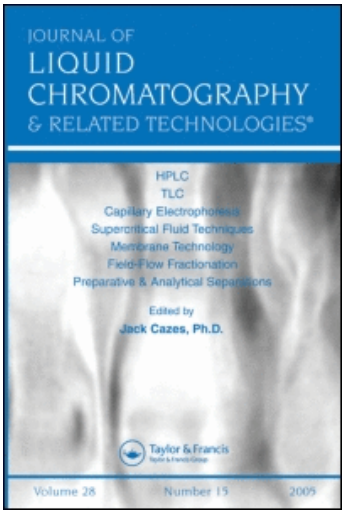

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Identification of 3-Hydroxykynurenine and Xanthurenic Acid and Quantitation of 3-Hydroxykynurenine Transaminase Activity Using HPLC with Electrochemical Detection

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IDENTIFICATION OF 3-HYDROXY-KYNURENINE AND XANTHURENIC ACID AND QUANTITATION OF 3-HYDROXYKYNURENINE TRANSAMINASE ACTIVITY USING HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Identification of 3-hydroxykynurenine and xanthurenic acid and determination of 3-hydroxykynurenine transaminase activity using HPLC with electrochemical detection (HPLC-ED) are described using mosquito larval samples as testing materials. Results indicate that 3-hydroxykynurenine and xanthurenic acid in crude biological samples can be identified based on their electrochemical and chromatographic behaviors during HPLC-ED analysis. Xanthurenic acid is produced through transamination of 3-hydroxykynurenine, and identification of xanthurenic acid in the mosquito larval extract also leads to the detection of 3-hydroxykynurenine transaminase activity in this insect species.

Using the same HPLC-ED technique, a method to detect and quantitate 3-hydroxykynurenine transaminase activity is developed using isolated mosquito larval protein as enzyme source, and the applicability of the HPLC-ED transaminase assay for samples from other resources is demonstrated using supernatant of mouse liver and kidney homogenates.

INTRODUCTION

A major electrochemically active compound was detected from mosquito eggs using HPLC with electrochemical detection (HPLC-ED) in our previous study,[1] and recently the same electrochemically active compound also was found to accumulate in the mosquito larvae. After an extensive analysis of mosquito larval extract using HPLC-ED under different conditions, the major electrochemically active compound from mosquito larvae was identified as xanthurenic acid (XA). XA is a metabolite in the tryptophan oxidation pathway, and 3-hydroxykynurenine (HKN) is the direct precursor for XA production. Identification of XA also resulted in the detection of HKN from mosquito larval extract. The detailed biochemical pathway from HKN to XA involves an initial transamination of HKN to a side chain ketoacid intermediate and subsequent intramolecular cyclization of the intermediate to XA. The finding that accumulation of XA occurs in mosquitoes during larval development also lead to the detection of high HKN transaminase activity in the isolated mosquito larval protein using HPLC-ED analysis.

Our experience with the identification of XA and HKN using HPLC-ED suggests that their oxidation profiles (or hydrodynamic voltammograms) and chromatographic behavior toward ion pairing reagent can aid greatly to the identification of HKN and XA in crude biological samples. Although there were described methods for the detection of XA and other related compounds using liquid chromatographic techniques,[2-4] the chromatographic behavior and the electrochemical characteristics of XA and HKN, which is critical for their identification, have not been discussed.

In this report, we provide data regarding the identification of XA and HKN in crude biological samples based on their oxidation profiles and their coelution with authentic standards at different chromatographic conditions using HPLC-ED with mosquito larval extract as testing sample, the determination of HKN transaminase activity of mosquito larval protein by the same HPLC-ED technique, and the applicability of the HPLC-ED transaminase assay for samples from other resources using supernatant of mouse kidney and liver homogenates as testing materials.

MATERIALS AND METHODS

Chemicals

N-acetyldopamine, 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris), ammonium sulfate, 3,4-dihydroxyphenylacetic acid, dopa, dopamine, epinephrine, glutathione, 3-hydroxykynurenine (HKN), 2-mercaptoethanol, norepinephrine, octopamine, pyridoxal-5-phosphate (PLP), serotonin, sodium phosphate, tryptophan, tyrosine, tyramine, and xanthurenic acid (XA) were from Sigma (St. Louis, MO).

Animals

Aedes aegypti mosquitoes used in this study were reared according to a described method.[5] *Drosophila melanogaster* (wild type) was obtained from North Carolina Biological Supply Company (Burlington, NC). Balb/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN).

Preparation of Mosquito and *Drosophila* Larval Extracts

Five-day-old mosquito larvae or 3- to 5-day-old *Drosophila* larvae were collected and lyophilized. The lyophilized mosquito or *Drosophila* larvae were ground to powder, and then the larval powder was treated with 0.4 M formic acid to extract the electrochemically active compounds. Briefly, dry mosquito or *Drosophila* larval powder (5.0 mg) was mixed with 1.0 mL of 0.4 M formic acid. The mixture was sonicated for 2.0 min, stored in a 4°C refrigerator for 60 min and then centrifuged at 20,000 g for 20 min at 4°C. The supernatant, thus obtained, was analyzed using HPLC-ED.

Separation and Identification of HKN, XA and Other Electrochemically Active Compounds in the Mosquito and *Drosophila* Larval Extracts Using HPLC-ED

The HPLC-ED apparatus consisted of a Gilson 302 pump, a Rheodyne (Cotati, CA) Model 7125 injector fitted with a 20-μL sample loop, and a Bioanalytical System (West Lafayette, IN) Model LC-4C amperometric detector fitted with a glassy carbon working electrode. A 5 μm spherical C_{18} reverse-phase column (4.6 x 150 mm) from Alltech (Deerfield, IL) was used for the separation of larval extracts.

The mobile phase consisted of 0.1 M citrate buffer (pH 3.0) containing 8% acetonitrile and 0.4 mM or 1.8 mM octyl sulfate. A flow rate of 0.5 mL min^{-1} was used during chromatography.

Identification of HKN, XA and other electrochemically active compound in the larval extract was based on both their oxidation profiles, obtained by hydrodynamic voltammetric analysis of the larval extract, and their retention times during HPLC-ED analysis at different conditions. During the hydrodynamic voltammetric analysis using HPLC-ED, the amount of sample injected and the sensitivity of the detector remained constant, while the potential of the working electrode was adjusted at each 25 or 50 mV interval from 500 - 875 mV.

HKN Transaminase Assay

Mosquito larval protein, used for HKN transaminase activity assay, was isolated by ammonium sulfate precipitation. Five-day-old mosquito larvae were collected and then homogenized in 20 mM phosphate buffer (pH 7.0) containing 30% saturated ammonium sulfate, 0.1 mM PLP and 10 mM mercaptoethanol. Supernatant was obtained by centrifugation of the homogenate at 15,000 g for 20 min at 4°C, and then the supernatant was brought to 60% saturation of ammonium sulfate. The 30 - 60% ammonium sulfate precipitated protein was collected by centrifugation, redissolved in a minimum volume of 20 mM tris buffer (pH 8.0) containing 10 mM mercaptoethanol, and dialyzed against the same buffer. Protein precipitated during dialysis was eliminated by centrifugation (15,000 g for 10 min at 4°C). Protein in the supernatant was determined by Lowry,[6] adjusted to 2.0 mg mL^{-1} using 100 mM tris buffer (pH 8.0), and then used as enzyme source for HKN transaminase activity assay.

The assay was initiated by mixing 0.4 mg larval protein in 0.2 mL tris buffer into a 0.8 mL of freshly prepared substrate preparation containing 3.75 mM HKN, 6,25 mM pyruvate and 0.63 mM PLP in 100 mM tris buffer (pH 8.0), which gave the final concentrations of 3.0 mM, 5.0 mM, and 0.05 mM for HKN, pyruvate, and PLP, respectively. The reaction mixture was incubated at 37°C, and the reaction was stopped by withdrawing 0.1 mL of the reaction mixture and mixing with an equal volume of 0.4 M formic acid. Supernatant was obtained by centrifugation of the formic acid treated reaction mixture at 20,000 g for 10 min at 4°C, and XA in the supernatant was determined using HPLC-ED with an applied potential of 800 mV. Separation of XA and substrates was achieved by reverse-phase chromatography on a 4.6 x 50 mm 3 μm spherical C_{18} column (Alltech, Deerfield, IL). A mobile phase consisting of

0.1 M citrate buffer (pH 3.0) containing 7% acetonitrile with a flow rate of 0.6 mL min^{-1} was used during the reverse-phase separation. Quantitation of XA formed in the reaction mixture was based on a standard curve generated by injecting increasing amounts of XA standard and measuring peak heights. Reaction mixtures containing heat-inactivated larval protein (2.0 min at 100°C) served as control. Reaction mixtures without either pyruvate or PLP were assayed in the same manner to determine their effects on the HKN transaminase activity of the mosquito larval protein. For some single time point assays, the volume of the reaction mixture was reduced to 0.1 mL.

Once the HKN transaminase activity assay was established using isolated larval protein, suitability of this HKN transaminase assay toward crude samples was determined using lyophilized larval powder. Typically, 2.0 mg of mosquito larval powder were incorporated into 1.0 mL of fresh substrate preparation containing 3.0 mM HKN, 5 mM pyruvate, and 0.05 mM PLP in 100 mM tris buffer (pH 8.0), and then the reaction mixture was incubated at 37°C. Termination of the enzymatic reaction and quantitation of XA produced in the reaction mixture were the same as those described for mosquito larval protein. An equivalent amount of larval powder mixed directly into 0.4 M formic acid or reaction mixture containing heat-inactivated larval powder served as controls to subtract endogenous XA in the powder and XA formed nonenzymatically.

HKN transaminase activity of the supernatant of mouse kidney or liver homogenate was assayed in the same manner as those described for mosquito larval protein. Mice were killed by cervical dislocation (consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association), and the kidney and liver were removed immediately, washed with ice-cold distilled water, and homogenized in 50 mM tris buffer (pH 8.0) containing 10 mM mercaptoethanol. Supernatant of kidney or liver homogenates, obtained by centrifugation of the homogenates at 20,000 g for 20 min at 4°C, was used directly as enzyme source for HKN transaminase activity assay.

RESULTS

Analysis of mosquito larval extract using HPLC-ED at 850 mV of the working electrode (versus Ag/AgCl reference electrode) resulted in the detection of two major and one minor electrochemically active compounds with retention times of 4.8 (peak 1), 6.2 (peak 3), and 5.2 min (peak 2), respectively (Fig. 1A). The two front peaks in the chromatogram (see Fig. 1A) also were observed when 0.4 mM formic acid, used to extract the electrochemically active

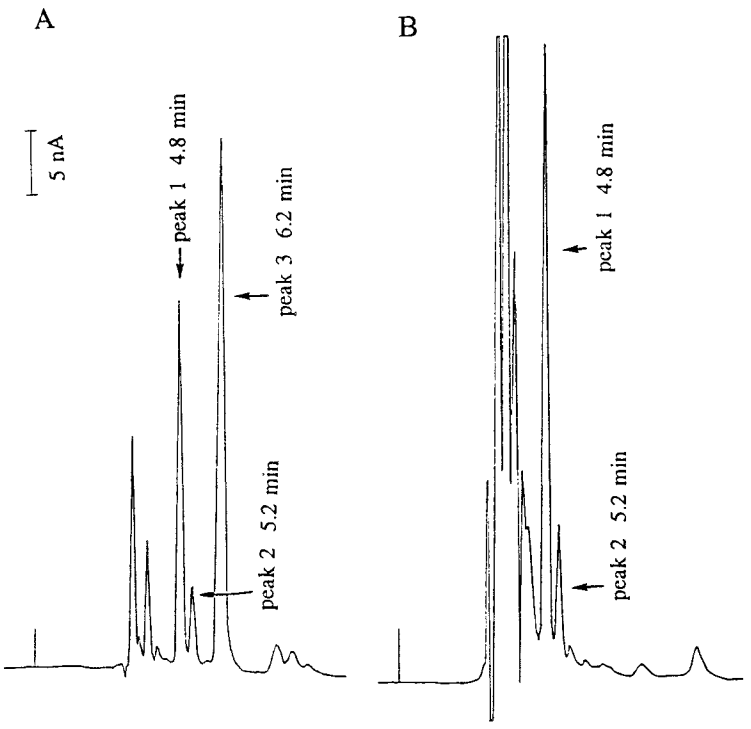

Figure 1. Separation and detection of the electrochemically active compounds in mosquito and *Drosophila* larval extracts using HPLC-ED. Chromatograms show the electrochemically active compounds detected in mosquito larval extract (A) and *Drosophila* larval extract (B), respectively.

compounds, was injected for HPLC-ED analysis. Therefore, no effort was made to analyze those two peaks. When larval powder extract from *Drosophila melanogaster* (wild type) was analyzed using HPLC-ED at the same conditions, both peak 1 and peak 2 were detected, but peak 3 was barely observed (Fig. 1B).

Tyrosine, dopa, dopamine, *N*-acetyldopamine, *N*-acetylnorepinephrine, glutathione, HKN, serotonin and tryptophan are the commonly detected electrochemically active compounds in insects, while low concentrations of epinephrine, tyramine, norepinephrine, octopamine and XA also are likely present in insects. Analysis of the above standards by HPLC-ED at the same conditions showed that tyrosine had the same retention time as peak 1 and

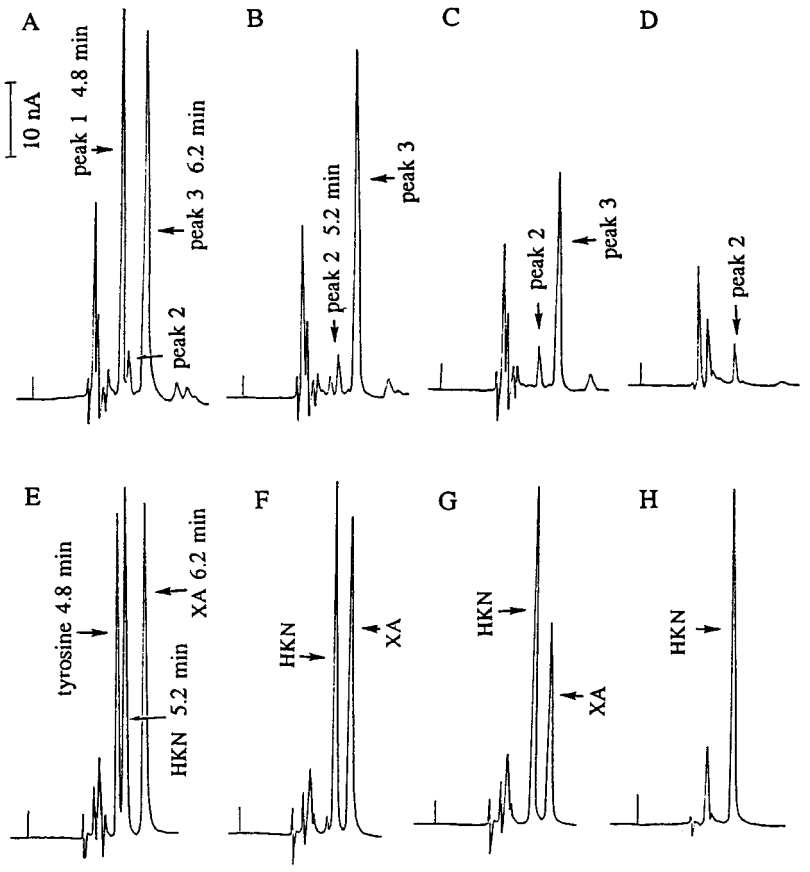

Figure 2. Identification of the electrochemically active compounds in mosquito larval extract by comparing their oxidation profiles with those generated using known standards. Chromatograms (top) show the results of HPLC-ED analysis of the same larval extract at 875 mV (A), 800 mV (B), 750 mV (C) and 650 mV (D) of the working electrode, and chromatograms (bottom) illustrate the results of HPLC-ED analysis of the same tyrosine, HKN and XA mixture at 875 mV (E), 800 mV (F), 750 mV (G) and 650 mV (H) of the working electrode.

HKN has the same retention time as peak 2, the minor electrochemically active compound detected in mosquito larval or *Drosophila* larval extract, while both dopamine and XA had the same retention time as peak 3 from the mosquito larval extract (not shown).

Coelution of tyrosine, HKN, dopamine and XA with the corresponding electrochemically active compounds in mosquito larval extract also was verified by mixing those standards with the larval extract and then analyzing by HPLC-ED (not shown).

Hydrodynamic voltammetric analysis of the mosquito larval extract showed that there were significant differences in the oxidation profiles among the peak 1, peak 2, and peak 3. The oxidation peak of the 4.8 min compound (peak 1) at 875 mV (Fig. 2A) was much greater than its oxidation peak at 850 mV (see Fig. 1A), and oxidation of this compound was barely observed when the oxidation potential was decreased to 800 mV (Fig. 2B). In contrast, the oxidation peak corresponding to the 6.2 min compound (peak 2) essentially was the same at the applied potentials from 800 - 875 mV (Fig. 2A-B). The oxidation peak of the 6.2 min compound (peak 3) was decreased considerably at 750 mV (Fig. 2C), and oxidation of this compound was not observed at 650 mV (Fig. 2D). These results demonstrate that peak 3 in the mosquito larval extract is not dopamine or other *o*-diphenolic compounds, because oxidation of the typical *o*-diphenolic compounds (such as dopa and dopamine) occurred easily at 600 mV of the working electrode (not shown). Oxidation of peak 2 at the working electrode was much easer than the oxidation of both the peak 1 and peak 3, because no decrease in the peak height of the peak 2 was observed as the decrease of the oxidation potentials from 875 to 650 mV (Fig. 2A-D).

At the described conditions of HPLC-ED analysis, tyrosine, HKN and XA had the same retention times as the peak 1, peak 2, and peak 3 in mosquito larval extract, respectively. When a mixture of tyrosine, HKN and XA standards was injected for HPLC-ED analysis in the same manner, the oxidation profiles of tyrosine, HKN and XA at the working electrode (Fig. 2E-H) essentially were the same as those observed for the peak 1, peak 2, and peak 3 in the mosquito larval extract, respectively (see Fig. 2A-D).

When the column was equilibrated with the same citrate buffer containing 1.8 mM octyl sulfate and then the larval extract was injected for HPLC-ED analysis, the retention times of peak 1, peak 2, and peak 3 were shifted from 4.8, 5.2, and 6.2 min to 5.8, 6.4, and 5.3 min, respectively (Fig. 3A). Analysis of tyrosine, HKN, and XA standards by HPLC-ED at the same conditions revealed that the retention times of tyrosine, HKN, and XA also were changed from 4.8, 5.2, and 6.2 min to 5.8, 6.4, and 5.3 min, respectively (Fig. 3B).

Based on the above results, it is concluded that the peak 1, peak 2, and peak 3 in the mosquito larval extract (Fig. 1) are tyrosine, HKN, and XA, respectively. XA is derived from HKN. Accumulation of XA in the mosquito larvae suggests that the enzyme responsible for transamination of HKN to XA

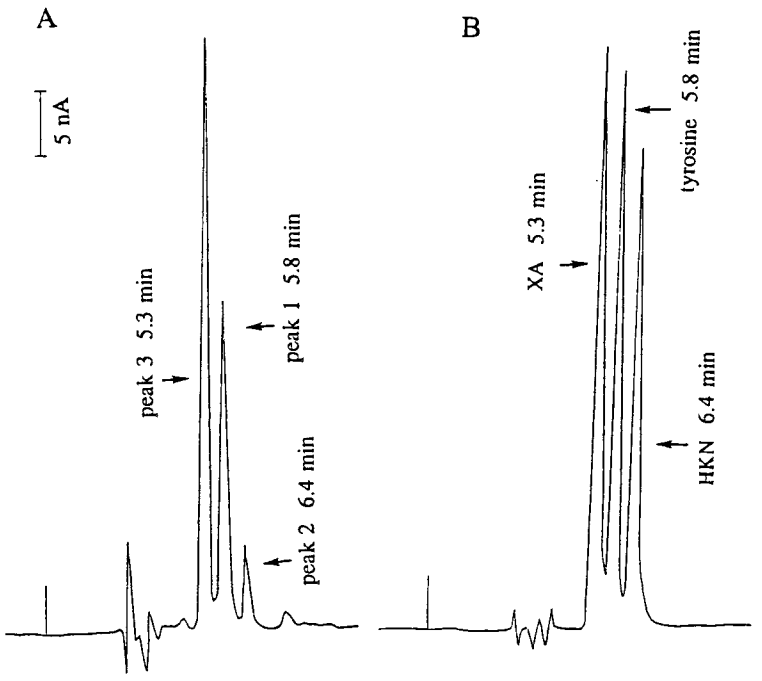

Figure 3. Identification of the electrochemically active compounds in mosquito larval extract by their chromatographic behavior toward ion pairing agent. Chromatogram (A) shows the retention time shifts of peak 1, peak 2 and peak 3 due to high concentration of octyl sulfate in the mobile phase, and chromatogram (B) illustrates the identical retention time changes of tyrosine, HKN and XA standards at the same conditions.

must be present in this insect species. When isolated mosquito larval protein was mixed with a substrate preparation containing HKN, pyruvate, and PLP, and then this reaction mixture was incubated at 37°C for 20 min, fairly high concentration of XA was produced in the reaction mixture (Fig. 4A).

In contrast, trace amount of XA was observed in a reaction mixture containing heat-inactivated larval protein (Fig. 4B). About 25% decrease in HKN transaminase activity of the larval protein was observed when PLP, cofactor of transaminase, was omitted in the reaction mixture (Fig. 4C), while very low concentration of XA was detected in a reaction mixture without pyruvate (Fig. 4D).

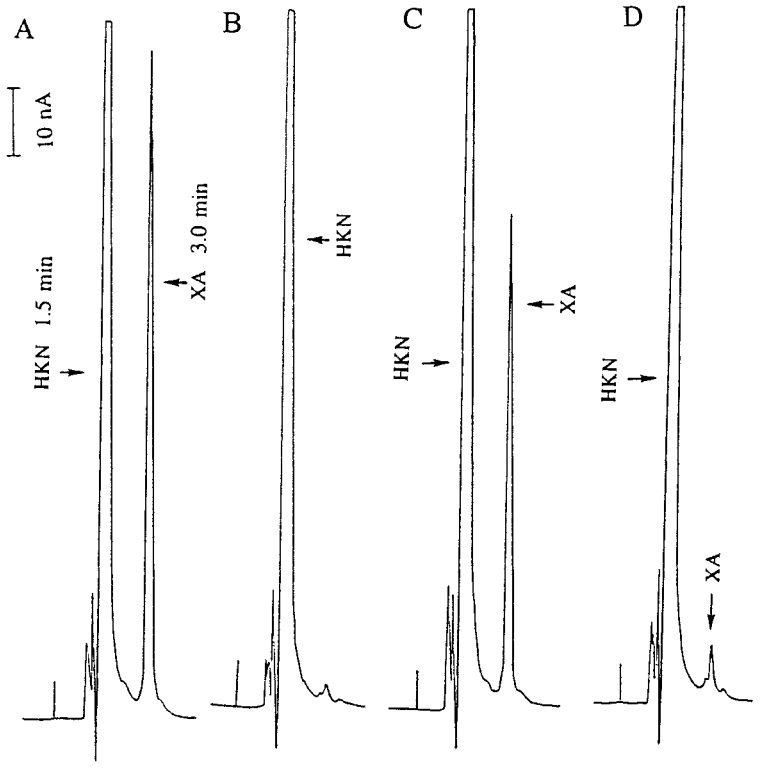

Figure 4. Determination of HKN transaminase activity of the isolated mosquito larval protein using HPLC-ED. Chromatograms illustrate the amounts of XA produced during a 20 min incubation in a reaction mixture containing either 0.4 mg larval protein (A) or 0.4 mg heat-inactivated larval protein (B), in a reaction mixture containing the same amount of larval protein in the absence of PLP (C) or in the absence of pyruvate (D).

The enzyme activity was found to be proportional both to the reaction time and to the concentration of mosquito larval protein during a 30 min incubation period (Fig. 5). Further kinetic analysis showed that increase in the final concentrations over 5.0 mM and 0.05 mM for pyruvate and PLP, respectively, did not increase the specific HKN transaminase activity of the larval protein (not shown). Increase in the concentrations of HKN above 3.0 mM in the reaction mixture slightly increased the specific HKN transaminase activity of the larval protein, but HKN did not solubilize well in the reaction buffer at higher concentrations. Based on these data, the reaction mixture containing 3 mM HKN, 0.05 mM PLP, 5.0 mM pyruvate, and varying amounts of protein

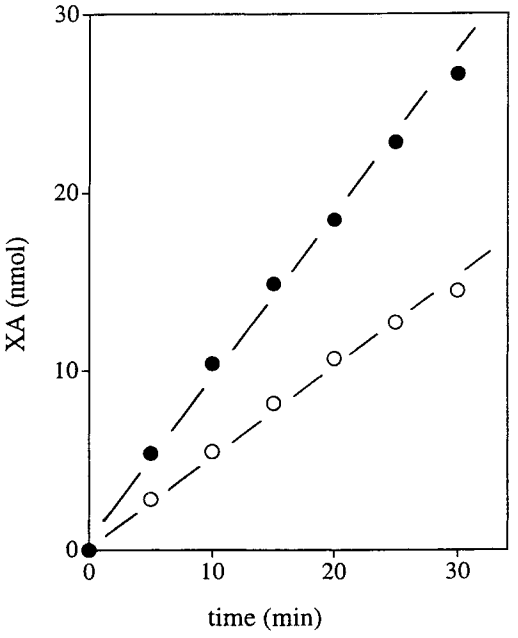

Figure 5. Dependence of HKN transaminase activity of the isolated larval protein measured with HPLC-ED assay as a function of time and protein concentration. XA formation was based on 1.0 ml of reaction mixture containing either 0.2 mg protein (open circle) or 0.4 mg protein (solid circle) with final substrate concentrations of 3.0, 0.05 and 5.0 mM for HKN, PLP and pyruvate, respectively.

samples in 1.0 mL of 100 mM tris buffer (pH 8.0) was used as standard reaction mixture. At the standard assay conditions, the specific HKN transaminase activity of the isolated larval protein was 2.7 nmol min^{-1} mg^{-1} protein.

After the HKN transaminase assay was validated using the isolated larval protein as enzyme source, larval powder, obtained from lyophilized larvae, was mixed directly with the substrate preparation for determining its HKN transaminase activity. Proportional increase in XA concentrations was observed in 1.0 mL reaction mixture containing 2 mg of larval powder and the standard substrate preparation during 15 and 30 min incubation periods (Fig. 6A-C). Although there was endogenous XA in the larval powder (Fig. 6A), quantitative evaluation of the larval powder HKN transaminase activity was achieved easily by subtracting the amount of XA in the heat-inactivated sample.

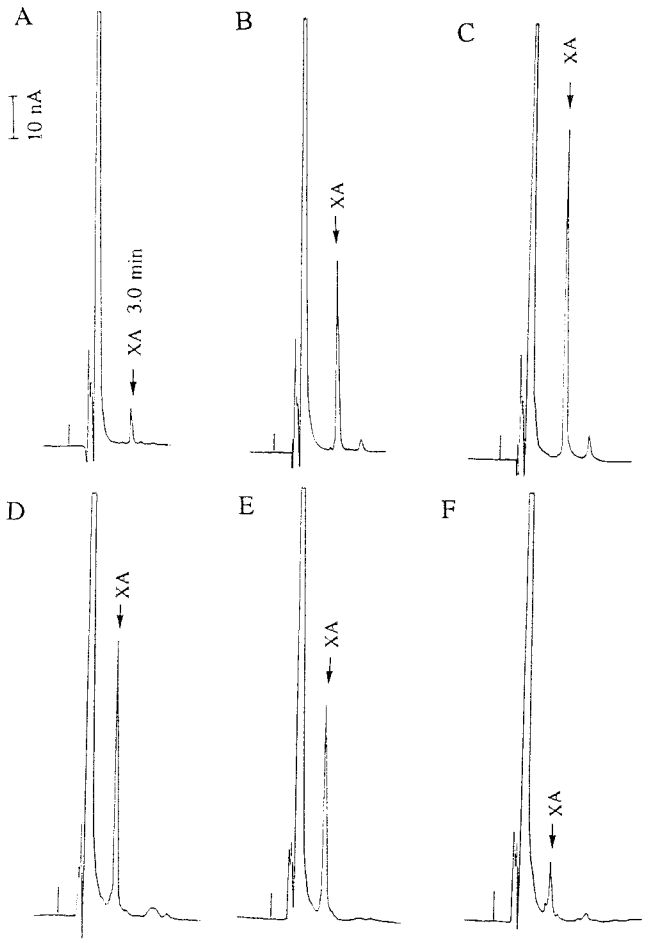

Figure 6. Determination of HKN transaminase activity of mosquito larval powder and supernatant of mouse kidney and liver homogenates using HPLC-ED. Chromatograms (top) illustrate the amount of XA in a reaction mixture containing 2 mg of heat-inactivated powder at 30 min after incubation (A), and the amounts of XA in a reaction mixture containing 2.0 mg larval powder at 15 min (B) and 30 min (C) after incubation. Chromatograms (bottom) show the amounts of XA produced in the reaction mixtures containing either 0.35 mg kidney supernatant protein (D) or 0.8 mg liver supernatant protein (E) during a 20 min incubation period, and the amount of XA produced during 20 min incubation in a reaction mixture containing kidney supernatant in the absence of PLP (F).

At the described assay conditions, the specific HKN transaminase activity of the mosquito larval powder was 0.45 nmol min^{-1} mg^{-1} dry powder. The applicability of this HKN transaminase activity assay for samples from other resources was demonstrated by using supernatant of mouse kidney and liver homogenates (Fig. 6D-E). However, when PLP was omitted in the reaction mixture, very low concentrations of XA were produced in the reaction mixture containing either the supernatant of kidney homogenate (Fig. 6F) or the supernatant of liver homogenate (not shown). Therefore, for both kidney and liver samples, it is essential to have exogenous PLP incorporated into the reaction mixture. In addition, endogenous XA was negligible in the supernatant of mouse liver or kidney (not shown).

DISCUSSION

Chromatographic separation of individual components in crude biological samples and their coelution with authentic standards may lead to the identification of the individual compounds without them being purified from the crude samples. But in this study it seems apparent that coelution of the peak 3 with XA is not sufficient to propose its XA identity, because (I) there have been no reports showing the accumulation of high concentration of XA in insects, (II) the same compound essentially is not observed in *Drosophila* larvae, and (III) the concentration of XA (the product) is 20-30 times greater than the concentration of HKN (the substrate) in the larval extract, which seems contrast to some of the enzymatic reactions. However, results from the hydrodynamic voltammetric analysis of the larval extract in conjunction with the coelution of peak 3 with XA standard at different conditions of HPLC-ED analysis provide convincing evidences for its XA identity.

During HPLC-ED analysis, individual electrochemically active compounds diffuse toward the surface of the working electrode, and oxidization or reduction of those compounds at the working electrode results in their detection. Depending upon the redox potentials of the electrochemical active compounds, some are oxidized at relatively low oxidation potentials, while some are oxidized at relatively high oxidation potentials. In general, the current intensity or peak height corresponding to the oxidation of individual electrochemically active compounds increases continuously as the increase of the oxidation potential, and then the peak height basically stays the same after the oxidation potential is raised to certain level (the oxidation peak no longer depends on oxidation potential). Under the described conditions of HPLC-ED analysis, oxidation of monophenolic compounds (such as tyrosine and tyramine) at the working electrode begins at $\geq$800 mV and oxidation of *o*-diphenolic compounds (such as, dopa and dopamine) occurs at about 450 mV

(not shown). Therefore, XA can be distinguished decisively from the commonly observed mono- and *o*-diphenolic compounds by its oxidation profile. Consequently, an identical oxidation profile of the peak 3 in larval extract with that generated using XA standard and its coelution with this standard under different chromatographic conditions seem sufficient to suggest its XA identity.

Tyrosine has one free amino group and HKN has two free amino groups, and these amino groups are positively charged under acidic conditions. Therefore, increase in the concentration of octyl sulfate (dynamic ion pairing agent) in the mobile phase should slow down these molecules during chromatography due to interaction between the positive charged amino groups on tyrosine and HKN and the negatively charged SO_3^- group of octyl sulfate on the surface of non-polar phase. In contrast, the ion pairing agent should have no effect on increasing the retention time of XA during chromatography because XA has no free amino group. The hydrocarbon chain of octyl sulfate partitions into the non-polar phase and leaves the hydrophilic SO_3^- group on the non-polar surface. The hydrophilic SO_3^- groups on the non-polar surface may decrease the hydrophobicity of the non-polar phase, which may explain the decrease in retention time for XA by octyl sulfate. Therefore, the retention time shifts of the peak 1, peak 2, and peak 3 due to ion paring reagent provide supporting evidence for their tyrosine, HKN and XA identities.

The HPLC-ED analysis also works well for HKN transaminase activity assay. The advantages of this enzyme assay are selectivity, sensitivity, and convenience. The stability of the baseline or the noise level often is a key factor that affects the sensitivity of a detection method. Because only the electrochemically active compounds are detected at the working electrode, the baseline is less affected by the presence of other components that are not electrochemically active. The sensitivity used in the HKN transaminase activity assay is 100 nA full scale. Depending upon the enzyme activity, the sensitivity can be increased to 5-10 nA without deteriorating the baseline. Because separation of the substrates and product is achieved using a short column, it takes only 3 - 4 min to analyze each sample. Currently, the HKN transaminase is being purified from mosquito larvae, and the described enzyme assay is used routinely to determine the active fractions and specific activity of the protein samples after each step of purification.

In summary, the described HPLC-ED method is useful for the identification of endogenous HKN and XA in crude biological samples, and also for the determination of HKN transaminase activity from a variety of biological sources.

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