Biochem. J. (1984) **222**, 407–411
Printed in Great Britain

Oxidation of tyrosine residues in proteins by tyrosinase

Formation of protein-bonded 3,4-dihydroxyphenylalanine and 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine

Shosuke ITO,* Toshiaki KATO, Kan SHINPO and Keisuke FUJITA
Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470-11, Japan

(*Received 5 March 1984/Accepted 15 May 1984*)

A simple and rapid method was developed for the determination of 3,4-dihydroxyphenylalanine (dopa) and 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine (5-*S*-cysteinyldopa) in proteins with the use of high-pressure liquid chromatography. With this method, it is demonstrated that mushroom tyrosinase can catalyse hydroxylation of tyrosine residues in proteins to dopa and subsequent oxidation to dopaquinone residues. The dopaquinone residues in proteins combine with cysteine residues to form 5-*S*-cysteinyldopa in bovine serum albumin and yeast alcohol dehydrogenase, whereas dopa is the major product in bovine insulin, which lacks cysteine residues.

Tyrosinase is a unique enzyme in melanocytes responsible for melanine formation (Jimbow *et al.*, 1976). It is a bifunctional enzyme catalysing both hydroxylation of tyrosine dopa and subsequent oxidation to dopaquinone (Hearing *et al.*, 1980). The high reactivity of dopaquinone leads to two major pathways. In the absence of thiol compounds, the intramolecular nucleophilic addition of the amino group takes place, giving ultimately the black melanin, eumelanin (Prota, 1980). However, in the presence of thiol compounds, an extremely rapid addition of the thiol (Tse *et al.*, 1976) leads to the production of thioether derivatives of dopa. For example, tyrosinase oxidation of dopa in the presence of cysteine affords 5-*S*-cysteinyldopa and its 2-*S* isomer in a ratio of 5:1 (Ito & Prota, 1977). Further oxidation of cysteinyldopas results in the formation of the reddish melanin, pheomelanin (Prota, 1980).

Takahashi & Fitzpatrick (1966) isolated large amounts of dopa from the acid hydrolysates of mouse melanomas, and Agrup *et al.* (1978) detected 5-*S*-cysteinyldopa in the hydrolysates of human melanomas. In our comprehensive study (Ito *et al.*, 1983*a*) we found high concentrations of the protein-bonded dopa and 5-*S*-cysteinyldopa not only in melanomas but also in black and yellow hair of mice, and suggested that tyrosinase is responsible for the conversion of tyrosine residues in proteins into dopa and subsequently into 5-*S*-cysteinyldopa residues as shown in Scheme 1.

In the present study, we developed a simple and rapid method to quantify the protein-bound dopa and 5-*S*-cysteinyldopa with the use of high-pressure liquid chromatography with electrochemical detection. With this method, we demonstrate that mushroom tyrosinase can catalyse the formation of dopa and 5-*S*-cysteinyldopa in proteins.

Experimental

Materials

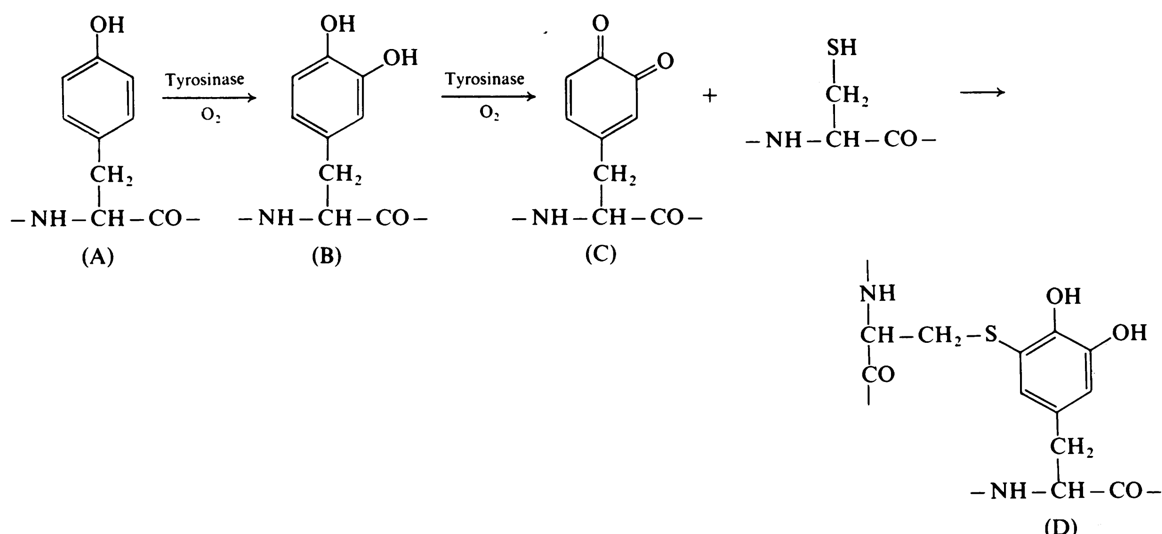
Mushroom tyrosinase (2000 units/mg), yeast alcohol dehydrogenase, bovine insulin, L-dopa, DL-α-methyldopa and glutathione were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin, thioglycollic acid and other chemicals were from Wako Pure Chemical Industries, Osaka, Japan. 5-*S*-Cysteinyldopa was prepared as described by Ito *et al.* (1981). Milli-Q-system (Millipore Corp., Bedford, MA, U.S.A.) ultrapure water was used throughout this study.

Oxidation of proteins by tyrosinase and acid hydrolysis of the products

Screw-capped test tubes were used for both oxidation and hydrolysis. A reaction mixture contained 100 nmol of protein, 50 or 100 μg of mushroom tyrosinase and 1 ml of 0.1 M-sodium phosphate buffer, pH 7.4. In some experiments 1 μmol

Abbreviations used: dopa, 3-(3,4-dihydroxyphenyl)-alanine; 5-*S*-cysteinyldopa, 3-(5-*S*-cysteinyl-3,4-dihydroxyphenyl)alanine; α-methyldopa, 2-methyl-3-(3,4-dihydroxyphenyl)alanine.

* To whom correspondence should be sent.



Scheme 1. *Modification of a tyrosine residue in protein as the result of tyrosinase action*
(A) Tyrosine residue; (B) dopa residue; (C) dopaquinone residue; (D) 5-*S*-cysteinyldopa residue.

of glutathione or 10 μmol of ascorbic acid were also included. After the addition of tyrosinase, the mixture was incubated at 30°C for 30, 60 and 120 min. The reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid, and the resulting precipitate was collected by centrifugation after being kept overnight at 4°C. The precipitate was washed twice with 1 ml of 5% (w/v) trichloroacetic acid, and 1 ml of 6 M-HCl containing 5% (w/v) thioglycollic acid was added to it. The test tube was screw-capped and heated in an oil bath at 110°C under argon atmosphere. After 16 h, 10 μl of 100 μg/ml α-methyldopa in 0.1 M-HCl was added as an internal standard and the hydrolysate was stored at −20°C until analysis.

Alumina extraction of catechols

In a capped test tube (10 ml volume) were placed 50 mg of acid-washed alumina, 1 ml of 0.2% $Na_2S_2O_5$ and 0.2 ml of either the hydrolysate or a standard solution containing 0.5 μg of dopa, 0.5 μg of 5-*S*-cysteinyldopa and 1 μg of α-methyldopa per ml of 6 M-HCl containing 5% (w/v) thioglycollic acid. Catechols were adsorbed on alumina at pH 8.6 by adding 1 ml of 2.7 M-Tris containing 2% (w/v) Na_2EDTA and immediately shaking for 5 min. The alumina was washed twice with 10 ml of water, and catechols were eluted with 0.3 ml of 0.4 M-$HClO_4$. The extract was analysed by high-pressure liquid chromatography with electrochemical detection as follows.

Chromatography

A Yanaco model L-2000 liquid chromatograph (Yanagimoto Manufacturing Co., Kyoto, Japan) was employed with a Yanaco model VMD-101 electrochemical detector. The detector was set at +750 mV against an Ag/AgCl reference electrode. A C_{18} reversed-phase column (Yanapak ODS-T, 4 mm x 250 mm) was used at 35°C with the mobile phase: 0.1 M-sodium citrate buffer, pH 4.0, containing 1 mM-sodium octanesulphonate/methanol (97:3, v/v). The flow rate was 0.7 ml/min.

Results

Acid hydrolysis of proteins and alumina extraction and chromatographic analysis of dopa and 5-S-cysteinyldopa

Acid hydrolysis of proteins is commonly performed in an evacuated sealed tube. However, this conventional method appeared not to be suitable for handling a large number of samples. Therefore we used screw-capped test tubes for the tyrosinase oxidation followed by acid hydrolysis.

Catechols are extremely susceptible to autoxidation. In a previous study (Ito *et al.*, 1983*a*) it was shown that catechols, especially 5-*S*-cysteinyldopa, are unstable in the absence of a reducing agent. In an attempt to prevent the oxidation, acid hydrolysis was carried out in the presence of thioglycollic acid under argon atmosphere, and the stability of dopa and 5-*S*-cysteinyldopa was examined. The recoveries (means ± S.D. for five determinations) of 0.5 μg each of dopa and 5-*S*-cysteinyldopa subjected to the hydrolytic conditions in the presence of 0.1 nmol of bovine serum albumin were 99.3 ± 4.0% and 83.4 ± 3.0% respectively. The 5-*S*-cysteinyldopa values in the present paper have been corrected for the recovery of 83%.

Table 1. *Formation of dopa and 5-S-cysteinyldopa from tyrosine and cysteine under hydrolytic conditions*
A mixture of the reactants was heated with 1 ml of 6 M-HCl containing 5% (w/v) thioglycollic acid at 110°C under argon atmosphere. After 16 h, 1 μg of α-methyldopa was added and the mixture was treated as described in the Experimental section. Data represent means ± S.D. for five determinations.

Expt. no.	Reactants	Products (nmol)	
		Dopa	5-*S*-Cysteinyldopa
1	Tyrosine (1 mg) + cysteine (1 mg)	0.286 ± 0.190	<0.005*
2	Tyrosine (1 mg) + cysteine (1 mg) + tryptophan (1 mg)	0.091 ± 0.061	<0.005

* Below detection limit.

The possibility was then examined that dopa and 5-*S*-cysteinyldopa were formed as artifacts from tyrosine and cysteine during acid hydrolysis. The results shown in Table 1 indicate that under the hydrolytic conditions the formation of dopa and 5-*S*-cysteinyldopa was minimal or undetectable. Furthermore, amino acids, such as tryptophan, have some preventive effect on the hydroxylation of tyrosine, as has also been shown in a previous study (Ito *et al*., 1983*b*).

The hydrolysate was subjected directly to alumina extraction to simplify the procedure; this was achieved by adjusting pH of the hydrolysate to 8.6 with 2.7 M-Tris. Under these conditions the recoveries (means ± S.D. for five determinations) of catechols in the standard solution were reasonably high: 69.1 ± 3.6% for dopa, 69.7 ± 4.7% for 5-*S*-cysteinyldopa and 66.1 ± 4.1% for α-methyldopa.

Thioglycollic acid contained in the hydrolysis medium caused some problems; it was extracted by alumina, although in low yields, and in chromatograms gave a broad peak that interfered with dopa. These problems were overcome with a solvent system containing octanesulphonate, which retarded the catecholic amino acids but not thioglycollic acid. Under the chromatographic conditions described in the Experimental section, dopa, 5-*S*-cysteinyldopa and α-methyldopa appeared at about 8, 11 and 13 min respectively.

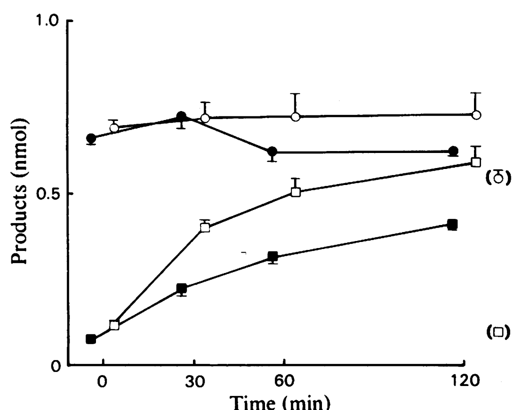


Fig. 1. *Oxidation of bovine serum albumin by tyrosinase*
The reaction mixture contained 100 nmol of bovine serum albumin, 50 μg (● and ■) or 100 μg (○ and □) of mushroom tyrosinase and 1 ml of 0.1 mM-sodium phosphate buffer, pH 7.4. Dopa (● and ○) and 5-*S*-cysteinyldopa (■ and □) were determined by high-pressure liquid chromatography with electrochemical detection after acid hydrolysis and alumina extraction (see the Experimental section for details). The results obtained by incubation for 120 min with boiled enzyme (100 μg) are shown in parentheses. Vertical bars denote S.E.M. for three separate experiments. Data points were shifted left or right for clarity.

Oxidation of proteins by tyrosinase

Bovine serum albumin contains 19 tyrosine and 1 cysteine residues per molecule. The protein was incubated with mushroom tyrosinase, and protein-bonded dopa and 5-*S*-cysteinyldopa were analysed after acid hydrolysis and alumina extraction (Fig. 1). Dopa showed no increase from the control values, but 5-*S*-cysteinyldopa exhibited a hyperbolic increase with time. These results indicate that tyrosinase can catalyse the hydroxylation of tyrosine residue(s) in bovine serum albumin and the subsequent oxidation to the dopaquinone form. Doubling the tyrosinase concentration almost doubled the 5-*S*-cysteinyldopa formation. Heat-denatured enzyme did not catalyse the formation of protein-bonded catechols (Fig. 1), indicating that the reaction was indeed tyrosinase-dependent. Although the protein-bonded 5-*S*-cysteinyldopa found in the samples without incubation (Fig. 1, 0 min) might arise as artifacts during acid hydrolysis, the increase after incubation must be ascribed to enzymic formation.

The effect of glutathione (1 μmol) was next examined to see whether the dopaquinone residue(s) in bovine serum albumin could react with the physiological thiol. The yields of 5-*S*-cysteinyldopa increased only slightly (1.4-fold) with the addition of glutathione (Fig. 2), indicating that the sole cysteine residue in the protein is capable of binding with the dopaquinone residue(s).

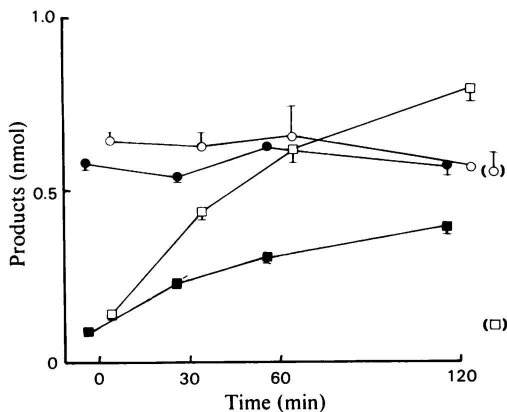


Fig. 2. *Oxidation of bovine serum albumin by tyrosinase in the presence of glutathione*
Glutathione (1 μmol) was added to the reaction medium described in Fig. 1. The other conditions were the same as in Fig. 1.

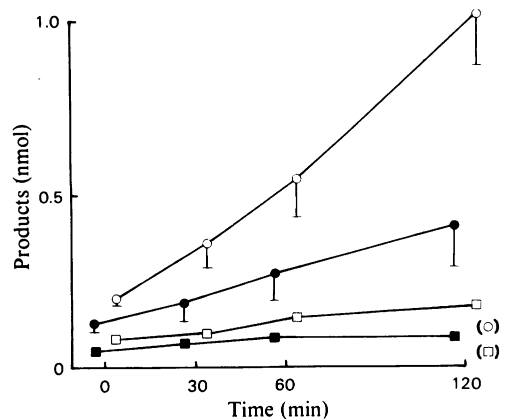


Fig. 4. *Oxidation of insulin by tyrosinase*
Bovine insulin (100 nmol) replaced bovine serum albumin. The other conditions were the same as in Fig. 1.

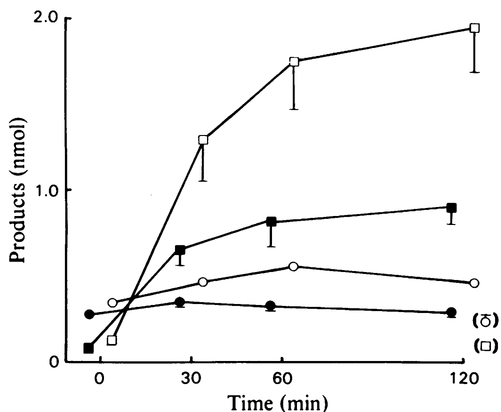


Fig. 3. *Oxidation of alcohol dehydrogenase by tyrosinase*
Yeast alcohol dehydrogenase (100 nmol as monomer) replaced bovine serum albumin. The other conditions were the same as in Fig. 1.

The effect of ascorbic acid (10 μmol) was also examined, inasmuch as the compound has the ability to reduce dopaquinone rapidly back to dopa (Tse *et al.*, 1976) and the formation of 5-*S*-cysteinyldopa was thus expected to be prevented. The formation of protein-bonded dopa, instead of 5-*S*-cysteinyldopa, was found to proceed rapidly and linearly with time (1.31 nmol/120 min with 100 μg of tyrosinase; results not shown). However, the reaction also occurred rapidly (1.00 nmol/120 min) with the heat-denatured enzyme. Thus it appeared that most of the dopa was produced non-enzymically, possibly by the Fenton-type hydroxylation reaction catalysed by Fe^{2+} (Breslow & Lukens, 1960) present in trace amounts.

Yeast alcohol dehydrogenase contains 14 tyrosine and 8 cysteine residues per monomer. As shown in Fig. 3, oxidation of the protein by tyrosinase gave results similar to those with bovine serum albumin, but the yields of protein-bonded 5-*S*-cysteinyldopa increased 3-fold. The rate of 5-*S*-cysteinyldopa formation was proportional to the amount of tyrosine added.

We then examined the behaviour of bovine insulin, which contained 4 tyrosine but no cysteine residues. Protein-bonded dopa increased almost linearly with time, whereas 5-*S*-cysteinyldopa showed little increase (Fig. 4). The slight increase of the latter may be ascribed to cysteine residues in tyrosinase.

Discussion

Oxidation of peptides and proteins by tyrosinase has been studied by several groups. Yasunobu *et al.* (1959) have reported spectroscopic evidence for the alteration of tyrosine-containing peptides on incubation with tyrosinase. Cory & Frieden (1967*a*) have shown that oxidized B-chain of insulin is oxidized by tyrosinase with a decrease in tyrosine. They have also shown that yeast alcohol dehydrogenase (Cory & Frieden, 1967*b*) and bovine serum albumin (Frieden *et al.*, 1959) serve as substrates for tyrosinase. In those studies, however, the reaction products were not identified. The present study demonstrates that tyrosinase can catalyse the hydroxylation of tyrosine residues in proteins to dopa and the subsequent oxidation to dopaquinone residues (Scheme 1). 5-*S*-Cysteinyldopa is the major product in bovine serum albumin and alcohol dehydrogenase, which contain cys-

teine residue(s), whereas dopa is formed in insulin, which lacks cysteine residues.

Agrup *et al* (1982) have reported that the oxidation of free tyrosine by tyrosinase gives cysteinyldopa, with little accumulation of dopa, as long as cysteine is present. Thus the behaviour of tyrosine in proteins is similar to that of free tyrosine. Although dopa or 5-*S*-cysteinyldopa is the major product identified by chromatography, the possibility cannot be ruled out that, in addition to these catechols, other products may be formed from dopaquinone residues in proteins. For example, the amino group of a lysine residue is also capable of the nucleophilic addition to dopaquinone, although the reaction should be much slower than that of the thiol group of a cysteine residue (Tse *et al.*, 1976).

The natural occurrence of dopa in proteins has rarely been reported. The protein-bonded dopa has been found in melanoma tissues (Takahashi & Fitzpatrick, 1966; Agrup *et al.*, 1978; Ito *et al.*, 1983*a*), melanosome preparations from melanomas and other melanin-producing tissues (Duchon *et al.*, 1973; Ito *et al.*, 1983*a*) and mouse hair (Ito *et al.*, 1983*a*). More recently, Waite (1983) has isolated from the marine mussel *Mytilus edulis* an adhesive protein containing a large amount (11%) of dopa. The occurrence of cysteinyldopa in proteins has been reported only by Agrup *et al.* (1978) and by Ito *et al.* (1983*a*).

The present study developed a simple and rapid method to identify and quantify dopa and 5-*S*-cysteinyldopa present in proteins. We hope that the method will facilitate study of the occurrence and significance of protein-bonded dopa and 5-*S*-cysteinyldopa in biological samples.

References

Agrup, G., Hansson, C., Rorsman, H., Rosengren, A.-M. & Rosengren, E. (1978) *Acta Derm.-Venereol.* **58**, 270–272

Agrup, G., Hansson, C., Rorsman, H. & Rosengren, E. (1982) *Arch. Dermatol. Res.* **272**, 103–115

Breslow, R. & Lukens, L. N. (1960) *J. Biol. Chem.* **204**, 741–749

Cory, J. G. & Frieden, E. (1967*a*) *Biochemistry* **6**, 116–120

Cory, J. G. & Frieden, E. (1967*b*) *Biochemistry* **6**, 121–126

Duchon, J., Borovansky, J. & Hach, P. (1973) in *Pigment Cell* (Montagna, W. & Hu, F., eds.), vol. 1, pp. 165–170, S. Karger, Basel

Frieden, E., Bigelow, C. C. & Ottensen, M. (1959) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **18**, 438

Hearing, V. J., Jr., Ekel, T. M., Montagnue, P. M. & Nicholson, J. M. (1980) *Biochim. Biophys. Acta* **611**, 251–268

Ito, S. & Prota, G. (1977) *Experientia* **33**, 1118–1119

Ito, S., Inoue, S., Yamamoto, Y. & Fujita, K. (1981) *J. Med. Chem.* **24**, 673–677

Ito, S., Homma, K., Kiyota, M., Fujita, K. & Jimbow, K. (1983*a*) *J. Invest. Dermatol.* **80**, 207–209

Ito, S., Jimbow, K., Kato, T., Kiyota, M. & Fujita, K. (1983*b*) *Acta Derm.-Venereol.* **63**, 463–467

Jimbow, K., Quevedo, W. C., Jr., Fitzpatrick, T. B. & Szabo, G. (1976) *J. Invest. Dermatol.* **67**, 72–89

Prota, G. (1980) *J. Invest. Dermatol.* **75**, 122–127

Takahashi, H. & Fitzpatrick, T. B. (1966) *Nature (London)* **209**, 888–890

Tse, D. C. S., McCreery, R. L. & Adams, R. N. (1976) *J. Med. Chem.* **19**, 37–40

Waite, J. H. (1983) *J. Biol. Chem.* **258**, 2911–2915

Yasunobu, K. T., Peterson, E. W. & Mason, H. S. (1959) *J. Biol. Chem.* **234**, 3291–3295