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Synthesis of 3,4-dihydro-3-amino-7-hydroxycoumarin from *S*-methyl-L-cysteine and resorcinol by crystalline β-tyrosinase

KAKIHARA AND ICHIHARA[1] have reported that phenol is produced from L-tyrosine and its derivatives in bacterial culture not through stepwise degradation but through primary fission of their side chains. The tyrosine-inducible enzyme responsible for this conversion was subsequently named β-tyrosinase (L-tyrosine phenol-lyase (deaminating)) by UCHIDA *et al.*[2]. YOSHIMATSU[3] demonstrated that β-tyrosinase catalyzes the stoichiometric conversion of L-tyrosine into phenol, pyruvate and ammonia and requires pyridoxal phosphate as a cofactor. An apparently homogeneous preparation of β-tyrosinase was recently prepared in our laboratory[4–6]. We reported that the crystalline preparation of β-tyrosinase catalyzes a series of α,β-elimination and β-replacement reactions[5,7].

L-Tyrosine + H_2O → pyruvate + NH_3 + phenol (1)

S-Methyl-L-cysteine + H_2O → pyruvate + NH_3 + CH_3SH (2)

L-Cysteine + H_2O → pyruvate + NH_3 + H_2S (3)

L-Serine → pyruvate + NH_3 (4)

L-Tyrosine + pyrocatechol → L-DOPA + phenol (5)

In this paper, further studies concerning the β-replacement reaction catalyzed by crystalline β-tyrosinase are described.

Crystalline β-tyrosinase was prepared from cells of *Escherichia intermedia* grown in a bouillon–peptone medium supplemented with L-tyrosine, according to the method of KUMAGAI *et al.*[5].

The formation of pyruvate from L-tyrosine, *S*-methyl-L-cysteine, L-cysteine or L-serine by recrystallized β-tyrosinase was inhibited by resorcinol. Fig. 1 shows the

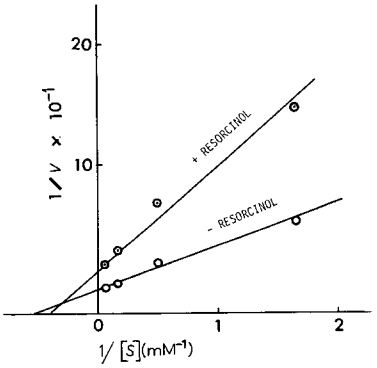

Fig. 1. Inhibition of pyruvate formation by resorcinol. Incubations were carried out at 30° for 10 min in reaction mixtures containing *S*-methyl-L-cysteine as indicated, 0.4 μmole of pyridoxal phosphate, 0.2 mmole of potassium phosphate buffer (pH 7.8), 0.0264 unit of the recrystallized enzyme and 1 μmole of resorcinol, in a total volume of 4.0 ml. The amount of pyruvate formed was determined by a modification of the method of FRIEDEMANN AND HAUGEN[8]. Velocity (v) was expressed as μmoles of pyruvate formed per min.

Lineweaver–Burk plots of pyruvate formation from *S*-methyl-L-cysteine in the presence or absence of resorcinol. Inhibition was of a mixed type and the K_i value was 0.160 mM.

Chromatographic examination of a similar reaction mixture on paper and ion-exchange resin showed the presence of a new ninhydrin-positive compound which chromatographed similarly to L-tyrosine. This product was isolated from a large-scale incubation mixture. Incubation was carried out at 30° for 12 h in a reaction mixture containing *S*-methyl-L-cysteine (7.5 mmoles), resorcinol (7.5 mmoles), pyridoxal phosphate (0.05 mmole), potassium phosphate buffer (25 mmoles, pH 7.8) and 24 mg of the recrystallized enzyme, in a total volume of 500 ml. Under these conditions, approx. 2.9 mmoles of the product were synthesized in the mixture based on ion-exchange chromatographic analysis. The mixture was applied to a Dowex 50-X8 column (70 cm × 1.9 cm, Na^+ form) and the column was eluted with 0.2 M sodium citrate buffer, pH 3.25. The elution of the product was followed by measuring its absorbance at 280 nm, as well as by the ninhydrin reaction. Fractions containing the product were then applied to a charcoal column (20 cm × 0.9 cm, activated). The column was first washed with distilled water, then eluted with 5% acetic acid. The acetic acid eluate was evaporated to dryness and the solid obtained was dissolved in ethyl alcohol containing a small amount of water. Colorless crystals of the product weighing 370 mg were obtained. Recrystallization was carried out from ethyl alcohol–water.

The enzymatically synthesized product was designated as 3,4-dihydro-3-amino-7-hydroxycoumarin based on the following physicochemical examination. The NMR spectrum of the product in ^2H_2O (Fig. 3) revealed an octet of AB protons (H_{4a} and H_{4b}) of the ABX type at δ 2.7–3.5 and a quartet of X proton (H_3) at δ 3.9–4.2 (J_{AB} = 14.2, J_{AX} = 4.5 and J_{BX} = 8.0 cycles/sec), suggesting the presence of nonequivalent protons. This indicates that an elimination reaction took place between *S*-methyl-L-

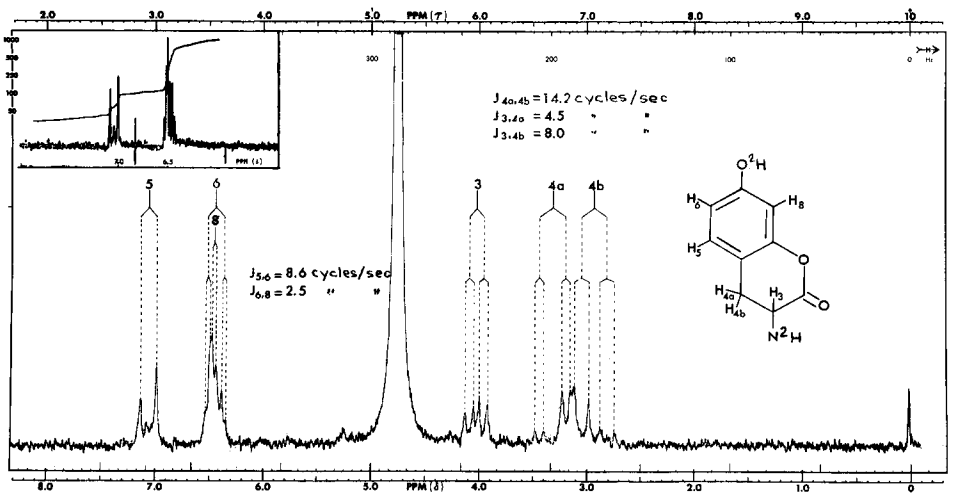

Fig. 2. NMR spectra of enzymatically synthesized 3,4-dihydro-3-amino-7-hydroxycoumarin. The spectra were taken with coumarin saturated in ^2H_2O, by Varian A-60 and H-100 (insert) spectrometers.

cysteine and resorcinol to give a product which was isolated in a δ-lactone form. This lactone structure was confirmed using a Hitachi RMS-4 mass spectrometer coupled with a Hitachi K-53 gas chromatograph. The presence of a molecular ion peak at *m/e* 263 was definitely observed in the mass spectrum of its diacetate derivative, $C_{13}H_{13}NO_5$. Signals in the lowest field of the NMR spectrum represent aromatic protons of H_5, H_6 and H_8. The sextet at δ 6.3–6.6 was assigned to H_6 and H_8 on the basis of the coupling constant $J_{6,8} = 2.5$ cycles/sec (*meta* coupling) and $J_{5,8} = 0$ cycles/sec (*para* coupling). The doublet was assigned to H_5 with $J_{5,6} = 8.6$ cycles/sec (*ortho* coupling). This assignment of the aromatic protons was also supported by 100-Mcycle measurements (Fig. 2, insert). Based on this information, the remaining hydroxyl group must be attached to the 7-position of the coumarin ring.

Synthesis of 3,4-dihydro-3-amino-7-hydroxycoumarin by β-tyrosinase was pyridoxal phosphate dependent and proceeded linearly with incubation time and enzyme and resorcinol concentrations.

Synthesis of coumarin from *S*-methyl-L-cysteine and resorcinol, by β-tyrosinase, is explainable by adopting the general mechanism for pyridoxal phosphate-dependent reactions proposed by BRAUNSTEIN AND SHEMYAKIN[9] and by METZLER *et al.*[10]. In catalysis of pyruvate formation (α,β-elimination), *S*-methyl-L-cysteine interacts with β-tyrosinase to form reversibly enzyme-bound α-aminoacrylate by eliminating H^+ and CH_3S^-. The enzyme-bound α-aminoacrylate hydrolyzes irreversibly to yield pyruvate and ammonia and regenerates β-tyrosinase. In the presence of resorcinol (β-replacement), addition of resorcinol to enzyme-bound α-aminoacrylate, rather than hydrolysis, can occur yielding 2,4-dihydroxyphenyl-L-alanine by reversal of the reactions. Ring closure of 2,4-dihydroxyphenyl-L-alanine to form coumarin may be spontaneous *via* intramolecular lactone formation (Fig. 3).

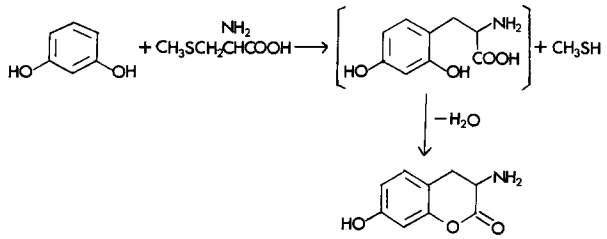

Fig. 3. Proposed scheme for enzymatic synthesis of 3,4-dihydro-3-amino-7-hydroxycoumarin.

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Purification and properties of *N*-acetylneuraminate lyase from beef kidney cortex

The enzyme *N*-acetylneuraminate lyase (*N*-acetylneuraminate pyruvate-lysase, EC 4.1.3.3, also known as neuraminic acid aldolase) isolated from *Clostridium perfringens* was shown by GANTT *et al.*[1] to be selectively inhibited by the substrate analogue 3-fluorosialic acid. Incubation of the enzyme in the presence of this analogue resulted in an analogue concentration-dependent irreversible inactivation. This inactivation was prevented by increasing amounts of the substrate *N*-acetylneuraminic acid. This communication describes the preparation of *N*-acetylneuraminate lyase from a bovine source and its properties, particularly with respect to the effect of 3-fluorosialic acid.

The enzyme was assayed by following the liberation of either pyruvate or *N*-acetylmannosamine resulting from the cleavage of the substrate *N*-acetylneuraminic acid. Each assay mixture contained 0.10 ml of 1.0 M potassium phosphate (pH 7.2), 0.10 ml of 0.10 M *N*-acetylneuraminic acid, 0.050–0.50 ml of enzyme preparation, and distilled water to a total volume of 1.0 ml. After 15-min incubation at 37° the reaction was terminated by heating at 100° for 2 min. Any precipitated protein was removed by centrifugation. The *N*-acetylmannosamine liberated was determined by the modified Morgan–Elson reaction as described by BRUNETTE *et al.*[2]. This assay was used throughout the purification to follow the increase in specific activity of the preparation. Pyruvate formed was determined by the coupled reaction with NADH and lactate dehydrogenase as described by BRUNETTE *et al.*[3]. A unit of activity is defined as the μmoles of either *N*-acetylmannosamine or pyruvate liberated per 15 min at 37°. Protein was determined spectrophotometrically[4]. Specific activity of the enzyme preparation is defined as units/mg protein.

Beef kidneys were obtained fresh from a local slaughterhouse. Unless otherwise stated, all preparative procedures were carried out at 4°, and centrifugations at 14 000 × *g*. A total of 900 g of kidney cortex was homogenized in 1800 ml of distilled