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THE EFFECT OF CATALASE ON THE INACTIVATION OF TYROSINASE BY ASCORBIC ACID AND BY CYSTEINE OR GLUTATHIONE

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Abstract. When tyrosine was incubated with tyrosinase in the presence of ascorbic acid, dopa and 5-hydroxydopa were formed and the enzyme was inactivated. In the presence of catalase, more dopa and 5-hydroxydopa were formed because enzyme inactivation was prevented. Incubation of dopa and cysteine with small amounts of mushroom tyrosinase led to rapid inactivation of the enzyme. This inactivation was accelerated in the presence of catalase. New systems developed have been useful in demonstrating the role of hydrogen peroxide in tyrosinase inactivation by several compounds of importance in melanin biochemistry. Cysteine and glutathione inactivated tyrosinase. Addition of catalase increased the inactivation at high thiol concentrations, but decreased the inactivation at low concentrations. Ascorbic acid and 5-hydroxydopamine also inactivated tyrosinase, but with these compounds inactivation was completely prevented by addition of catalase. The inactivation by dopamine was negligible under the experimental conditions. Inactivation of tyrosinase by ascorbic acid and by 5-OH-dopamine was found dependent on oxygen, whereas inactivation by cysteine and glutathione was independent of oxygen. Large amounts of serum albumin protected tyrosinase from inactivation by ascorbic acid and 5-OH-dopamine, but did not prevent inactivation by cysteine and glutathione. The presence of substrate had a protective effect on the inactivation of tyrosinase by cysteine.

Tyrosinase, the enzyme responsible for melanin production in most tissues, has been extensively investigated, but many aspects of its function remain obscure. Copper is essential in tyrosinase; and thiols, which combine with copper, inhibit the activity of the enzyme (12, 14, 22). The inactivation of tyrosinase by sulfhydryl-containing substances has become of central interest because the regular occurrence of cysteinyldopas in melanin-forming cells indicates that tyrosinase acts in an environment containing cysteine or cysteine-containing peptides such as glutathione (16).

A striking characteristic of tyrosinase is that it is inactivated by its own substrate. This inactivation has been the subject of many studies, and the early work on tyrosinases of varying origin has been reviewed by Nelson and Dawson (15). In a series of investigations Seiji, Tomita, and collaborators have explored the inactivation of tyrosinase obtained from mouse melanomas (17, 19). They found that scavengers for superoxide anion, singlet oxygen, and hydroxyl radical did not prevent inactivation of the enzyme. Nor did catalase, the enzyme required for cleaving hydrogen peroxide, have any effect on tyrosinase inactivation (21).

Ascorbic acid has often been used as a reducing agent in experiments on tyrosinase inactivation in order to prevent melanin formation. The rapid reduction of dopaquinone by ascorbic acid prevents intramolecular cyclization of the quinone with subsequent polymer formation. There are contradictory reports on the effect of ascorbic acid itself on tyrosinase activity (5, 9, 11, 12, 21). Nothing is known about the effect on tyrosinase of 5-OH-dopa, which accumulates in large amounts when dopa is oxidized by tyrosinase in the presence of ascorbic acid (8).

Tyrosinases from a large variety of sources have been isolated and studied, but mushroom and Neurospora tyrosinases have been examined in particular detail, and studies on these enzymes often serve as models for work on vertebrate tyrosinases (13). Our work has been concerned with mushroom tyrosinase and with human tyrosinase obtained from cultured melanoma cells. The present report deals with the inactivation of mushroom tyrosinase in the presence of thiols, ascorbic acid, and 5-OH-dopa. The results form a background for a study on human tyrosinase in the presence of naturally occurring inhibitors.

MATERIAL AND METHODS

Material

The chemical used were L-tyrosine (Sigma), L-dopa (Merck), L-cysteine (Merck), D-cysteine (Merck), L-

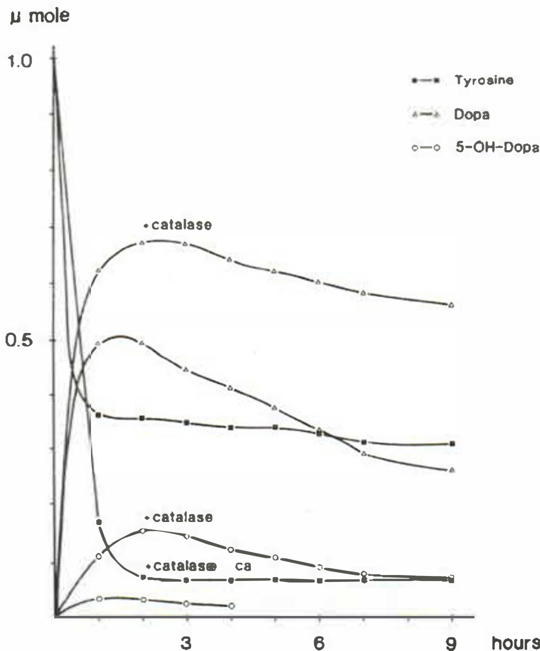

Fig. 1. The effect of catalase on the inactivation of tyrosinase incubated with tyrosine and ascorbic acid.

ascorbic acid (Merck), and 5-OH-dopa (Hoffmann-La Roche). The preparation of 2-S-cysteinyldopa and 5-S-cysteinyldopa is described elsewhere (2, 3). Catalase (Sigma), glutathione peroxidase (a gift from Karl-E. Arfors, Pharmacia AB, Uppsala, Sweden), and albumin (Kabi). The enzyme used for method 2 experiments was mushroom tyrosinase (2 230 U/mg, Sigma). For method 1 experiments a further purified mushroom enzyme was used. 50 mg mushroom tyrosinase (Sigma) was dissolved in 20 ml of 10^{-2}M sodium phosphate, pH 7.8, and centrifuged at 30 000 *g* for 1 h. The solution was put on a column (0.9×29 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals AB, Uppsala), and washed with 5 ml of the buffer. Gradient elution was performed with 250 ml of 10^{-2}M sodium phosphate, pH 7.8, in the mixing chamber and with 10^{-2} M sodium phosphate +0.3 M NaCl, pH 7.8, in the reservoir. 10-ml fractions were collected and examined for tyrosinase activity by measuring 5-S-cysteinyldopa formation over 2 min in a system containing dopa 10^{-3} M and cysteine 3×10^{-3} M.

Fractions 18–22, which contained the tyrosinase activity, were pooled and further purified on a G-200 column (2×90 cm). Elution was done with 0.1 M sodium phosphate, pH 7.8, in 20-ml fractions. Fractions 6–9 contained the tyrosinase activity, and were used for the assays.

Measurement of tyrosinose inactivation

Two different methods were used for analysis of tyrosinase inactivation.

Method 1. Recording of decreased rate of product formation in the course of incubation with substrate. Relevant data are given in the description of each experiment.

Method 2. Recording of initial velocity of product formation by tyrosinase after preincubation of the enzymes with inactivating compounds. The standard procedure for measuring tyrosinase activity in such experiments was as follows:

Step A: Preincubation of 1 mg of tyrosinase in 1 ml of 0.5 M phosphate buffer, pH 6.5, at 25° for 30 min with the substance to be studied for inactivation.

Step B: 5 µl of incubate A was added to 1 ml 0.5 M phosphate buffer, pH 6.5, at 0°C, containing L-cysteine 10^{-2} M and L-dopa 10^{-3} M. After 45 sec at 0°C the incubation was interrupted by dilution with 24 ml of 0.4 M perchloric acid, and the amount of 5-S-cysteinyldopa formed was measured. Tyrosinase activity at the start of incubation A served as the control in each experiment.

In method 2 experiments the inactivating effect of different compounds were studied also in the absence of substrate in step A.

Performance of analyses

All catecholic amino acids were determined by high-pressure liquid chromatography using a model 6000 A (Waters Ass., Milford, Mass.) high-pressure liquid chromatograph with a Model 7120 100 µl sample valve injector (Rheodyne, Berkeley, Calif.) and electrochemical detector Model LC-10 (Bioanalytical Systems, West Lafayette, Ind.). The detection potential was set at +0.75 V against an Ag/AgCl reference electrode. The electrode was prepared from CPO graphite material. The column packing material was Nucleosil C_{18} (5 µm, Macherey, Nagel & Co, Düren, G. F. R.). Columns were 250×4.6 mm. The mobile phase contained 6 g of methane sulphonic acid and 2.9 g phosphoric acid per litre water. The pH was adjusted to 1.75 with 5 M NaOH. Isocratic elution was used, and the flow rate was 1.5 ml per min.

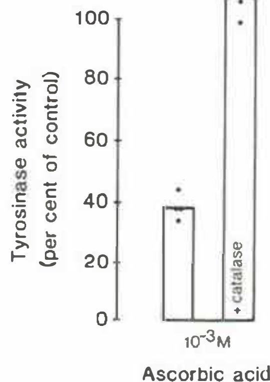

Fig. 2. Inactivation of tyrosinase by ascorbic acid, and prevention by catalase.

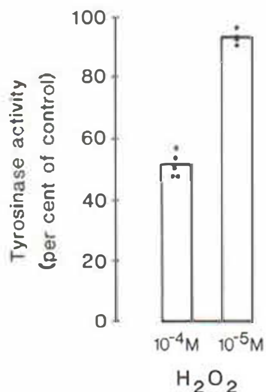

Fig. 3. Inactivation of tyrosinase by hydrogen peroxide.

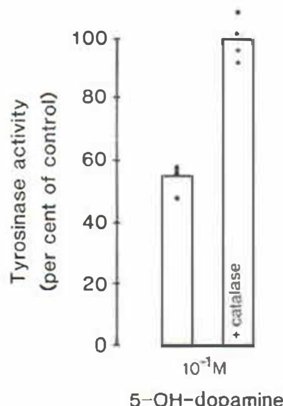

Fig. 5. Inactivation of tyrosinase by 5-OH-dopamine, and prevention by catalase.

Experiments

Inactivation of tyrosinase incubated with tyrosine and ascorbic acid (Method 1). When tyrosine (10^{-3} M) was incubated in 1 ml of the tyrosinase eluted from the G-200 column in the presence of ascorbic acid (2×10^{-2} M), the concentration of tyrosine showed a pronounced decrease after 1 h, but subsequently the concentration remained largely unchanged, indicating inactivation of the tyrosinase. Dopa reached a high level after 1 h, remained high for the next hour, and then declined slowly (Fig. 1). After 1 h the concentration of 5-OH-dopa was about 5% of that of dopa, and the decrease after this time was slow.

Effect of catalase. When catalase (10 µg) was present in the incubate the course of reactions was different. Tyrosine concentrations continued to decrease for 2 h, indicating the persistence of some intact tyrosinase. Delayed inactivation of tyrosinase in the presence of catalase was also indicated by the fact that the levels of dopa and 5-OH-dopa after 2 h were higher than after 1 h. With catalase, much higher concentrations of 5-OH-dopa were obtained than without the enzyme. The oxidation of dopa was slower in the presence of catalase than when catalase was absent.

Inactivation of tyrosinase by ascorbic acid, and prevention by catalase (Method 2). Incubation of tyrosinase with ascorbic acid alone (10^{-3} M) for 30 min led to 60% inactivation of the enzyme (Fig. 2). This inactivation was prevented completely by catalase (10 µg), which indicated that the inactivation was mediated by hydrogen peroxide formed on the oxidation of ascorbic acid.

Inactivation of tyrosinase by hydrogen peroxide (Method 2). The quantities of hydrogen peroxide necessary for tyrosinase inactivation were tested. With hydrogen peroxide (10^{-4} M) tyrosinase inactivation was about 50% (Fig. 3).

Inactivation of tyrosinase by 5-OH-dopamine, and prevention by catalase (Method 2). Because incubation of dopa with tyrosinase and ascorbic acid leads to considerable accumulation of 5-OH-dopa (8), the possibility was considered that under certain circumstances the inactivation of tyrosinase by ascorbic acid could be mediated by 5-OH-dopa or products formed by oxidation of this compound. For technical reasons this was investigated by comparing the inactivation of tyrosinase by dopamine and 5-OH-dopamine instead of by the corresponding catecholic amino acids. The inactivation of tyrosinase by dopamine was negligible under our experimental conditions, and there was no significant effect of catalase (Fig. 4). In contrast, high concentrations of 5-OH-dopamine led to an inactivation which was inhibited by 10 µg of catalase (Fig. 5).

Inactivation of tyrosinase by cysteine and glutathione, and the effect of catalase. Method 1. When dopa was incubated with tyrosinase in the presence of cysteine the tyrosinase activity, measured as 5-S-cysteinyldopa, decreased rapidly, and the decrease was related to the concentration of cysteine. In the presence of catalase (10 µg) the tyrosinase inactivation occurred more rapidly (Fig. 6). Boiled catalase had no effect.

Method 2. When *cysteine* alone was incubated with tyrosinase, a dose-dependent inactivation of the enzyme

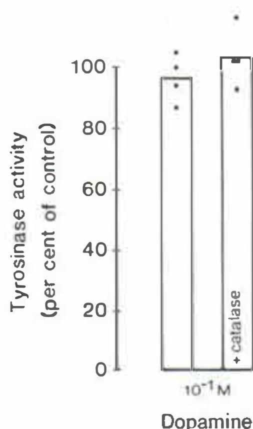

Fig. 4. Negligible inactivation of tyrosinase by dopamine.

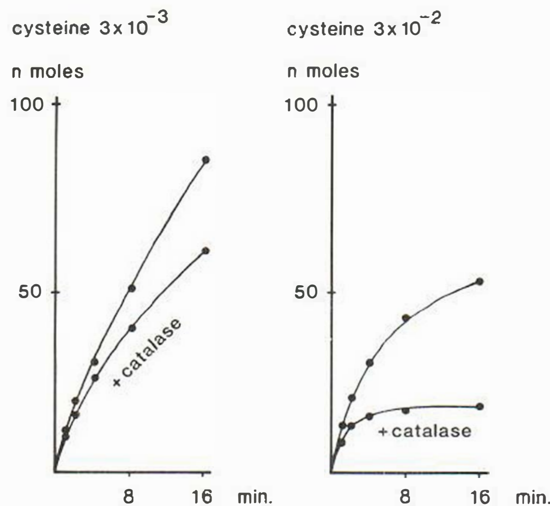

Fig. 6. Inactivation of tyrosinase by cysteine and promotion of inactivation by catalase.

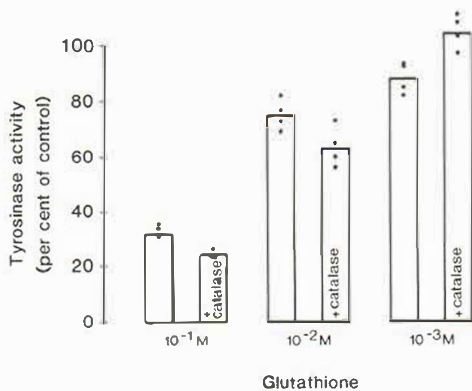

Fig. 8. Inactivation of tyrosinase by glutathione in various concentrations and the effect of catalse on the inactivation.

was observed. The inactivation was promoted by catalase (10 μg) at high cysteine concentrations (10^{-2} M and 10^{-3} M), but at 10^{-4} M cysteine concentration catalase prevented the inactivation by cysteine (Fig. 7). The effects of L-cysteine and D-cysteine were alike. Similar results were obtained with *glutathione*, but higher concentrations were needed for inactivation than with cysteine (Fig. 8). The effect of glutathione peroxidase (100 μg) was also studied for 10^{-2} M glutathione: a more pronounced inactivation of tyrosinase was obtained with 76% activity in the absence of glutathione peroxidase, and with 52% activity in the presence of glutathione peroxidase.

Inactivation of tyrosinase by cysteine. Protection by substrate (Method 2). Inactivation of tyrosinase by cysteine also occurs in the presence of substrate, as demonstrated by method 1 (Fig. 6). However, with the larger amounts of enzyme available in method 2 experiments, presence of dopamine (10^{-2} M) in step A reduced the inactivation by cysteine (10^{-2} M) so that it was no longer measurable.

Effect of albumin on the inactivation of tyrosinase by cysteine and ascorbic acid (Method 2). Addition of 10 mg of bovine serum albumin to incubates of cysteine (10^{-2} M) and tyrosinase or to incubates of ascorbic acid (10^{-2} M) and tyrosinase did not inhibit the inactivation of tyrosinase by cysteine, whereas albumin protected tyrosinase from inactivation by ascorbic acid.

The importance of oxygen for inactivation of tyrosinase (Method 2). The inactivation of tyrosinase by ascorbic acid was completely prevented when step A was performed with nitrogen or argon instead of oxygen. In contrast, the inactivation of tyrosinase by cysteine took place when oxygen was replaced by argon or nitrogen.

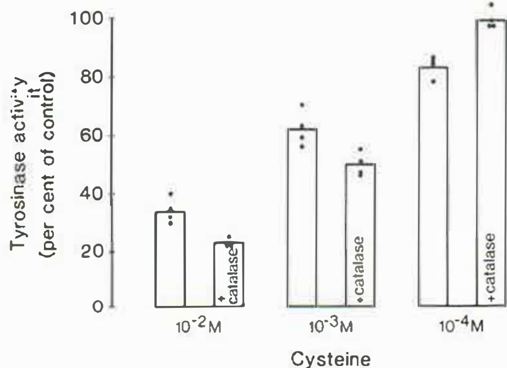

Fig. 7. Inactivation of tyrosinase by cysteine in various concentrations and the effect of catalase on the inactivation.

DISCUSSION

The results show that oxidation of naturally occurring compounds such as cysteine, glutathione, and ascorbic acid leads to the formation of hydrogen peroxide, which can react with and inactivate tyrosinase. In the case of ascorbic acid this inactivation is counteracted by catalase and by protein, and the inactivation by ascorbic acid can be fully explained by hydrogen peroxide formation. It is well known that hydrogen peroxide is formed during oxidation catalysed by cupric ions. Oxidation of ascorbic acid in the presence of the copper-containing tyrosinase apparently also involves formation of hydrogen peroxide, and this will oxidize additional ascorbic acid. Accumulation of hydrogen peroxide at the end of the reaction may

therefore be insignificant (20). Catalase has been reported to possess L-dopa-peroxidase activity (4), but under our conditions this effect is minimal (unpublished observations).

The inactivation of tyrosinase by cysteine or glutathione is more complex. Thiols can bind to the copper of tyrosinase, thereby inactivating the enzyme (12, 14, 22).

The oxidation of thiols that occurred in our system also led to the production of hydrogen peroxide, which further accelerated oxidation of cysteine. However, the increased inactivation of tyrosinase seen with catalase at higher cysteine concentrations is probably not caused by the slightly higher concentrations of cysteine that may result from the consumption of hydrogen peroxide by catalase. Another explanation could be that oxytyrosinase formed in the presence of hydrogen peroxide (10) has less reactivity to thiols than have mettyrosinase or deoxytyrosinase. This is contradicted by the finding that Neurospora tyrosinase, completely oxygenated by aerobic reduction with H_2O_2, formed a complex to the same extent as did the native enzyme on treatment with 2-mercaptoethanol (1). Nevertheless, it is conceivable that hydrogen peroxide formed by the oxidation of mercaptoethanol in the experiment of Aasa et al. may have induced oxygenation of the enzyme just as did addition of extraneous H_2O_2. In that case further addition of H_2O_2 would have no effect on the oxygenation of the enzyme, and complex formation with the mercapto compound would occur to the same extent with or without addition of hydrogen peroxide. At a low cysteine concentration, catalase protected tyrosinase against inactivation. It is conceivable that inactivation by copper-thiol binding at the lower cysteine concentration is insignificant, and that the hydrogen peroxide inactivation is dominant.

The role of hydrogen peroxide for protection of tyrosinase was further illustrated by the experiment with glutathione as inactivating substance. Glutathione peroxidase had the same effect as catalase, and both enzymes increased the inactivation by glutathione.

It should be noted that the inactivation of tyrosinase in the model systems of method 2, containing cysteine, glutathione, ascorbic acid, and 5-OH-dopamine, are much more pronounced than the reaction inactivation occurring with the substrates tyrosine and dopa or dopamine. Actually, the substrate protects tyrosinase against cysteine inactivation, as illustrated by the method 2 experiment, where cysteine and dopamine were present in the same concentrations (10^{-2} M). Some of the protection is certainly attributable to the binding of cysteine to quinone, but the very pronounced enzyme-protecting effect of dopamine must have other explanations. It seems probable that less H_2O_2 is formed in the presence of proper substrate, and that 'natural' oxygenation of the copper atoms reduced by the substrate may endow protection against the SH-groups.

For clarity we preferred to compare the inactivation of tyrosinase by dopamine and 5-OH-dopamine instead of by dopa and 5-OH-dopa, since dopa in step A would interfere with the cysteinyldopa determination of step B. From Figs. 4 and 5 it is evident that tyrosinase is inactivated much more by oxidation of the 5-OH-derivative than of the unsubstituted substrate. Since 5-OH-dopa is produced on incubation of dopa with tyrosinase, reaction inactivation may be due to oxidation of 5-OH-dopa formed in the incubate. It has been reported that reaction inactivation is promoted by ascorbic acid added to incubates in order to prevent melanin formation (21). Such inactivation could be due to oxidation of ascorbic acid and also to some extent to oxidation of 5-OH-dopa, which is present in increased amounts in incubates containing ascorbic acid. Oxidation of either substance may give H_2O_2 the effect of which would depend on the presence of proteins (cf. the protection of tyrosinase by serum albumin) and catalase or other enzymes catalysing the reduction of H_2O_2.

It has long been thought that reaction inactivation of tyrosinase could be due to binding of formed quinones to nucleophilic groups in the enzyme (23). Such binding close to the active centre might result in loss of copper (7). It is also known that autooxidation of 5,6-dihydroxyindole, an intermediary in melanin formation, results in the production of hydrogen peroxide (6). Many investigators have tried to avoid reaction inactivation of tyrosinase in incubates by adding reductants such as ascorbic acid, which procedure diminishes the lifetime of dopaquinone and prevents formation of indoles, yet reaction inactivation has nevertheless occurred. The present finding of hydrogen peroxide production on adding different reductants to model systems of tyrosinase suggests that hydrogen peroxide and radicals formed from it may cause tyro-

sinase inactivation also in experimental systems where melanin formation is inhibited by reducing compounds.

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