An Improved Method of Light-Induced Pigmentation

By KENNETH N. KUAN,* Y. Y. LEE* and PAUL MELIUS†

*Department of Chemistry and †Department of Chemical Engineering, Auburn University, Auburn, AL 36830, U.S.A.

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An improved procedure was developed whereby a primary light signal can be intensified and made visible by activation of a pre-tyrosinase (pre-phenoloxidase) enzyme [isolated from silkworm (*Bombyx mori*)] by α -chymotrypsin; this activation results from the light-activated conversion of the inactive *cis*-cinnamoyl- α -chymotrypsin.

Kaufman *et al.* (1968) first introduced the concept that enzymes that are not directly affected by light can be made light-sensitive by using an effector molecule. The investigations by Berezin *et al.* (1970) and Varfolomeyev *et al.* (1971) (in which α -chymotrypsin was inhibited with *cis*-cinnamoylimidazole and re-activated by light) verified this idea. Kuan *et al.* (1979) have demonstrated that α -chymotrypsin is capable of intensifying a light signal, which can then be made visible by coupling to tyrosinase. This study was undertaken to improve further and refine the tyrosinase reaction, which is coupled to the α -chymotrypsin as shown:

Experimental

Materials

α -Chymotrypsin (bovine pancreas; 3 $\times$ crystallized and freeze-dried; type II; Sigma Chemical Co., St. Louis, MO, U.S.A.) was used without further purification. *N-trans*-Cinnamoylimidazole (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was used as the acylating agent. DL-Dopa (DL- β -3,4-dihydroxyphenylalanine), Folin & Ciocalteu phenol reagent and bovine albumin (fraction V) were all obtained from Sigma Chemical Co. Nitrophenyl *p*-guanidino-

benzoate was obtained from ICN Pharmaceuticals, Irvine, CA, U.S.A.

Preparation of pre-tyrosinase

Pre-tyrosinase from the larval haemolymph of the silkworm (*Bombyx mori*) was prepared essentially by the method of Ashida (1971) with some modification. Haemolymph was collected into 99.2% satd. (NH₄)₂SO₄, pH 6.5. Crude pre-tyrosinase, obtained from between 25 and 42% satd. (NH₄)₂SO₄ solution was dissolved in 20% satd. (NH₄)₂SO₄ containing 0.1M-potassium phosphate, pH 6.5, and 1mM-nitrophenyl *p*-guanidinobenzoate. The (NH₄)₂SO₄ concentration was then raised to 38% saturation. Precipitate obtained from this step was subsequently dialysed, heat-treated and applied to a DEAE-cellulose column as described by Ashida (1971). Those fractions containing the pre-tyrosinase were pooled, concentrated, and used for experiment without further purification.

Assay of tyrosinase (monophenol mono-oxygenase; EC 1.14.18.1)

Tyrosinase activity was assayed colorimetrically at room temperature as described by Horowitz & Shen (1952) and measured at 575 nm in a Beckman DU spectrophotometer. The reaction mixture consisted of 0.25 ml of 0.02M-DL-dopa in 0.1M-sodium citrate buffer, 1 ml of 0.1M-sodium citrate buffer, pH 6.0, and 50 μ l of enzyme solution.

Inhibition and re-activation of α -chymotrypsin

These were done by methods previously reported (Kuan *et al.*, 1979).

Protein assay

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and Discussion

As reported by Ohnishi *et al.* (1970), the partially purified pre-tyrosinase from silkworm (*Bombyx mori*) can be activated by chymotrypsin as shown in Fig. 1. Excess chymotrypsin appears to have a detrimental effect on tyrosinase activity, whereas a low concentration does not fully liberate the activity.

When *cis*-cinnamoyl-inhibited chymotrypsin is exposed to u.v. light, the activated α -chymotrypsin almost instantaneously acts on pre-tyrosinase to convert it into the active tyrosinase, which, in turn, reacts with dopa to give a black pigment (melanin), as expected from the schematic reaction. This is in sharp contrast with the control (*cis*-cinnamoyl-inhibited chymotrypsin not exposed to u.v. light), as shown in Fig. 2. This, in effect, shows that the primary light signal has been intensified and made visible by the formation of black pigment by the activated tyrosinase.

Chymotrypsin may not play a physiological role in the tanning system of silkworm, since a pre-tyrosinase-activating enzyme has been reported (Dohke, 1973*a,b*). The fact that pre-tyrosinase can be activated by a light-sensitive molecule through the mediation of chymotrypsin is noteworthy, however, and may be similar to some other systems photo-regulated *in vivo*.

The mechanism by which light energy affects living organisms at the molecular level remains obscure. Hug *et al.* (1971) reported that urocanase from *Pseudomonas putida* slowly loses its activity when stored in the dark, but is restored to full activity when briefly exposed to u.v. irradiation. Macko *et al.* (1970, 1971), on the other hand, discovered that two

Fig. 1. Effect of different concentrations of α -chymotrypsin on the activation of pre-tyrosinase

Pre-tyrosinase (3.8 mg/ml) was incubated with an equal volume of chymotrypsin at different concentrations in 0.08 M-Tris/HCl, pH 7.8, with 0.1 M-CaCl₂ at room temperature, and 50 μ l samples of the mixture were assayed at different time intervals. Tyrosinase activity is expressed as the change in A_{575} /min of incubation with DL-dopa. Chymotrypsin concentrations: ●, 0.1 mg/ml; △, 1 mg/ml; □, 2 mg/ml; ○, 4 mg/ml.

Fig. 2. Pigmentation of DL-dopa by u.v.-induced α -chymotrypsin activation of pre-tyrosinase

(a) U.v.-re-activated chymotrypsin (4 mg/ml in 0.8 M-Tris/HCl buffer, pH 7.8)+pre-tyrosinase (3.8 mg/ml in 0.01 M-potassium phosphate buffer, pH 7.0) was incubated for 10 min, then DL-dopa and sodium citrate buffer, pH 6.0, added. (b) Control.

inhibitors of uredospore germination in the rust fungi are methyl 3,4-dimethoxycinnamate and methyl ferulate. Furthermore, these compounds exist in *cis*- and *trans*-forms and are interconvertible by u.v. radiation. Subsequently, it was found that only the *cis*- and not the *trans*-isomers inhibit germination (Allen, 1972). There are reasons to believe that an enzyme is present with the inhibitors during digestion of wall material (Hess *et al.*, 1975, 1976). Whether or not the pre-tyrosinase-activating enzyme is light-regulated through effector molecules in order to control the tanning system of silkworm remains to be seen. It is interesting to speculate, however, that this may be the mechanism for activation reactions controlled by light, which causes formation of pigment and other polymeric materials in insects and other organisms.

The work reported here was undertaken to develop an enzymic process for preserving a light signal. It has the advantages over the old procedure (Kuan *et al.*, 1979) of shortening colour-development time and giving sharper contrast against the control.

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