Phytochemistry, 1980, Vol. 19, pp. 1613–1617. © Pergamon Press Ltd. Printed in England 0031 9422/80/0801 1613 $02.00/0

INHIBITION BY OXALATES OF SPINACH CHLOROPLAST PHENOLASE IN UNFROZEN AND FROZEN STATES

MITSUHIKO SATÔ

Department of Biology, Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo, 158 Japan

(*Revised received* 2 *January* 1980)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; enzyme inhibition; inhibitors; oxalates; ammonium oxalate; chloroplasts.

Abstract—Spinach chloroplast phenolase was inhibited by oxalic acid and its salts. Complete inhibitions were induced instantly in the acidic region (e.g. by 1 and 5 mM oxalate at pH 5 and 5.5, respectively), and in the neutral region pre-incubation of the enzyme with oxalates could also lead to complete loss of activity. The inhibition mode was non-competitive for phenol substrate with K_i of 0.9 mM pH 6.8. Reduction of enzyme activity in a crude extract of chloroplasts induced by freezing at neutral pH was due to the presence of ammonium oxalate. With 0.5 mM oxalate, the inhibition attained 75% under frozen conditions, whilst no inhibition could be detected in the enzyme which had not been frozen. Free oxalic acid and K^+ and Na^+ salts also caused freezing inhibition. Glyoxylic and oxamic acids acted as inhibitors with less efficiency. With a pure mushroom tyrosinase (phenolase), essentially the identical results were obtained using the same conditions.

INTRODUCTION

In spite of the wide occurrence in the plant kingdom of phenolase (*o*-diphenol:oxygen oxidoreductase), no comprehensive understanding has yet been reached of its physiological role [1, 2]. Phenolase of spinach leaf cells, which is predominantly located in the chloroplasts [3], especially in the thylakoid membranes [3–5], is a latent enzyme [3–6], like phenolase in broad bean [7, 8], sugar beet [9] and grape [10]. The author suggested that this latency partly results from the association of a low MW substance with active enzyme in the form of an inactive complex, which was produced by freezing [5, 6]. Meanwhile, it was found that spinach leaves contain two low MW substances, one acting as a stimulant on the enzyme, and the other exhibiting an inhibitory effect. The latter substance was identified as ammonium oxalate and free oxalic acid also acted as inhibitor [11]. This paper describes further studies on the phenolase inhibition by oxalates as well as inhibition induced by low concentrations of this natural inhibitor, which could be observed only by freezing, but not in the unfrozen state.

RESULTS

Inhibition by oxalates of spinach chloroplast phenolase (in liquid media)

Oxalate moiety as inhibitory factor. The activity reduction of spinach chloroplast phenolase by oxalates (free oxalic acid and its NH_4^+, K^+ and Na^+ salts) was examined and it was found that almost independent of the compounds *ca* 30 40% inhibition was induced with 10 mM at pH 6.8, whilst no enzyme activity remained at pH 5.5. The inhibition is thus due to the oxalate moiety, but not to the cations.

pH-Dependency of inhibition. The effect of various pH values on the enzyme activity by 10 mM oxalic acid was subsequently examined. In the region from *ca* pH 6.5 to 8, the inhibition rate is small (25–40%), whilst in the acidic region it increased markedly. In Fig. 1 is shown the effect at various pHs of the oxalate concentration on the inhibition rate. The concentration required for inducing complete inhibition decreased as the pH shifted to acidic values, i.e. it was 10, 5 and 1 mM at pH 6, 5.5 and 5, respectively.

Effect of pre-incubation of enzyme with oxalates. In a previous paper [11], it was reported that a longer contact

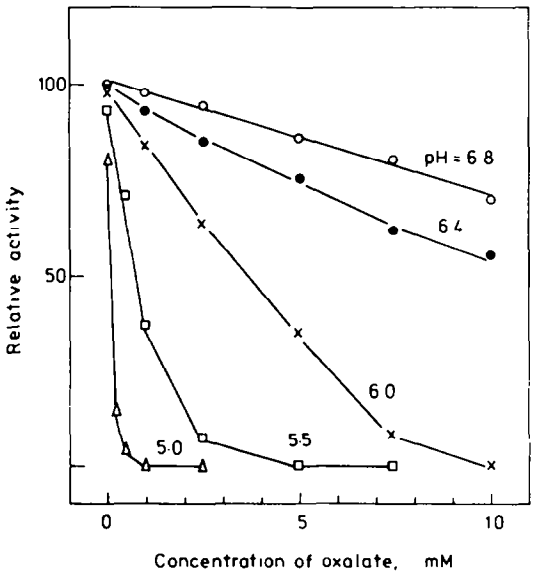

Fig. 1. Effect of pH and oxalate concentration on the inhibition rate of phenolase. Activities are expressed as % of that determined at pH 6.8 without oxalic acid.

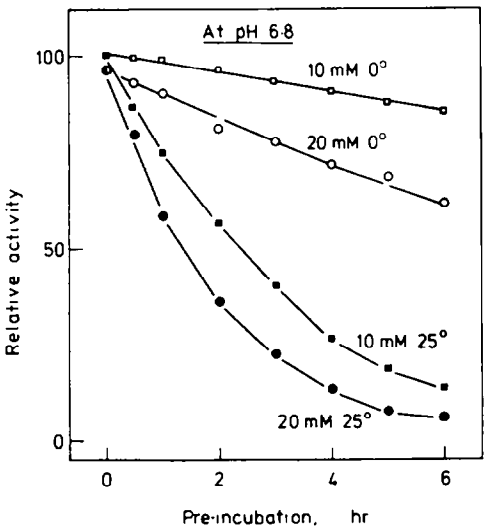

Fig. 2. Effect at pH 6.8 of pre-incubation of the phenolase–oxalate mixtures on the reduction in enzyme activity.

(18 hr at 0°) of the enzyme with oxalates buffered at pH 6.8 resulted in a remarkable reduction in activity. Subsequently, the effect at the same pH by a shorter period of pre-incubation (up to 6 hr) was examined at 0° as well as at 25° with 10 and 20 mM oxalic acid (here notice that when the enzyme activity was estimated, the final concentration of the inhibitor was diluted to one tenth, i.e. 1 and 2 mM, respectively, and the inhibition rate measured at these concentrations without prior incubation did not exceed 5%, as seen in Fig. 1). The results in Fig. 2 show that progressive decreases in activity were brought about even at this neutral pH, and these were dependent on the inhibitor concentration and the temperature, especially on the latter. The activity reduction, by standing for 6 hr at 25° without oxalate, was found to be less than 5%. Such a pre-incubation effect was similarly induced by salts of oxalic acid.

Freeze-induced inhibition by low concentrations of oxalates

pH-Dependency of inhibition for a crude enzyme solution. It was reported previously [5, 6] that, on freezing a crude enzyme solution prepared from acetone powder of spinach chloroplasts, the phenolase activity was decreased. This freezing effect was studied at pH 6.8. In Fig. 3(a) are shown the enzyme activities after the crude enzyme was stood for 18 hr at pH 5–7.5 in frozen as well as in the non-frozen states. It is noticeable that, while in the non-frozen enzyme, the activity reduction was observed only lower than *ca* pH 6, freezing brought about inhibition at neutral pH. Essentially the same results were obtained when a dialysed enzyme solution whose phenolase activity is no longer affected by pre-exposure to various pHs [Fig. 3(b)], was subjected to standing with a diffusate from the crude enzyme, and this showed, as previously suggested [5, 6], that the inhibition can be ascribed to the action of a low MW substance present in the crude enzyme solution *in situ*.

Freezing effect of oxalates. Since the effect of pH on the activity change of the enzyme solution after freezing appeared to resemble that by oxalate in ordinary liquid media, this was tentatively examined with ammonium oxalate which had been found to accumulate in spinach leaves [11], and almost identical results were obtained [Fig. 3(c)]. In the neutral pH region, no inhibition occurred with 0.5 mM oxalate on standing in liquid media, whilst a striking activity drop (75%) was induced after freezing. Freezing resulted in an exponential decrease of activity with low concentrations of oxalate (e.g. 50% with 0.25 mM), but this was only 20% even with 2 mM oxalate without freezing [Fig. 4(a)].

Detection of ammonium oxalate in crude enzyme. The above results strongly suggest the presence of oxalate in the original crude enzyme solution. This was subsequently detected with diffusate and its concentration in the enzyme solution in Fig. 3(a) was estimated to be *ca* 0.1 mM. It was previously postulated that the compound responsible for the freezing inhibition is extractable with ethyl acetate, positive to bromocresol green and probably volatile, and several low MW fatty acids could decrease

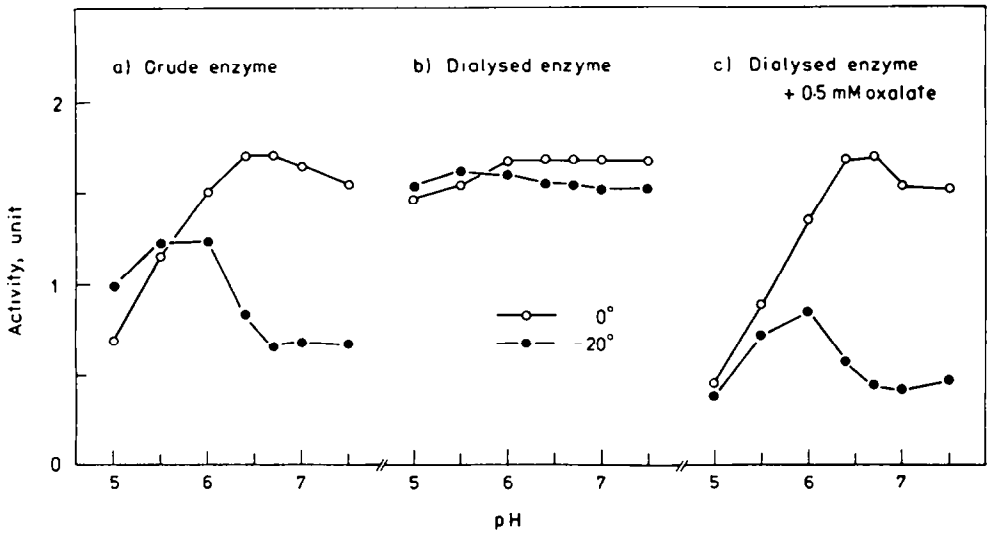

Fig. 3. Phenolase activity after standing for 18 hr at varied pH in frozen (−20°) and non-frozen (0°) liquid states, (a) in a crude enzyme solution from spinach chloroplasts, (b) in a dialysed soln of the crude enzyme (overnight against 100 vol. of 10 mM Na–Pi buffer pH 6.8), and (c) in a mixture of the dialysed enzyme and 0.5 mM ammonium oxalate. Activities were measured at pH 6.8.

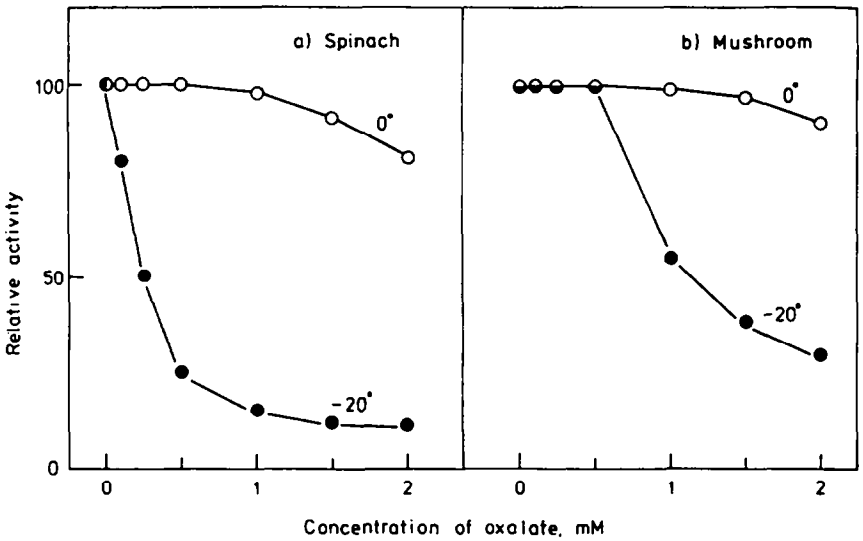

Fig. 4. Phenolase activity after standing with ammonium oxalate for 18 hr at pH 6.8 in frozen and non-frozen states. (a) Dialysed enzyme from spinach; (b) mushroom tyrosinase (Sigma Grade III: 1 ml contained 12 units of enzyme).

the enzyme activity on freezing [6]. It appears that free oxalic acid is an artefact derived from ammonium oxalate.

Substances inducing freeze-inhibition. Free oxalic acid and its K^+ and Na^+ salts exhibited essentially the same effect as ammonium oxalate (data not shown). Related compounds of oxalate without the carboxyl group, i.e. glyoxal and oxamide, some monocarboxylic acids, including glycolic, glyceric and pyruvic acids, and fumaric, malic, malonic, succinic and tartaric acids—all tested at 2 mM (a saturated solution was used for oxamide)—were ineffective. Unlike in liquid media [11], glyoxylic acid could inhibit with lesss efficiency, together with oxamic acid, i.e. *ca* 40% inhibition was induced with 2 mM of both compounds. It was reported that a series of fatty acids from formic to caproic acids could act as freezing inhibitors and propionic and butyric acids were most effective with *ca* 90% inhibition at 2 mM [6]. It seems that at least one carboxyl group is necessary, and a medium MW of the compounds is favourable for inhibition.

Oxalate inhibition with mushroom tyrosinase (phenolase). The effect of oxalate was studied with a pure phenolase preparation (mushroom tyrosinase; Sigma Grade III), and essentially the same results were obtained with oxalates, pH and pre-incubation effect (data not shown). Reciprocal plots of reaction velocity vs oxalate concentration at fixed catechol concentrations showed that the inhibition is of a non-competive type for mushroom enzyme as well as for spinach phenolase, and the K_i value of 1.1 mM at pH 6.8 of the former was comparable to that of 0.9 mM of the latter. Freeze-inhibition was also observed with the mushroom enzyme [Fig. 4(b)]. Unlike the spinach enzyme, this was not affected by oxalate up to *ca* 0.5 mN, but strong inhibition was brought about with increased concentration.

DISCUSSION

The results presented above clearly show that oxalates are potent inhibitors of chloroplast phenolase of spinach

Table 1. Summary of phenolase inhibition by oxalates

	Oxalate concn (mM)	pH	Inhibition
Without pre-incubation*	(10)†	6.0	100
	(5)	5.5	100
	(1)	5.0	100
	(10)	N‡	30
	(1)	N	0
With pre-incubation			
6 hr, 0°	10 (1)	N	15
6 hr, 25°	10 (1)	N	85
18 hr, 0°	0.5 (0.05)	N	0
18 hr, −20°	0.5 (0.05)	N	75

* The enzyme activity was measured after standing the enzyme system for a maximum time of 5 min which was required for attaining temperature-equilibrium at 25°.

† Values in parentheses represent the final concn when the enzyme activities were subjected to assay.

‡ pH 6.5–7.5.

in which ammonium oxalate is accumulated as the neutral salt [11]. The inhibition pattern is summarized in Table 1. Under ordinary assay conditions without pre-incubation, strong inhibition is observed in acidic media, whilst in the neutral region, this occurs only to a limited extent (Fig. 1). The inhibitory activity of oxalates at neutral pH can be enhanced, however, by longer exposure of the enzyme to the inhibitor, especially at elevated temperature (Fig. 2). It was further found that even at oxalate concentration lower than 0.5 mM where the activity did not diminish merely by pre-incubation in liquid media, a striking activity reduction could be induced by freezing [Figs. 3(c) and 4(a)].

It has been suggested that phenolase activity in spinach leaf cells, both in a latent and an active form, is predominantly located in the chloroplasts [3], mainly as membrane-bound enzyme [3–5]. On the other hand, the concentration of oxalates in mature cells, calculated from previous data on the oxalate content per dry wt [12] and our estimation on the dry/fr. wt ratio, might amount to as high as *ca* 0.1–0.2 M. If the enzyme was constantly in contact with such a high, or even with one order lower oxalate concentration, its activity would never exhibit *in vivo* at physiological temperatures (Fig. 2) and at pH values not only of the acidic side, but also in the neutral region (pre-incubation effect; Fig. 2). Furthermore, if freezing is regarded as a process which results in intimate contact of the enzyme with the inhibitory substances, it is probable that in highly dehydrated membrane parts an efficient suppression of phenolase can occur with a small amount of oxalate to make the enzyme inactive. Detailed studies on the intracellular location of oxalates as well as on the *in vivo* pH are therefore required. It is of interest in this connexion, that *p*-coumaroyl tartarate has been found in a chloroplast fraction of this plant [13].

Concerning the freezing effect, our results contrast with those of Lieberei and Biehl who found activation of spinach chloroplast phenolase in both stroma and thylakoid membranes [14], although their experimental conditions were different. Activation seems to arise also by the action of a low MW substance detected in this plant [11].

The activity reduction with oxalates is greatly influenced by pH (Fig. 1), and this is in accordance with previous reports on the phenolase inhibition by carboxylic acids, showing that a critical drop in activity was brought about on the acidic side of *ca* pH 6 [15–18]. Kreuger suggested that anionic species of acids may combine with the cationic moiety (i.e. imidazole group of histidine) at the active centre of the enzyme [15], whilst Robb and his co-workers have claimed that the undissociated acid molecule, but not the anion, may chelate with functional copper of the enzyme to form a complex and thereby cause an inhibition [17]. The present results are unlikely to be explained exclusively by the occurrence of the undissociated species, because a weaker, but a definite and time-dependent inhibition was induced also at neutral pH where oxalic acid is fully ionized. A strongly pH dependent inhibition by halides of a catechol oxidase (phenolase) has been ascribed to the formation of a 'complex' between copper and the anion which is stabilized as the pH decreases [19].

Mushroom tyrosinase (phenolase) was similarly affected by oxalates as has been shown in a previous paper [15]. The inhibition was essentially the same according to pH, mode, enhancement by pre-incubation as well as freezing. The possibility cannot be ruled out, therefore, that a common mechanism underlies phenolase inhibition by oxalates and by other carboxylic acids [15–18].

EXPERIMENTAL

Preparation of enzyme soln from chloroplasts. Chloroplast Me_2CO powder containing phenolase which had been activated and rendered soluble, was prepared as described previously [5]. This was suspended in 100 vol. (w/v) of 10 mM Na–Pi buffer pH 6.8, the suspension stirred for 1 hr, and the supernatant of centrifugation at 26 000 *g* for 20 min was used as enzyme soln, unless otherwise stated.

Estimation of enzyme activity. This was carried out by a colorimetric method using catechol and sulphanilic acid [5, 6, 11]. Reaction mixtures in total vol. of 5 ml were made by mixing 0.1 ml of enzyme, 2 ml of 0.2 M Na–Pi buffer of various pHs (0.1 M citrate 0.2 M Na_2HPO_4 buffer was used for pH 5), 0.4 ml of H_2O, 1 ml of oxalates of known concns, 1 ml of 10 mM suphanilic acid and 0.5 ml of 10 mM catechol, respectively. The reaction at 25° was started by adding catechol, and the reaction rate was determined from the initial linear portion recorded at 500 nm within 1 min. The expts in Figs. 2–4 and others, where pre-incubation was run before the activity estimation, were carried out as follows. To 0.1 ml of enzyme were added 0.3 ml 0.2 M Na–Pi buffer of known pH, then 0.1 ml of appropriate concns of oxalates, other substances or H_2O, and the mixtures (0.5 ml) were left to stand at −20, 0 or 25° for the required time. They were then added to 2 ml 0.2 M Na–Pi buffer, pH 6.8, and 2 *ml* 5 *mM* sulphanilic acid and allowed to attain a rapid temp. equilibrium at 25° within 6 min (the concn of oxalates and other substances in the preincubated mixtures was finally diluted to one tenth when the enzyme activity was subjected to assay). One unit of enzyme activity is defined as the amount of enzyme which increases *A* by 0.01/min.

Detection of ammonium oxalate. Crude enzyme soln (10 ml, equivalent to 100 mg of chloroplast Me_2CO powder) were thoroughly dialysed against H_2O, the diffusate evapd to dryness under red. pres. below 50°, and the residue taken up in 0.5 ml of H_2O. PC of the aq. sample (neutral) revealed a substance positive to Nessler's reagent, whose behaviour in solvents *n*-BuOH–HOAc–H_2O (4:1:2) and *n*-BuOH–Py–HOAc–H_2O (50:33:1:40) coincided with those of an authentic sample of NH_4 oxalate (neither free oxalic acid nor other Nessler's reagent positive substances could be detected). The sample evolved NH_3 by gently warming in dilute alkaline soln. An acidic substance remained which was soluble in EtOAc. This was identified as oxalic acid by co-chromatography in the above solvents. The amount of NH_4 oxalate was determined for an aliquot of the above sample by a colorimetric method with Nessler's reagent using this salt as standard.

REFERENCES

1. Stafford, H. A. (1974) *Annu. Rev. Plant Physiol.* **25,** 459.
2. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18,** 193.
3. Tolbert, N. E. (1973) *Plant Physiol.* **51,** 234.
4. Lieberei, R. and Biehl, B. (1976) *Ber. Dtsch. Bot. Ges.* **89,** 663.
5. Satô, M. and Hasegawa, M. (1976) *Phytochemistry* **15,** 61.
6. Satô, M. (1977) *Phytochemistry* **16,** 1523.
7. Kenten, R. H. (1957) *Biochem. J.* **67,** 300.
8. Swain, T., Mapson, L. W. and Robb, D. A. (1966) *Phytochemistry* **5,** 469.
9. Mayer, A. M. (1966) *Phytochemistry* **5,** 1297.
10. Lerner, H. R., Mayer, A. M. and Harel, E. (1972) *Phytochemistry* **11,** 2415.
11. Satô, M. (1979) *Plant Sci. Letters* **16,** 355.

12. Gutz, W. (1957) *Chem. Abstr.* **48,** 2946.
13. Ottemeier, W. and Heupel, A. (1972) *Z. Naturforsch. Teil B* **27,** 177 and 586.
14. Lieberei, R. and Biehl, B. (1978) *Phytochemistry* **17,** 1427.
15. Kreuger, R. C. (1955) *Arch. Biochem. Biophys.* **57,** 52.
16. Kuttner, R. and Wagreich, H. (1953) *Arch. Biochem. Biophys.* **43,** 80.
17. Robb, D. A., Swain, T. and Mapson, L. W. (1966) *Phytochemistry* **5,** 665.
18. Duckworth, H. W. and Coleman, J. E. (1970) *J. Biol. Chem.* **245,** 1613.
19. Ben-Shalom, N., Kahn, V., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16,** 1153.