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Note

Rapid and sensitive thin-layer chromatographic assay procedure for measuring xanthine dehydrogenase activity from tissue extracts

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There are a variety of assay procedures available for measuring xanthine dehydrogenase (XDH) activity from tissue extracts [1—8]. Several of the more sensitive assays involve fluorometric detection of the conversion of pterin (2-amino-4-hydroxypteridine, AHP) to isoxanthopterin (IXP), which is one of the reactions catalyzed by XDH [1, 2]. That fluorometry was a sensitive way of measuring XDH activity was first suggested by Burch et al. [3] and subsequently applied to the analysis of XDH-deficient mutants in *Drosophila melanogaster* by Glassman and Mitchell [4], Chovnick et al. [5], Sayles et al. [6], Browder and Williamson [7], and others. More recently, we have become interested in assaying XDH activity in tissues of pigment mutants of the Mexican axolotl (*Ambystoma mexicanum*) that were suspected to be deficient in XDH activity [9].

Logically, amphibian skin, which is rich in pteridines that function as xanthophore pigments [10, 11], should contain significant amounts of XDH activity. However, amphibian skin, unlike other tissues such as liver and kidney that are rich sources of XDH activity [2], has been difficult to assay for this enzyme [12, 13], possibly because XDH activity might only be present in xanthophores and because xanthophores comprise a relatively small proportion of the total cells found in skin. Previous attempts at measurement suggest that there is either no activity [13] or that activity levels in skin are unpredictable and perhaps unreasonably low [12]. The following assay procedure represents a significant modification of the assay described by Browder and Williamson [7] and has been used successfully for comparative measurements of XDH activity from a variety of tissues (skin included) in mutant and wild-type axolotls.

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EXPERIMENTAL

Wild-type axolotls were obtained from the Indiana University Axolotl Colony (Bloomington, IN, U.S.A.). They were kept in 20-cm culture bowls containing 50% Holtfreter's solution [14], which was replaced every other day. Animals were fed daily; young axolotls were fed live brine shrimp, while older axolotls ate beef liver [15]. All chemicals used in these experiments were purchased from Sigma (St. Louis, MO, U.S.A.), unless otherwise specified.

Six animals, 20—27 weeks old, were used for the XDH assay. To obtain fresh tissue, animals were sacrificed by overdosing with MS-222 (ethyl *m*-aminobenzoate, methanesulfonic acid salt) and the liver and skin were dissected. The entire liver and 0.4—0.5 g of the skin (cut in small pieces) were each homogenized in a volume of buffer (0.05 *M* Tris—HCl; 0.001 *M* EDTA; pH 8.0) equivalent to 5 ml/g wet weight of tissue. The homogenization buffer also contained 0.75% (w/v) Norit A (Nutritional Biochemicals, Cleveland, OH, U.S.A.) to adsorb endogenous pteridines, and 0.086 ml/g (original tissue weight) Aprotinin to inhibit endogenous protease inactivation of XDH. The resulting homogenates were allowed to sit on ice for 10 min to facilitate pteridine adsorption [7]. They were then centrifuged at 30 900 *g* in a Beckman J21 centrifuge equipped with a JA20 rotor for 30 min at 4°C. Supernatants were drawn off and saved, and pellets were discarded.

The skin protein sample was further concentrated by centrifugation twice at 2000 *g* and 15—20°C in Centricon 30 microconcentrator tubes (Amicon, Danvers, MA, U.S.A.). The first centrifugation was for 25 min, after which the microconcentrator filter was rinsed by gently pipetting the retentate against the filter. The sample was then centrifuged for an additional 20 min at the same settings, after which the microconcentrator tube was inverted and the retentate was recovered by centrifugation for 5 min at 700 *g* and 15—20°C.

The liver and skin protein samples (0.3 ml) were combined with 0.9 ml of pterin reaction mixture ($6 \cdot 10^{-5}$ *M* AHP, 0.02% NAD in 0.1 *M* Tris—HCl, pH 8.0 [7]), placed in 10 × 75 mm culture tubes, and incubated in the dark for 2 h at room temperature. A portion of the remaining protein sample was retained for protein assay. After 2 h, the reaction was terminated by adding 0.3 ml of 100% ethanol to each culture tube and vortexing. The above mixtures were then centrifuged in microconcentrator tubes for 40 min at 2000 *g* and 15—20°C to separate pteridines from proteins and other high-molecular-weight substances. The filtrates, containing pteridines, were then stored in darkness at 4°C until application to thin-layer chromatography (TLC) plates.

Protein concentration of the samples was determined using the Bradford assay [16], with bovine serum albumin as the standard. The volume of pteridine filtrate spotted on TLC plates was adjusted for each tissue so that each sample was roughly comparable in protein quantity. The amount of IXP produced was thus directly comparable to the XDH activity present in the various tissues.

TLC was performed on combination plates of silica gel (Type G) and cellulose (Sigmacell, Type 100) as described by Frost and Bagnara [17]. Samples were applied to the plates with a Hamilton syringe and plates were run 10 cm in one dimension under a sodium vapor safelight. The solvent used

was *n*-propanol—7% ammonia (2:1). Chromatograms were viewed under UV light (360 nm), and some were photographed (Kodak Panatomic-X) using a Kodak dark blue filter over the UV light source and a 2B Wratten filter over the camera lens. TLC plates were scanned in the dark in a Turner double-beam transmittance fluorometer (Model 111) equipped with a TLC scanner and connected to a Fisher Recordall (Series 5000 recorder). Because some pteridines are photolabile [18], TLC plates were always scanned within 2 h of the completion of development. Scans of the TLC plates produced peaks representing each of the two pteridines (AHP and IXP). The identity of IXP and AHP has been verified by co-chromatography with authentic compounds (purchased from Sigma) and by UV absorption spectra obtained for each compound in question as described in previous publications [15, 17].

The amount of IXP formed during the 2-h incubation period was used as a measure of XDH activity. To be able to quantify the amount of IXP present in peaks obtained from fluorometric scans, standards (20 μl) of five different concentrations of IXP ($1 \cdot 10^{-9}$, $8 \cdot 10^{-10}$, $6 \cdot 10^{-10}$, $4 \cdot 10^{-10}$, and $2 \cdot 10^{-10}$ mol per 20 μl) were applied to TLC plates and run as described before. The weight of the IXP peaks (obtained by the cut and weigh method) was used to determine the amount of IXP present. A regression line was obtained by plotting the logarithm of the number of moles of IXP versus the logarithm of the weight of the peak produced by scanning. The r^2 of the regression line obtained was calculated to be 92%. Thus, the relationship between the number of moles of IXP and the weight of the peak produced as expressed by the regression equation was considered to be a valid way to predict the amount of IXP present from the weight of the peaks obtained.

To attempt to increase the accuracy of the data, identical amounts of liver and skin samples from each animal were run twice on different TLC plates, and the average weight of the IXP peaks was used to calculate XDH activity.

The computations were made in a Honeywell 66 DPS-3E computer, using the Minitab software package (Statistics Department, Pennsylvania State University, release 82.1).

RESULTS

The formation of reaction product is time-dependent and the kinetics of the reaction are shown in Fig. 1. The reaction reaches saturation within the first hour of incubation. Thus, a 2-h incubation period ensures that the reaction is complete. In no case was substrate the limiting factor as some AHP was always detectable on chromatograms.

The results of a typical TLC run are shown in Fig. 2A. Each reaction mixture produces two fluorescent peaks. The faster-migrating, blue-fluorescing peak is the substrate, pterin (comigrates with the pterin standard), and the slower-migrating, purple-fluorescing peak is the reaction product, isoxanthopterin (comigrates with its standard). The plate is then scanned in the fluorometer and two clearly separable peaks are obtained (Fig. 1B). The isoxanthopterin peak is cut out, weighed, and the weight is used to calculate the amount of IXP produced as follows: log (mol IXP) = [log (peak weight) − 6.82]/0.891. This equation was obtained by linearly regressing the results of known quantities of IXP (see Experimental) over the range 10^{-9}—$8 \cdot 10^{-10}$ mol IXP.

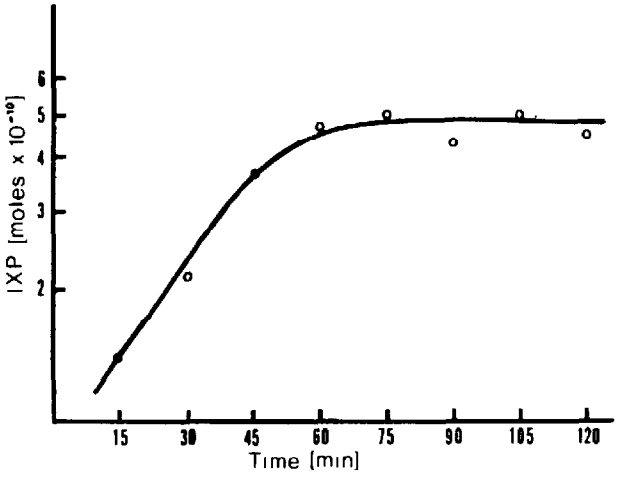

Fig. 1. Kinetics of XDH-catalyzed production of isoxanthopterin. Note that by 60 min the reaction had reached saturation (plateau) levels.

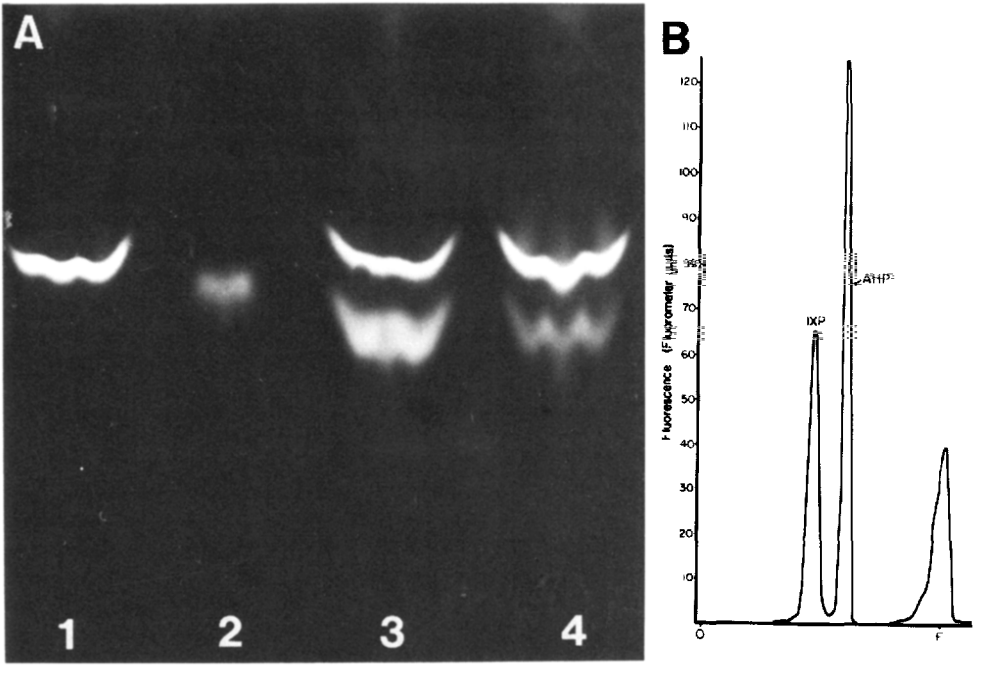

Fig. 2. (A) Results of TLC analysis of pterin standard (substrate, column 1), isoxanthopterin standard (product, column 2), wild-type axolotl liver extract (column 3) and wild-type axolotl skin extract (column 4), viewed and photographed under UV light. (B) Fluorometric scan of wild-type liver extract (from A, column 3). IXP = isoxanthopterin; AHP = pterin, O = origin; F = solvent front.

The results of assays performed on liver and skin extracts from six different axolotls are presented in Table I. These data were pooled and averaged and are summarized in Fig. 3. Units of enzyme activity are thus equivalent to 10^{-10} mol IXP produced per 2-h incubation period.

TABLE I

AMOUNT OF IXP PRODUCED DURING THE 2-h INCUBATION PERIOD FROM TISSUE SAMPLES OF EACH OF THE SIX ANIMALS

Animal	Age (weeks)	IXP produced ($\times 10^{-10}$ mol)					
		Liver			Skin		
		Run 1	Run 2	Mean*	Run 1	Run 2	Mean*
1	20	5.98	13.57	9.78	2.75	4.49	3.69
2	20	8.55	12.11	10.31	4.01	3.06	3.53
3	21	13.02	10.67	11.93	2.75	2.29	2.44
4	26	9.07	11.39	10.13	4.33	5.15	4.66
5	26	15.23	18.61	16.91	4.66	4.17	4.33
6	27	11.57	9.25	10.49	8.03	7.68	7.85
Mean		10.57	12.60	11.59	4.42	4.47	4.42

*Mean values were calculated from averaged peak weight values, not from the values of IXP production listed here.

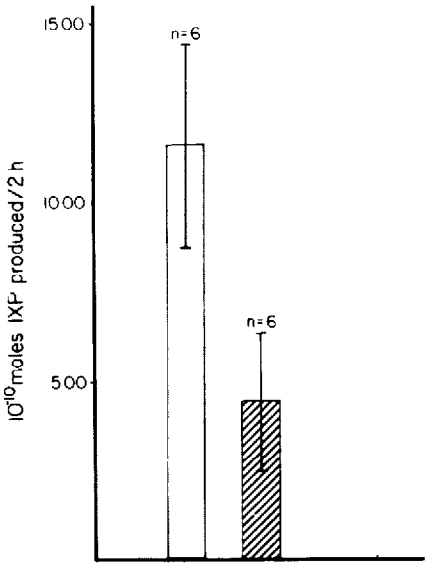

Fig. 3. Summary of the data from Table I. Values from liver extracts (shaded) and skin extracts (cross-hatched) were averaged. Bars represent 95% confidence intervals (mean ± S.D.). Enzyme activity is expressed as 10^{-10} mol IXP produced per 2 h.

Clearly, there is significant enzyme activity in both the liver and skin. In general XDH activity is greater in the liver (x = 11.59 U) than in skin (x = 4.42 U). However, it is notable that there is *always* detectable activity in *both* tissues.

DISCUSSION

None of the standard spectrophotometric or fluorometric assays for XDH

activity [1–8] were suitable for reliably measuring enzyme activity from axolotl skin extracts. Consequently, we developed the assay described herein and have used it to measure XDH activity in tissues of axolotls of different ages, sizes and phenotypes (unpublished results). This assay is sensitive enough that individual animals can be assayed, and even axolotls only a few centimeters in length will yield enough material to allow determination of enzyme activity from at least the liver and skin.

The major features of the assay procedure described herein that differ significantly from other published assays include:

(1) Addition of Aprotinin to the homogenization buffer. Without Aprotinin the results obtained were often inconsistent and highly variable from sample to sample even among animals of the same age and phenotype.

(2) The use of Centricon 30 microconcentrator tubes both during the preparation of enzyme extracts and subsequently in recovery of the reaction product. The initial microconcentration step is not necessary for liver samples. However, it is essential if enzyme activity is to be detected in the skin. Microconcentration of skin samples effectively removes all low-molecular-weight proteins (< 30 000 daltons) and a substantial amount of the buffer as well. The retentate is thus enriched for high-molecular-weight proteins, among which is XDH. Microconcentrator tubes are also used (for both liver and skin samples) following the incubation of the tissue extracts with appropriate substrate (AHP). In this case the enzyme and other proteins remain as retentate and the substrate and reaction product (IXP) pass through the filter (collected as filtrate). Thus, microconcentration facilitates spotting samples on TLC plates, because most of the proteins that can interfere with TLC separation remain in the retentate. In several cases, the retentates were checked for the presence of either substrate or reaction product (by TLC) to ensure that the filtrate indeed accounted for 100% product recovery. In no case was IXP detected in the retentate; however, some substrate (AHP) was observed in the retentate and may have been protein-bound.

(3) Browder and Williamson [7] were able to assay individual fly larvae by a similar TLC procedure. However, they did not quantitate their results, but relied on visualization of reaction product (IXP) for determining whether there was more or less enzyme activity in any individual animal. We quantify results by using a fluorometer equipped with a TLC scanner and chart recorder. Enzyme activity is thus a direct function of the moles of IXP produced during the 2-h incubation period. The regression equation obtained from measurements of known quantities of IXP provided a confident (95%) means of determining enzyme activity.

(4) To illustrate the general reliability of this assay, we subjected each sample to more than one (usually only two) TLC run. The data in Table I show that, in general, there is very little difference between one run and another in terms of enzyme activity measurements. The differences that are observed are probably due primarily to the differences in the surfaces of the TLC plates used. Once the reaction has been terminated, the reaction product is relatively stable and can be frozen and stored in the dark indefinitely. TLC plates are scanned as soon as possible after completion of the run because the intensity of the spots fades with time.

In conclusion, the procedure described herein is simple and sensitive. The only special equipment needs are a fluorometer equipped with TLC scanner. Use of Centricon tubes adds expense to the procedure (ca. US$ 2.30 per tube), but the sensitivity and reproducibility gained seem well worth the investment. At the present time we are investigating whether Centricon tubes can be washed and recycled or whether we can fashion a similar version of the Centricon tube which will work as effectively but cost less.

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