussed.

Materials and Methods

Chemical reagents. 2-Methoxy-5-nitrotropone was purchased from Sankyo Co. (Japan). Other chemicals were obtained from Nakarai Chemicals (Japan). All reagents were of analytical grade and used without further purification.

Enzyme assay. Sinigrin, obtained from NBC Chemicals (Ohio, U.S.A.), was used as the substrate for myrosinase; *p*-nitrophenyl β-D-glucoside (Sigma) was used as the substrate for β-glucosidase.

The assay mixture contained 2.5 μmol substrate and enzyme solution in a total volume of 1 ml. Myrosinase activity was measured by the liberation of glucose and sulfate. β-Glucosidase activity was measured by the liberation of *p*-nitrophenol, as described previously [4,5].

Enzyme preparation. Myrosinase (fraction F-IA) was prepared from yellow mustard powder (*Brassica juncea*) by the methods described in a preceding paper [1], and was confirmed as homogeneous by chromatography, polyacrylamide gel electrophoresis and ultracentrifugation.

Four proteins (F-IA, B, F-IIA and F-IIB), having myrosinase activity, were separated and purified from yellow mustard powder. Molecular weights calculated by gel-filtration and sedimentation equilibrium were 153 000 (F-IA, F-IB and F-IIA) and 125 000 (F-IIB). Sedimentation coefficients were 6.8 S (F-IA, B and F-IIA) and 5.8 S (F-IIB). Stokes radii (Å), diffusion coefficients (cm^2/s) and frictional ratio (f/f_0) were 47, $4.28 \cdot 10^{-7}$ and 1.33 (F-IA, B and F-IIA) and 43, $4.67 \cdot 10^{-7}$ and 1.29 (F-IIB), respectively. Isoelectric points were 4.6 (F-IA, B and F-IIA) and 4.8 (F-IIB). The enzymes were glycoprotein with 9—22% carbohydrate. Amino acid composition of F-IA, B and F-IIA were very similar, but in the case of F-IIB, glutamic acid, arginine and methionine contents were higher and aspartic acid and histidine contents lower than others. The molecular weights estimated from SDS-polyacrylamide gel electrophoresis were 40 000 (F-IA, B and F-IIA) and 30 000 (F-IIB), respectively.

Protein concentration was determined by the method of Lowry et al. [6].

Inhibition experiments. Unless stated otherwise, 50—300 μg myrosinase were incubated with the inhibitors 0.1 mM *p*-chloromercuribenzoate or 1 mM

2-methoxy-5-nitrotropone in 500 μl 200 mM potassium phosphate buffer. 5—20 μl were removed at specific times for the activity assay.

Chemical modifications. The state of the amino groups of myrosinase was determined by 2-methoxy-5-nitrotropone [7]. 5 μl 100 mM 2-methoxy-5-nitrotropone in *N,N'*-dimethylformamide was added to 1.5 ml enzyme solution (1 mg protein) in 0.2 M phosphate buffer (pH 8.5). After the mixture had been incubated at 25°C for 60 min, it was passed through a Sephadex G-50 column eluted with water to remove excess reagent and byproducts. Yellow-colored fractions were collected. The number of mol amino groups which reacted with 2-methoxy-5-nitrotropone in myrosinase were calculated using a $\Delta\epsilon$ value of $2.07 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 420 nm.

The modification of tryptophan residues was determined with 2-hydroxy-5-nitrobenzyl bromide [8]. 5 μl 100 mM 2-hydroxy-5-nitrobenzyl bromide in acetone was added to 205 μl enzyme solution (500 μg protein) in 0.5 M acetate buffer (pH 5.0), then the mixture was incubated at 35°C for 45 min. After removal of excess reagent and byproducts using the above method, yellow-colored fractions were collected. The number of mol tryptophan residues which reacted with 2-hydroxy-5-nitrobenzyl bromide in myrosinase were calculated using a $\Delta\epsilon$ value of $1.80 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 410 nm (pH 10).

Photometric measurements. Absorption spectra and differential spectra were measured with a Hitachi dual wavelength spectrometer (356 B). For measuring difference spectra, two reference cells containing the enzyme (or chemically modified enzyme) and L-ascorbic acid, but not mixed, were used. The sample cell containing the enzyme solution was mixed with L-ascorbic acid.

Results

Conformational change of the enzyme by L-ascorbic acid

As shown in Table I, the activation effect seemed to be specific for L-ascorbic acid. Nagashima and Uchiyama [9] showed that myrosinase was not activated by most reducing agents (e.g. gluthatione, cysteine, dimercaprol and gallic acid), except L-ascorbic acid. These results show that ascorbic acid was not acting as a conventional reducing agent.

TABLE I

EFFECTS OF THE ANALOGS OF ASCORBIC ACID ON MYROSINASE ACTIVITY

Enzymic activities were measured by sulfate liberation.

Ascorbic acid analog (10^{-3} M)	Relative activity (%)
None	100
L-Ascorbate	2560
Dehydro-L-ascorbate	122
D-Araboascorbate	140
Glucoascorbate	92
Ascorbyl palmitate *	170
Ascorbyl stearate *	85
Ascorbyl 2,6-dipalmitate *	47

* Ascorbic acid analogs were dissolved in 10^{-1} M dimethylformamide.

In a previous ultracentrifuge analysis [3], we also reported that the activation mechanism of myrosinase by L-ascorbic acid is not due to the dissociation and association mechanism of myrosinase.

Difference spectra of myrosinase. Fig. 1 shows the differential spectra of myrosinase with or without L-ascorbic acid. When the enzyme was mixed with L-ascorbic acid, a negative absorbance was observed in the ultraviolet region. This shows that some chromophores of the enzyme protein were changed by adding L-ascorbic acid. But, the absorbance of the high concentration of ascorbic acid in ultraviolet region is unreliable, because ascorbic acid has a considerable absorbance itself. Therefore, 2-methoxy-5-nitrotropone and 2-hydroxy-5-nitrobenzyl bromide were employed as the colored indicators for photometric measurement in order to ascertain whether that was true.

Similar phenomena were also observed for the 2-methoxy-5-nitrotropone or 2-hydroxy-5-nitrobenzyl bromide bound-enzyme. Fig. 2 shows the absorption and differential spectra of myrosinase chemical modified by 2-methoxy-5-nitrotropone. Approx. 1.5 amino residues appeared on the surface of the enzyme after adding 1 mM L-ascorbic acid. The spectra of 2-hydroxy-5-nitrobenzyl bromide modified myrosinase are shown in Fig. 3. About 2.3 tryptophan residues were buried in the molecule when 1 mM L-ascorbic acid was added. These results show that the myrosinase is conformationally changed by the addition of L-ascorbic acid.

Optimum temperature for myrosinase. The optimum temperature for myrosinase changed with the addition of L-ascorbic acid as shown in Fig. 4.

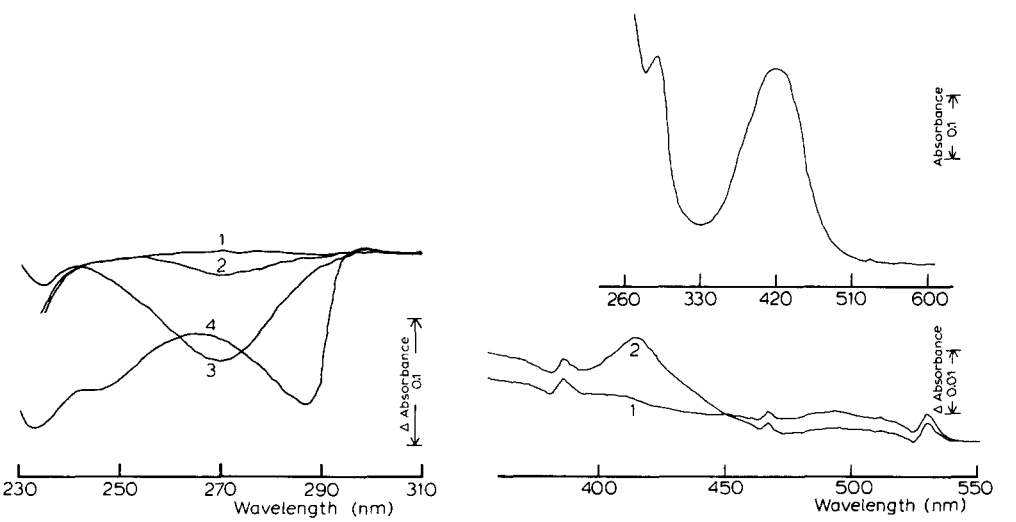

Fig. 1. Differential spectrum of myrosinase with and without L-ascorbic acid. Protein concentration is 1 mg/ml in 50 mM phosphate buffer and 1 mM EDTA, pH 7.0, 20°C, in both compartments. Concentrations of ascorbic acid are: (1) none; (2) $5 \cdot 10^{-5}$ M; (3) $1 \cdot 10^{-4}$ M; (4) $1 \cdot 10^{-3}$ M.

Fig. 2. Absorption and differential spectra of the myrosinase chemically modified by 2-methoxy-5-nitrotropone. Protein concentration is 0.25 mg/ml in 50 mM phosphate buffer, pH 8.5, 20°C. Concentrations of ascorbic acid are: (1) none; (2) $1 \cdot 10^{-3}$ M. The upper curve is an absorption spectrum of 2-methoxy-5-nitrotropone bound-enzyme.

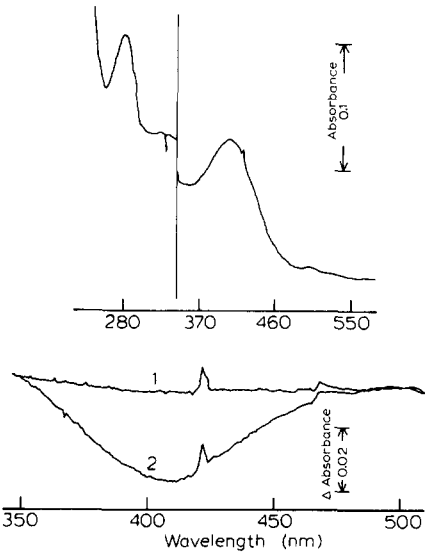

Fig. 3. Absorption and differential spectra of the myrosinase chemically modified by 2-hydroxy-5-nitrobenzyl bromide. Protein concentration is 0.15 mg/ml in 50 mM carbonate buffer, pH 10, 20° C. Concentrations of ascorbic acid are: (1) none; (2) $1 \cdot 10^{-3}$ M. The upper curve is an absorption spectrum of 2-hydroxy-5-nitrobenzyl bromide bound-enzyme.

The optimum temperature for myrosinase activity was about 55°C without L-ascorbic acid, but only about 35°C with L-ascorbic acid. This change suggests that the structure of the myrosinase protein was altered by the addition of L-ascorbic acid. However, the optimum temperature for β-glucosidase activity was the same with or without L-ascorbic acid (about 55°C) which was about

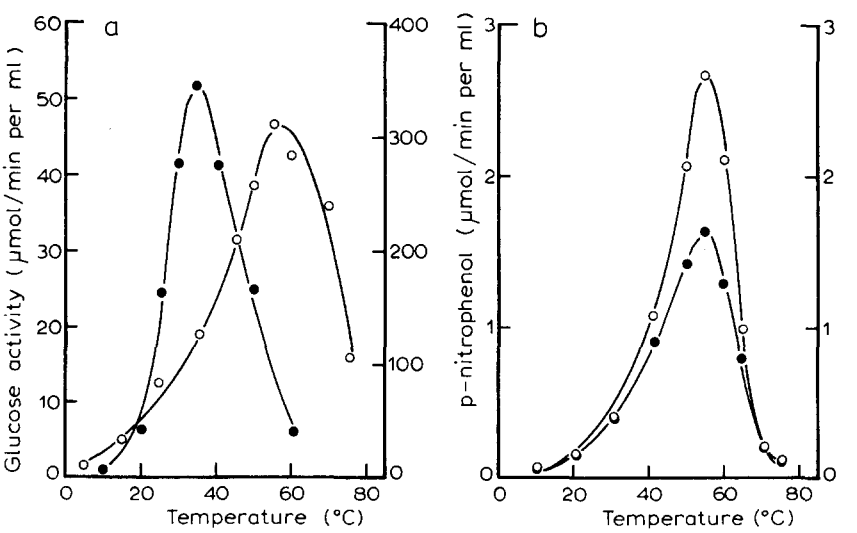

Fig. 4. Optimum temperature curves of myrosinase (a) and β-glucosidase (b) activities of plant myrosinase. Reactions were carried at pH 7 for 15 min without ascorbic acid (○) or for 6 min with 1 mM ascorbic acid (●).

the same as that for myrosinase without L-ascorbic acid. This shows that the binding of L-ascorbic acid did not affect the β-glucosidase activity in spite of affecting myrosinase activity.

Effects of chemical modification on the interaction of L-ascorbic acid with myrosinase

It has been reported [2] that sulfhydryl and amino groups are important for myrosinase activity. In the case of sulfhydryl groups, the enzyme activity was strongly inhibited by 0.1 mM *p*-mercuribenzoate and 1 mM Ellman reagent at 37°C for 15 min and in the case of amino groups, dinitrofluorobenzene, trinitrobenzenesulfonic acid, monochlorotrifluoro-*p*-benzoquinone and 2-methoxy-5-nitrotropone (all at 1 mM concentration) all strongly inhibited the activity at 37°C for 30 min, but it was impossible to distinguish the change with or without L-ascorbic acid in the above conditions. Here, the effects of chemical modification by these groups on the interaction of L-ascorbic acid was investigated by following up both myrosinase and β-glucosidase activities simultaneously in the considerable mild conditions.

Effect of p-mercuribenzoate. As shown in Fig. 5, both myrosinase and β-glucosidase activities were strongly inhibited by 0.1 mM *p*-mercuribenzoate at 25°C for 30 min in all cases. However, β-glucosidase activity was more strongly inactivated than myrosinase activity. From these results, we postulate that sulfhydryl groups are situated at the active center of myrosinase.

Effect of 2-methoxy-5-nitrotropone. Myrosinase and β-glucosidase activities were completely inactivated by incubation with 2-methoxy-5-nitrotropone at 37°C for 120 min with or without L-ascorbic acid, but an interesting result was obtained when this experiment was repeated at 25°C. Inactivation of myrosinase by 2-methoxy-5-nitrotropone is shown in Fig. 6. The inactivation rates for myrosinase and β-glucosidase activities by this reagent were almost the

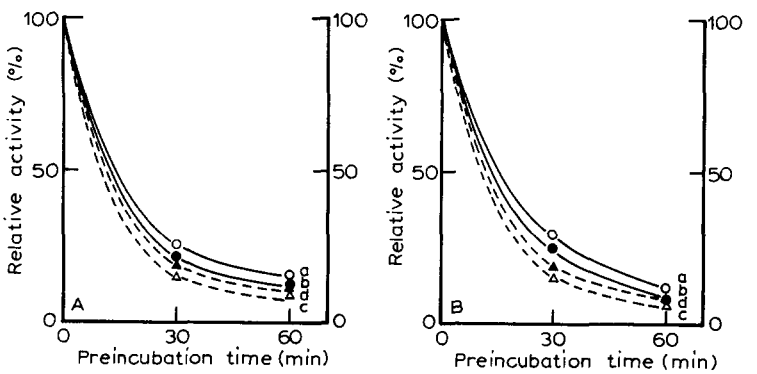

Fig. 5. Inactivation of plant myrosinase by *p*-mercuribenzoate. 100 μl (300 μg) myrosinase were preincubated at 25°C with 0.1 mM *p*-mercuribenzoate in 200 mM potassium phosphate buffer, pH 7, and 1 mM ascorbic acid (omitted in Fig. 5A), in a total volume of 0.5 ml. Aliquots (5—20 μl) were removed at different times and assayed. Enzyme reactions were carried out in a system containing 2.5 μmol substrate (sinigrin as the substrate for myrosinase activity, ○———○ or ●———●, and *p*-nitrophenyl β-glucoside as that for β-glucosidase activity, △- - - - -△, or ▲- - - - -▲), 0.2 nmol phosphate buffer, 20 μl of the sample in curves a (○), c (△), 5 μl in curves b (●), d (▲), pH 7, and in curves b, d, 1 mM of ascorbic acid was added.

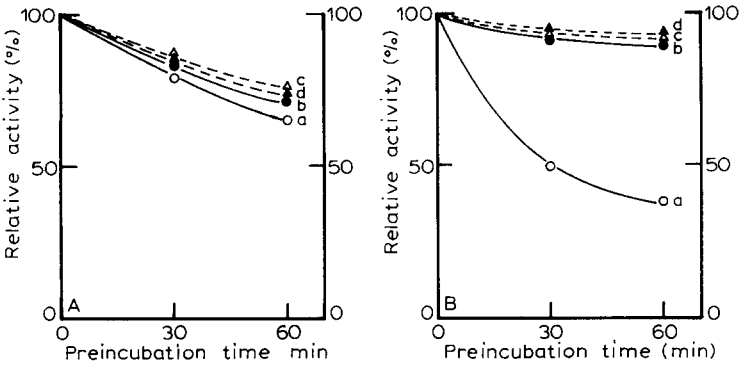

Fig. 6. Inactivation of plant myrosinase by 2-methoxy-5-nitrotropone. 100 μl (300 μg) myrosinase were preincubated at 25°C with 1 mM 2-methoxy-5-nitrotropone in 200 mM potassium phosphate buffer, pH 8.5, and 1 mM ascorbic acid (omitted in Fig. 6A), in a total volume of 0.5 ml. Enzyme reactions were carried out as described in Fig. 5.

same, but in B an interesting results was obtained. In contrast to results with the other reagents, myrosinase activity was strongly inhibited only when it was measured without L-ascorbic acid, but the activity was not affected by this reagent when measured with L-ascorbic acid. This phenomenon was not observed for β-glucosidase activity. This shows that the amino residues in the protein are also located in the region which was altered by L-ascorbic acid.

Discussion

In a previous paper [10], we assumed the presence of one action site for the substrate and two sites for ascorbic acid. The substrate site has two moieties, one for the glycon and one for the aglycon part of mustard oil glucoside [4,11]. The conformation of the binding site for the aglycon moiety is altered when the ascorbic acid site is occupied. However, the binding of ascorbic acid to the effect site exerts no influence on the hydrolysis of *p*-nitrophenyl β-glucoside, because it differs from mustard oil glucoside in its aglycon moiety. Ettlinger et al. [12] reported that the hydrolysis of 2,4-dinitrophenyl β-thioglucoside and desulfoglucocapparin by mustard myrosinase is not accelerated in the presence of ascorbic acid. This observation can be similarly explained. The above explanation is also support by the fact that the optimum temperature for sinigrin hydrolysis was changed by the addition of L-ascorbic acid, while that for *p*-nitrophenyl β-glucoside hydrolysis was the same in the presence or absence of L-ascorbic acid. Björkman and Lönnerdal [13] also reported that the stability of the enzyme-substrate complex is reduced by the addition of ascorbic acid, and this change in stability may be cuased by a conformational change in the usbstrate site, that arises when effector molecules are attached to a certain site or locus on the enzyme molecule.

The experimental results obtained in the present work can be explained as follows (see Figs. 4, 5 and 6). Since both myrosinase and β-glucosidase activities were strongly inhibited by *p*-chloromercuribenzoate, with or without ascorbic acid, sulfhydryl groups are considered to be essential to the catalytic action of

myrosinase. Myrosinase activity with 2-methoxy-5-nitrotropone was more strongly inhibited than was β-glucosidase activity, and the enzyme treated with 2-methoxy-5-nitrotropone together with ascorbic acid was strongly inactivated when its enzymic activity was measured without ascorbic acid. The modified enzyme, however, was not inhibited when the activity was determined with ascorbic acid. No such phenomenon was observed for the β-glucosidase activity. Based on these facts, we believe that the amino residues are situated in the region which is altered by the addition of ascorbic acid (close to the aglycon moiety), and their locations are changed to accelerate the enzyme action by the binding of ascorbic acid to the effector site. On the other hand, since the sulfhydryl reagent strongly inhibited both myrosinase and β-glucosidase activities, the sulfhydryl groups are considered to be situated close the glycon moiety and to be essential to the catalytic action of plant myrosinase. This also shows that *p*-nitrophenyl β-glucoside combines with the enzyme only in the area for the glycon moiety in the substrate site, hence the hydrolysis of *p*-nitrophenyl β-glucoside was not influenced by 2-methoxy-5-nitrotropone.

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