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Note

Semi-micro quantitative assay for tryptophan oxygenase by high-performance liquid chromatography

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Tryptophan oxygenase (L-tryptophan oxygen-2,3-oxidoreductase, EC 1.13.11.11) has been studied in bacteria [1], invertebrates [2] and mammals [3]. Often it is desirable to examine the reactivity of this enzyme in crude extracts where the spectrophotometer assay procedure [4] is precluded by turbidity and the situation is further complicated by the presence of a formamidase that converts the product, N′-formylkynurenine, into kynurenine. Baglioni [2] adapted a method for measuring kynurenine by the diazotization procedure of Bratton and Marshall [5]. Thereafter, Kaufman [6] and Marzluf [7] improved this procedure by reducing the interference and unwanted side-reactions. Kaufman [6] and Mischke et al. [8] assayed this enzyme in crude extracts by employing paper or thin-layer chromatography, respectively. The kynurenine was eluted from the developed chromatogram and quantified on the basis of its absorption at 360 nm. In 1973, Casciano and Gaertner [9] developed another assay, which used two partially purified enzymes from *Neurospora crassa* to convert N′-formylkynurenine into anthranilate. The strong fluorescence of anthranilate allowed detection of low concentrations.

Certain eye color mutants of *Drosophila melanogaster* are unable to convert tryptophan into xanthommatin, the brown eye pigment. The enzyme lesion in the vermilion mutant is tryptophan oxygenase (less than 2% of wild type); the ennzyme activity is restored to higher levels (10–50%) when the genotype includes vermilion and the suppressor mutation $su(s)^2$ [10]. For the assay of the enzyme in *D. melanogaster* a reducing agent is required to stimulate activity (ascorbate [6] or 2-mercaptoethanol [7]); in some studies methemoglobin stimulated the

enzyme two-fold [11]. With regard to the time course of the enzymatic reaction, a 30-min lag in activity was observed in some cases [11], but in other cases no lag occurred [7,10].

The immediate product of tryptophan oxygenase is not kynurenine but N'-formylkynurenine, and the assay procedures listed above measure either kynurenine or anthranilate. For those that measure kynurenine, the assay depends on the presence of formylkynurenine formamidase to provide quantitative removal of the formyl group. Both Glassman [12] and Kimmel, Jr. [13] reported that this formamidase is very active in extracts of *D. melanogaster* and that this enzyme is not rate-limiting in kynurenine formation.

This note describes a simple and sensitive quantitative assay for tryptophan oxygenase that is based on reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and chemicals

L-Tryptophan, L-kynurenine, Trizma base and Sephadex G-25-120 were purchased from Sigma (St. Louis, MO, U.S.A.). N'-Formylkynurenine was obtained from Calbiochem (San Diego, CA, U.S.A.), 2-mercaptoethanol from Fischer Scientific (Pittsburgh, PA, U.S.A.) and guaranteed-grade methanol from Merck (Rahway, NJ, U.S.A.). Triply glass-distilled water was used for enzyme reactions and chromatography.

Enzyme source and extract

The enzyme extracts were prepared from the adult forms of wild-type strain Oregon-R of *D. melanogaster* and four mutants *bw, v; bw, su(s)^{2}v; bw* and *pr^{bw}cn* that are described in Lindsley and Grell [14]. The flies were grown in 180-ml glass bottles on a cornmeal–sucrose–agar medium seeded with yeast and acidified with propionic acid. The adult flies were collected at two-day intervals and then stored at −60°C.

The extracts were prepared in a ground-glass tube and pestle at 0–4°C by homogenization of the flies with 10 m*M* Tris–HCl (pH 7.5), using 4 ml per gram of flies, and subsequent centrifugation at 15 000 *g* for 30 min. The protein in the supernatant that precipitated between 40 and 65% saturated ammonium sulfate (4°C) was collected at 20 000 *g* for 30 min and dissolved in 1 ml of buffer per gram of flies. The enzyme solution was passed through Sephadex G-25-120 using the centrifuge procedure of Neal and Florini [15] to desalt the protein solution and to remove several substances that elute near kynurenine on the C_{18} column. The enzyme assays described have employed this desalted and depigmented enzyme preparation.

Enzyme assay conditions

The conditions for the enzyme assay are a modification of the methods of Baglioni [2] and Jacobson et al. [10] for tryptophan oxygenase. The reaction mixture contained 80 m*M* Tris–HCl (pH 7.5), 0.4 m*M* 2-mercaptoethanol, 10 m*M*

L-tryptophan and up to 160 μl of enzyme extract in a total volume of 250 μl. After incubation at 37°C, the sample was heated at 100°C for 5 min and centrifuged (Eppendorf) for 2 min. This supernatant was analyzed by HPLC.

Apparatus and HPLC conditions

Analysis of kynurenine and N′-formylkynurenine was performed by injection of the supernatant obtained from the assay procedure (50 μl) onto a μBondapak C_{18} column (30×0.4 cm I.D., 10-μm particles) protected by a 5×0.2 cm I.D. guard column. Isocratic elution was carried out with 8% methanol–1 m*M* potassium phosphate (pH 4.0) at a flow-rate of 1.5 ml/min. The chromatography was performed with a Waters (Milford, MA, U.S.A.) 6000A pump and a U6K injector. A Schoeffel (Westwood, NJ, U.S.A.) 770 UV monitor was used at 365 nm and a sensitivity of 0.1 a.u.f.s., in conjunction with a linear recorder at 1 mV and a chart speed of 10 or 20 cm/h. The peak height was used for quantitative measurements of kynurenine.

RESULTS

The elution position of kynurenine varied with the methanol concentration as follows: at 10% methanol it eluted in 2.86 ml, at 8% in 3.27 ml and at 6% in 3.55 ml. The addition of 1 m*M* potassium dihydrogenphosphate (pH 4.0) to 8% methanol improved the resolution of kynurenine from other substances that are present after the enzyme assay, and the elution volume was 3.84 ml.

The absorbance of kynurenine is greater at 365 nm than at 280 nm. Fig. 1 shows the chromatogram of the three compounds, kynurenine, N′-formylkynurenine, and tryptophan, as detected at 280 nm or 365 nm. All three are well resolved; at 365 nm the presence of tryptophan is indicated by a negative deflection.

When the kynurenine concentration was varied, the 365 nm peak height varied linearly over the concentration range 0.02–0.25 m*M* when 2-μl samples were injected. When samples of kynurenine from each concentration were determined in triplicate, the standard deviation was less than 5% (data not shown).

The identification of kynurenine as the product was accomplished by demonstrating that the enzymatically produced substance eluted in the same volume as the standard (Fig. 2). When the enzyme product was co-chromatographed with the kynurenine standard, a single peak was obtained that was the summation of the two individual peaks (Fig. 2). In addition, we demonstrated that kynurenine was produced when N′-formylkynurenine was incubated in the enzyme assay from which tryptophan was omitted (data not shown). When N′,Nα-diformylkynurenine [16] was incubated in the enzyme assay, no kynurenine was produced. Finally, we have purified tryptophan oxygenase and the formylkynurenine formamidase so that each is free of the other, and have shown that a peak that corresponds to N′-formylkynurenine accumulates when tryptophan oxygenase is free of the formamidase and that this peak disappears and one that corresponds to kynurenine appears when the two enzymes are incubated together with tryptophan (data not shown). These observations indicate that the product seen in Fig. 2 is kynurenine.

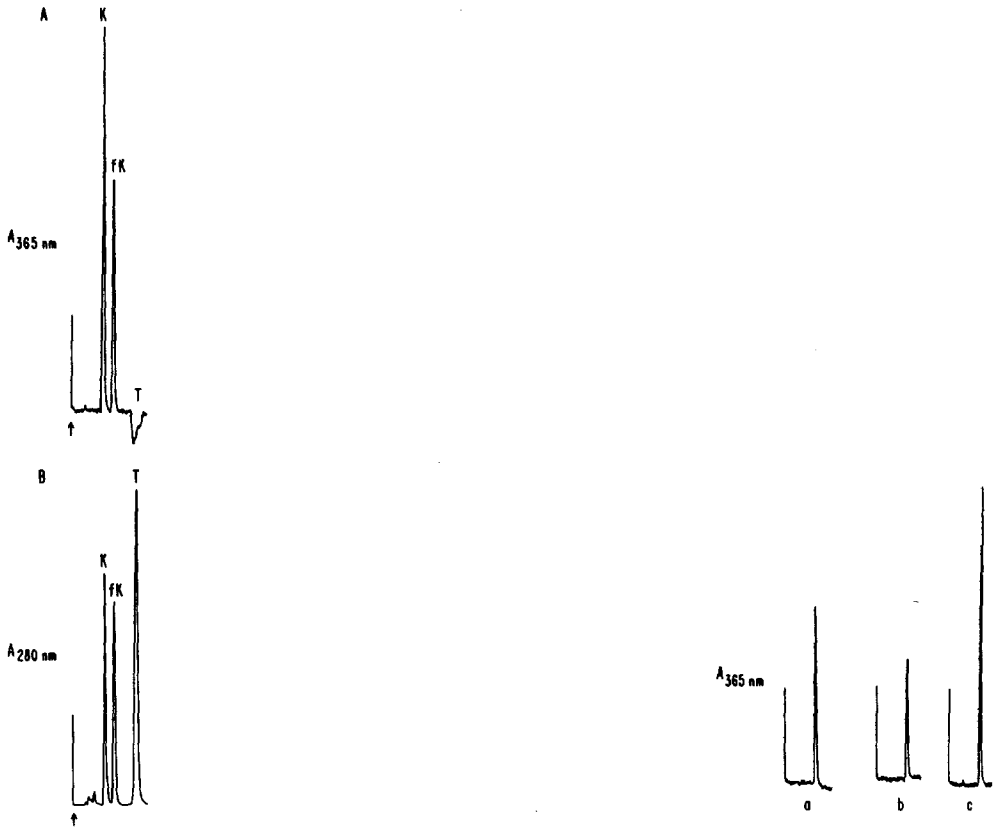
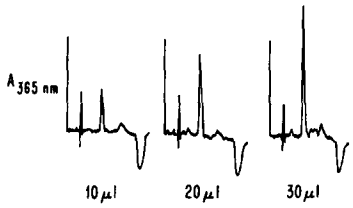


Fig. 1. Resolution of kynurenine (K), N′-formylkynurenine (fK) and tryptophan (T). The absorption was determined at 365 nm (A) or 280 nm (B). The left-most vertical line in the chromatogram, indicated by an arrow, represents the time of sample injection. The C_{18} reversed-phase column was eluted isocratically with 8% methanol–1 m*M* phosphate (pH 4.0), as described in Experimental.

Fig 2. Co-injection of kynurenine with the product of the tryptophan oxygenase–formamidase coupled enzyme system. The samples injected onto the column were as follows: (a) kynurenine; (b) product obtained by incubating tryptophan under standard assay conditions with the depigmented 40–65% saturated ammonium sulfate fraction; (c) samples a and b combined.

A number of substances that absorb at 365 nm are present in the crude enzyme extract but most are adsorbed on Sephadex G-25. As shown in Fig. 3, only a minor amount of 365 nm-absorbing material is detected in the chromatogram of a sample from the enzyme assay that elutes just prior to kynurenine. A small broad peak that appears between kynurenine and tryptophan, was shown to emerge after formylkynurenine (data not shown). Fig. 3 also demonstrates that the peak height of kynurenine increases linearly with the concentration of the enzyme contained in the 40–65% saturated ammonium sulfate fraction. This linear relationship was shown over the range 10–160 μl of the enzyme (data not shown). The absence of formylkynurenine in Fig. 3 demonstrates again that the formamidase is present in excess and converts all of the immediate product of tryptophan oxygenase into kynurenine, which is stable and accumulates.



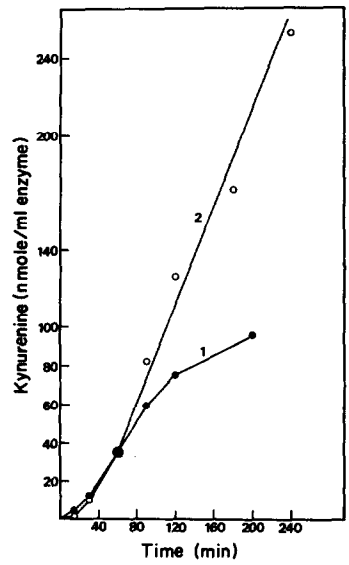


Fig. 3. Kynurenine produced at various enzyme concentrations. The standard assay conditions were used with 10, 20 or 30 μl of the enzyme fraction.

Fig. 4. Time course of the tryptophan oxygenase reactions. Either the crude extract (curve 1) or the 40–65% saturated ammonium sulfate fraction (curve 2) was desalted and depigmented by Sephadex G-25 and used in the standard assays. Samples were removed at various times, and kynurenine was determined by HPLC.

This chromatographic analysis was used to examine the time course of the reaction, and the amount of tryptophan oxygenase in known mutants was determined. The time course of the rate of kynurenine formation is shown in Fig. 4 for the crude extract (curve 1) and for the fraction obtained at 40–65% saturated ammonium sulfate. In both cases a lag occurred during the first 30 min. The crude extract did not maintain a linear rate for more than 1 h, but the ammonium sulfate fraction catalyzed the reaction at a linear rate for over 3 h after the lag. The demonstration of a linear relation between kynurenine production and enzyme concentration was based on a 0–100 min time interval.

Different mutants of *Drosophila* are known that have reduced concentration of tryptophan oxygenase. Flies that contained vermilion (*v*) were shown earlier to contain little or no activity, but when a suppressor mutation is present along with vermilion [$su(s)^2v$] the activity was restored to 10–50% of normal levels according to the age of the adult fly [10]. The mutations brown (*bw*) and purple (pr^{bw}) and cinnabar (*cn*) affect other metabolic pathways and were not expected to have an effect on tryptophan oxygenase. These genotypes were assayed, and the data in Table I show that the results are in agreement with the results reported in earlier studies [6,7,10,17]. Furthermore, the range of values obtained for several assays in reasonably small except for the assay of $pr^{bw}cn$.

Marzluf [7] earlier, and Tartof [17], reported that tryptophan oxygenase was partially purified by fractional precipitation between 43 and 57% saturated ammonium sulfate. Table II shows that the specific activity of 40–60% fraction

TABLE I *

TRYPTOPHAN OXYGENASE ACTIVITY IN VARIOUS STRAINS OF *DROSOPHILA*

Genotype	*n*	Tryptophan oxygenase (nmol kynurenine per 100 mg fresh weight per 3 h)		Percentage of wild type
		Mean	Range	
Oregon-R	5	62.6	56.7 -67.0	100
v; bw	4	0.07	0.02- 0.15	1
su(s)²v; bw	4	7.55	2.8 -10.7	12
bw	4	55.2	50.9 -58.4	88
pr^bw cn	3	74.7	62.2 -97.0	119

was increased ca. three-fold in specific activity compared with the crude extract. When the enzyme extract is centrifuged in a table-top Eppendorf centrifuge for 3 min, the extract is comparable with that obtained from a normal procedure (20 000 *g* for 30 min). These experiments demonstrate that the present assay gives results that are compatible with those from earlier studies based on genetic variation and on enzyme fractionation. This assay was employed during the purification of *Drosophila* tryptophan oxygenase to homogeneity and proved to be reliable [19].

DISCUSSION

Several procedures have been reported for the measurement of tryptophan oxygenase activity. The spectrophotometric detection of kynurenine at 365 nm [4] is subject to interference by turbidity; other compounds, such as pterins in *Drosophila*, also absorb at this wavelength.

The Bratton–Marshall method used by Baglioni [2] requires meticulous attention to the timing of the two steps needed to produce the chromophore. Tryptophan and ascorbate also give rise to colored products that interfere.

The method of Casciano and Gaertner [9] is based on the quantitative conversion of N′-formylkynurenine into anthranilate, a highly fluorescent compound that can be detected very sensitively. This requires two partially purified enzymes from *Neurospora crassa*, formylkynurenine formamidase and kynuren-

TABLE II

COMPARISON OF SPECIFIC ACTIVITIES OF TRYPTOPHAN OXYGENASE PREPARED BY DIFFERENT METHODS

Protein concentrations were determined by the method of Lowry et al. [18].

Method	Specific activity (nmol kynurenine per mg protein per
Eppendorf centrifugation*	3.98
Standard centrifugation (20 000 *g* for 30 min)**	4.13
Ammonium sulfate fraction (40–60%)	12.43

*The homogenate was centrifuged in Eppendorf centrifuge at top speed for 3 min and the supernatant served as the enzyme preparation.

**This enzyme extract was prepared as described in Experimental.

inase. This method not only avoids interference by other substances but is more sensitive than other previous methods. However, it is time-consuming and requires several manipulations, as well as the growth of *N. crassa* and the preparation of the two enzymes. Furthermore, substances that interfere with the detection of fluorescence of anthranilate can be a problem.

The assay method described here can eliminate the above problems. There is no coupling reaction needed to detect kynurenine or N′-formylkynurenine since the procedure is based on the direct determination of the product. By using reversed-phase chromatography, kynurenine can be separated from other endogenous compounds simply and conveniently. Using the present method sufficient kynurenine is produced for accurate determination by 20 μl of the enzyme preparation, an amount that is equivalent to 5 mg of flies. Assuming that a fly weighs 1 mg, only a few flies are required to provide sufficient enzyme for accurate determination.

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