

FLUOROMETRY OF A DOPA PEPTIDE AND ITS THIOETHER

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Abstract. Incubation of L-tyrosyl-glycyl-glycine with polyphenoloxidase for 2 hours leads to pigment formation. The intermediate product 3,4-dihydroxyphenylalanyl-glycyl-glycine (DGG) can be adsorbed to Al_2O_3 . This compound has maximum ultraviolet absorption at 282 nm, it stains red with potassium ferricyanide and gives a yellow fluorescence in UV-light after formaldehyde treatment. After oxidation with periodate it has a maximum fluorescence at 380 nm and a maximum excitation at 335 nm. Peptides with dopa in N-terminal position can thus be fluorometrically determined by this method.

Incubation of L-tyrosyl-glycyl-glycine with polyphenoloxidase in the presence of L-cysteine for 2 hours does not result in pigment formation. A new compound, cysteinyl-dihydroxyphenylalanyl-glycyl-glycine (CDGG), is formed and this substance can be adsorbed to Al_2O_3 . CDGG has maximum ultraviolet absorption at 255 and 294 nm, it stains red with potassium ferricyanide and gives a yellow fluorescence in UV-light after formaldehyde treatment. After oxidation with periodate it has a fluorescence peak at 485 nm with maximum excitation at 335 nm. Many characteristics of CDGG are the same as those of a compound previously observed in human malignant melanomas and our results are evidence for the presence of thioethers of dopa in these tumours.

Human melanomas contain not only dopa, but also other catechol derivatives (3, 4). Paper chromatograms of perchloric acid extracts of human melanomas developed in phenol:HCl have shown one such compound (4) with a lower Rf value than that of dopa. The substance fluoresced green-yellow after treatment with formaldehyde and became red after oxidation with potassium ferricyanide. When the melanoma extract was oxidized with periodate (1), two emission peaks appeared on excitation at 335 nm. One of the emissions corresponded with that of dopa, but the composition of the compound that fluoresced at a higher wavelength has remained obscure. Addition of acid (1) suppressed the dopa peak, but

had only a minor effect on the fluorescence of the unknown substance.

We have observed that a compound similar to that found in melanomas is formed during the oxidation of a tyrosine peptide by polyphenoloxidase in the presence of sulfhydryl inhibitors.

This paper reports evidence suggesting that the substance previously described in human melanomas is a thioether of dopa.

MATERIAL AND METHODS

Formation, isolation and fluorometry of 3,4-dihydroxyphenyl-alanyl-glycyl-glycine (DGG)

2 mg of L-tyrosyl-glycyl-glycine (Fluka AG) was incubated with 0.3 mg of polyphenoloxidase from mushrooms (Sigma Chem. Corp.) containing 1 100 enzyme units/mg in 2.3 ml of a 0.1 M phosphate buffer, pH 6.5, at room temperature for 2 hours. Ninhydrin treatment of chromatograms of L-tyrosyl-glycyl-glycine incubated with the polyphenol oxidase at pH 6.5 for 2 and 24 hours revealed no glycine, tyrosine or free dopa.

Incubation was stopped by addition of 25 ml of 0.4 N perchloric acid.

Catechols were adsorbed onto Al_2O_3 and eluted with 0.1 N HCl (Anton & Sayre, 1964).

Samples of eluates were used for spectrophotometry. Other samples of eluates were developed in descending chromatograms on Whatman paper no. 1 in the following solvents:

- (A) *n*-Butanol/acetic acid/water (60:15:25).
- (B) Methyl ethyl ketone/acetic acid/water (75:25:30).

Chromatograms were treated in 3 different ways: (a) Placed in a jar containing paraformaldehyde and heated at 80°C for 1 hour. These chromatograms were then examined in UV-light at a wavelength of 350 nm. (b) Sprayed with a 0.4% solution of potassium ferricyanide, heated at 80°C for 5 min and examined in visible light and UV-light at 350 nm. (c) Strips situated at different distances from the starting line were eluted with 0.1 N HCl and analysed according to Anton & Sayre.

Table 1. *R_f* values of substances adsorbed to Al₂O₃

	Butanol/ acetic acid/water	Methyl- ethylketone/ acetic acid/water
Dopa	0.30	0.42
DGG	0.22	0.36
CDGG	0.06	0.16
Spot with spontaneous blue fluorescence	0.38	0.71

The excitation and the emission spectra of oxidized eluates from chromatograms were compared with those of L-3-(3,4-dihydroxyphenyl)alanine (Fluka AG).

Formation, isolation and fluorometry of cysteinyl-dihydroxyphenylalanyl-glycyl-glycine (CDGG)

2 mg of L-tyrosyl-glycyl-glycine (Fluka AG) was incubated with 1.2 mg of L-cysteine-hydrochloride (E. Merck AG) and 0.3 mg of polyphenoloxidase (Sigma Chem. Corp.) in 2.3 ml of a 0.1 M phosphate buffer, pH 6.5, at room temperature for 2 hours.

Incubation was stopped by addition of 25 ml of 0.4 N perchloric acid.

Adsorption onto Al₂O₃, elution, spectrophotometry, chromatography, treatment of chromatograms and fluorometry were performed as for DGG. Dopa as well as 5-S-cysteinyl dopa (11) was used as reference substances for fluorometry. The latter substance was kindly supplied by Professor G. Prota, Istituto di Chimica Organica, University of Naples, Italy.

Formation, isolation and measurement of radioactive CDGG

Incubation of peptide, cysteine and polyphenoloxidase as for formation of unlabelled CDGG but with addition of 1 μ Ci ¹⁴C-L-cysteine (U) hydrochloride (Amersham) specific activity 18.6 μ Ci/mM, or of 4 μ Ci L-cysteine S-35 hydrochloride (Amersham) with a spec. act. of 34.6 μ Ci/mM. Adsorption, elution, chromatography, treatment of chromatograms and fluorometry were performed as for DGG. Radioscanning of formaldehyde-treated chromatograms was performed.

RESULTS

DGG

Incubation of L-tyrosyl-glycyl-glycine with polyphenoloxidase for 2 hours at room temperature resulted in pigment formation. Adsorption to Al₂O₃ and elution with 0.1 N HCl gave a catechol substance which was best separated from dopa at chromatography in butanol/acetic acid/water (Table 1). After formaldehyde treatment this compound was yellow in UV-light; and when

sprayed with potassium ferricyanide it turned red in visible light and fluoresced blue in UV-light. A compound with spontaneous blue fluorescence, probably a 5,6-dihydroxyindolylpeptide, which disappeared after treatment with potassium ferricyanide was also adsorbed to Al₂O₃, and demonstrable in chromatograms.

Spectrophotometry of the compound eluted from Al₂O₃ gave the same maximum adsorption as dopa at 282 nm (Fig. 1).

Spectrophotofluorometry after oxidation according to Anton & Sayre showed the same excitation and emission maxima as dopa, i.e. at 335 and 380 nm (Fig. 2). The fluorescence intensity decreased as for dopa after treatment with acid.

CDGG

Incubation of L-tyrosyl-glycyl-glycine with L-cysteine and polyphenoloxidase at room temperature for 2 hours did not result in pigment formation. Adsorption to Al₂O₃ and elution with 0.1 N HCl gave a catechol substance, which in both solvents moved more slowly on chromatograms than dopa and DGG. It fluoresced yellow when treated with paraformaldehyde and stained red with potassium ferricyanide.

Spectrophotometry of the eluate from Al₂O₃ gave two absorption maxima, one at 255 and the other at 294 nm (Fig. 1).

Spectrophotofluorometry according to Anton & Sayre showed an excitation maximum of the slowly moving catechol at 335 nm and emission

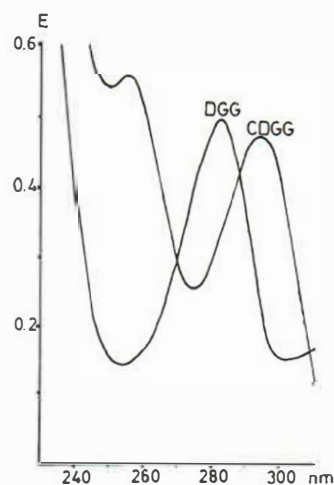


Fig. 1. Absorption spectra of DGG and CDGG in 0.1 N HCl.

maximum at 485 nm (Fig. 2). On spectrophotofluorometry according to Anton & Sayre 5-S-cysteinyl dopa (Protá) showed the same maxima, but also an emission peak at 390 nm.

Radioscanning of chromatograms showed that the new catechol compound formed by incubation of L-tyrosyl-glycyl-glycine and radioactive L-cysteine in the presence of polyphenoloxidase contained large amounts of labelled cysteine (Fig. 3).

DISCUSSION

Incubation of L-tyrosyl-glycyl-glycine with polyphenoloxidase resulted in the formation of a dopa peptide, 3,4-dihydroxyphenylalanyl-glycyl-glycine (DGG).

The oxidation of tyrosine-containing peptides by tyrosinase has been extensively studied (15). Three clear-cut types of spectral changes have been observed: a dopachrome pattern, a dopa quinone pattern and a protein pattern. N-terminal tyrosine peptides exhibit the dopachrome pattern characterized by the formation of an intermediate oxidation product with absorption maxima at 305 nm and 480 nm in the initial stages of the reaction. This absorption spectrum corresponds to that of aminochromes, e.g. dopachrome. In our experiments adsorption of catechols to Al_2O_3 was used for isolation of the intermediate dopa peptide formed. By this method a compound with an ultraviolet absorption at 282 nm was isolated after oxidation for 2 hours. Pigment formation was pronounced after this time. The tyrosine peptide, which has a maximum adsorption at 275 nm, is not adsorbed to Al_2O_3 . The substance isolated af-

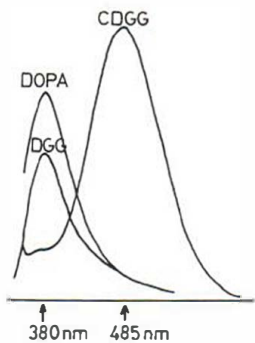


Fig. 2. Fluorescence spectra of Dopa, DGG and CDGG after oxidation according to Anton & Sayre. Excitation at 335 nm.

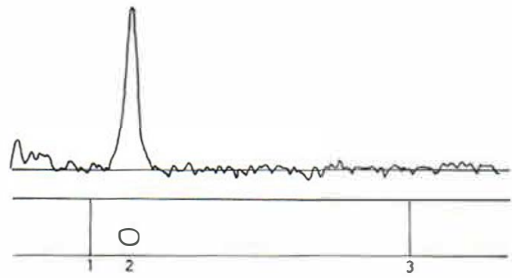


Fig. 3. Radioscanning of Al_2O_3 -adsorbed catechol formed at incubation of L-tyrosyl-glycyl-glycine and ^{14}C -L-cysteine with polyphenoloxidase. Chromatography in butanol/acetic acid/water. Treatment with formaldehyde at 80°C. (1) Starting line. (2) Spot with yellow fluorescence. (3) Solvent front.

ter elution from Al_2O_3 differed from dopa when chromatographed in butanol/acetic acid/water, but reacted in the same way as dopa when treated with potassium ferricyanide and formaldehyde. When the Al_2O_3 -adsorbed substance was oxidized according to Anton & Sayre a compound was formed with maximum excitation at 335 nm and maximum emission at 380 nm at fluorometry. These maxima are the same as for dopa, and the method of Anton & Sayre therefore seems to be useful for detection not only of free dopa but also of N-terminal dopa peptides.

Since dopa in N-terminal position in peptides fluoresces in the same way as dopa after oxidation, fluorometry must be supplemented by other methods if it is to confirm the presence of free dopa in a tissue. The occurrence of free dopa in human melanomas (3, 4) has been proved by the finding of dopamine formation after incubation of tissue extracts with dopa decarboxylases.

The new catechol peptide formed when tyrosyl-glycyl-glycine was incubated with polyphenoloxidase in the presence of cysteine is probably a thioether of dopa, cysteinyl-dihydroxyphenylalanyl-glycyl-glycine, CDGG.

It has previously been shown that quinones formed in melanogenesis may combine with sulphhydryl-containing compounds. Bouchilloux & Kodja (2) and Roston (12) have reported a characteristic UV-absorption of these compounds, a spectrum also demonstrated by us.

CDGG can be adsorbed to Al_2O_3 and this adsorption facilitates the determination of the substance. Chromatography and reaction with potassium ferricyanide or formaldehyde may be

used for isolation and identification of thioethers of dopa.

Incubation with radioactive cysteine was used for identification of the new product formed under our experimental conditions, and this procedure may also be used for quantitation of the enzymatically synthesized thio compound. Fluorometry of the oxidation product of the new cysteine-containing compound by the method of Anton & Sayre showed excitation at the same wavelength as dopa and DGG, but the emission of CDGG was at 485 nm. This spectrum was also shown by the oxidation product of 5-S-cysteinyl dopa (11) and fluorescence at 485 nm seems to be characteristic of thioethers of dopa. This fluorescence corresponds to that previously found in human malignant melanomas (4). The melanoma catechol compound with formaldehyde-induced fluorescence which could be chromatographically separated from dopa may thus represent a thioether of dopa or of a dopa peptide. Cysteine or glutathione may be the sulphur-containing moiety of the molecule.

The formation of 5-S-cysteinyl dopa is the first step in the biogenesis of pheomelanin (5, 6). Compounds containing sulphhydryl groups are believed to be essential in the regulation of melanin formation (7, 9, 13). The inhibitory effect of sulphhydryl-containing substances may be due to their ability to bind copper (9), but inhibition of melanin formation may also be explained by the formation of bonds between intermediate products in melanin synthesis and the sulphhydryl-containing inhibitors (2, 8, 10, 12, 14).

The characteristic fluorescence spectrum exhibited by thioethers of dopa and of dopa peptides permitting detection of very small amounts of these substances will surely be most helpful in the search for sulphur-containing catechols in tissues.

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