ANALYTICAL BIOCHEMISTRY 82, 573–579 (1977)

A Rapid Assay for Insect Juvenile Hormone Esterase Activity

BRUCE D. HAMMOCK[1] AND THOMAS C. SPARKS

University of California, Riverside, California 92521

Received January 31, 1977; accepted June 10, 1977

A rapid new assay is proposed for analyzing insect juvenile hormone esterase activity. The assay is based on > 99% of radiolabeled juvenile hormone being extracted into an isooctane phase while > 99% of the juvenile hormone acid remains in the basic aqueous-methanol phase. The assay is more rapid and less expensive than conventional chromatographic assays while yielding almost identical values of similar precision. This is the first rapid assay proposed for isomerically pure, commercially available juvenile hormone I.

The juvenile hormones (JHs) of insects are conjugated methyl esters of farnesoic acid 10,11-epoxide (JH III) or its homo- or dihomoanalogs (JH II, I). There has been intense interest in these compounds over the last few years because the JHs are important in regulating insect development and in some cases reproduction, and some JH mimics or juvenoids hold promise as insect pest control agents [see (1) for reviews].

Slade and Zibitt (2) first indicated that the major routes of JH metabolism were ester cleavage to JH acid, epoxide hydration to JH diol, and/or a combination of these pathways. Subsequent metabolism studies have verified the importance of these pathways [see (3) for review]. Correlations of blood esterase and JH levels with insect development (4–7), especially with the discovery of a blood-borne JH carrier molecule, have indicated the involvement of JH metabolism in hormone regulation. The finding of potent inhibitors of JH esterases (8,9) has raised the possibility that such inhibitors may themselves be pest control agents. Thus, there is a need for a rapid, inexpensive assay for JH esterases.

Until recently the assay for JH esterase activity involved multiple extraction of the enzyme fraction with an organic solvent, drying (Na_2SO_4) and evaporating the solvent, spotting with cold standards and isolation by thin-layer chromatography (tlc), visualizing the cold spots with ultraviolet light or labeled materials by proportional flow scanning, and scraping the appropriate bands followed by liquid scintillation counting (lsc). In

[1] Direct reprint requests to author at this address: Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, California 92521.

ISSN 0003-2697

addition to the time and expense involved in tlc assays for routine measurements of JH esterase, there are potential errors involved. JH acid is deceptively difficult to synthesize in high yields to use as a cold standard, and it is quite unstable on storage. This laboratory routinely uses juvenoid mimics as cold reference standards, but the tlc R_f of labeled JH acid may change drastically relative to these cold standards and JH diol depending on the activity and pH of the tlc plate and the amount of water cospotted with the enzyme extract even on different channels of the same tlc plate. Additionally, spotting artifacts, such as air oxidation or epoxide rearrangement, may occur.

Several rapid assay schemes for JH esterase activity have recently been proposed, but all have attendant disadvantages. JH esterases appear highly specific, so the use of model substrates must be cautious. Monitoring the release of ethanol from ethyl ester analogs of JH spectrophotometrically with an alcohol dehydrogenase system has so far proven unsuccessful because of the JH esterase specificity for methyl esters (8). An electrophoretic assay (9) simply substitutes one chromatographic assay for another and requires extensive controls. The most rapid published method, suggested by Sanburg *et al.* (6), monitors the release of radioactive methanol from methyl-labeled JH by charcoal precipitation (6,8) or extraction by a Folch distribution of unreacted JH (10). Although superb, this method requires methyl-labeled JH which is not commercially available. The JH esterase assay discussed here is sensitive, reproducible, and rapid, while utilizing commercially available chain-labeled JH I which was labeled at C-10 by a pathway which may shortly result in the availability of other chain-labeled JH isomers (11).

MATERIALS AND METHODS

Chemicals

Labeled JH I (~10 Ci/mmol; >96% pure by tlc; >98% E,E,Z; ^{3}H at C-10) was obtained from New England Nuclear Corporation, while a cold isomer mixture of JH I was provided by A. J. Manson (Ayerst Laboratories). Purity was verified by tlc and high-resolution liquid chromatography. The polar impurities in the labeled JH I were removed by the partition method described below to give >99% purity by tlc when necessary. Labeled JH acid was prepared by a modification of the method of Metzler *et al.* (12) by incubating labeled JH with esterases from fifth instar Day 2 *Trichoplusia ni* (cabbage looper) (13). A small amount of unreacted starting material was removed by partitioning, and the structure of the JH acid was supported by previously described microderivatization methods (14,15). All solvents were spectral grade except acetonitrile which was distilled before use.

Analytical Procedures

No plastic materials were allowed to come into contact with JH solutions, and glassware in contact with JH was treated with Siliclad (Clay Adams) or Carbowax (polyethylene glycol; 50,000 MW) to reduce JH binding to the glass (15). Classical tlc assays for JH esterase activity were performed as described in the introduction utilizing ethyl acetate as the extracting solvent after saturating the enzyme incubation with sodium chloride. The extracts were run on silica gel F_{254} chromatoplates (0.25-mm gel thickness, EM Laboratories) in hexane:ethyl acetate (2:1). Although three extracts are required for near quantitative removal of JH and JH acid from the incubation mixture, JH:JH acid ratios remain relatively constant for each extract. The JH:JH acid ratio was determined by lsc of the appropriate band, and the rest of the tlc plate was also analyzed to insure that JH acid was the only metabolite.

Partition assays consisted of halting the enzymatic reaction (carried out in 10 × 75-mm glass tubes utilizing phosphate buffer 0.1 M, pH 7.4, with 0.01% phenylthiourea) by the addition of a water-soluble organic solvent followed by the addition of a water-insoluble organic solvent. The phases were mixed on a Vortex, separated by centrifugation (2000*g*, 3–5°C), and aliquots of the organic and aqueous fractions were removed and analyzed by lsc in a toluene:Triton cocktail. The counts were corrected for quench and for differences in the final volumes of the two phases.

For routine analysis the substrate (in 1 μl of ethanol) was added to the enzyme preparation (100 μl) with a Hamilton repeating dispenser equipped with a 50-μl syringe. The standard deviation of the amount of label added was ±6%. To avoid this error while devising the analytical method, large enzyme incubations were run and multiple samples (100 μl) were taken for analysis which gave a standard deviation of total counts of ±2%. Varying incubation times and substrate levels were utilized to form different ratios of JH to JH acid.

RESULTS

Partition experiments similar to the ones illustrated in Table 1 were run between isooctane, hexane, carbon tetrachloride, and chloroform as the organic phases with methanol, dimethyl sulfoxide, and acetonitrile as the aqueous cosolvents. The chlorinated solvents had the advantage of causing the aqueous layer to be the hyperphase, but illustrated poor partition characteristics with JH and JH acid and resulted in such high levels of quenching that both phases could not be counted. Hexane and isooctane gave essentially identical results, but the higher-boiling isooctane was used since it resulted in fewer errors due to the evaporation of the hyperphase. Both acetonitrile and dimethyl sulfoxide resulted in separation of the phases in the scintillation fluid causing unstable count rates and varying

TABLE 1

DISTRIBUTION OF JUVENILE HORMONE AND JUVENILE HORMONE ACID BETWEEN AN ISOOCTANE (250 μl) AND AN AQUEOUS METHANOL PHASE (150 μl)[a]

MeOH in aqueous fraction (%)	NH_4OH (μl)	JH in aqueous phase (%)	JH acid in aqueous phase (%)
0	0	0.65	93.9
0	5	0.53	99.8
17	0	0.85	95.3
17	5	0.89	99.9+
33	0	1.33	96.4
33	5	1.20	99.9+
43	0	2.7	97.3
43	5	2.3	99.9+
50	0	3.1	98.3
50	5	2.8	99.9+
60	0	5.5	99.2
60	5	5.1	99.9+
67	0	6.0	99.9+
67	5	5.6	99.9+

[a] Counted to <0.5% error for total counts above background. Average of three replications on different days with different preparations of JH and JH acid. Each individual experiment showed the same trends illustrated in this table.

quench (16). Aliquots of the isooctane or methanol–water phases (100 μl) caused negligible quench, and the count rates were relatively stable over time.

The use of ammonium hydroxide increases the percentage of JH acid in the aqueous phase, while slightly reducing the amount of JH in the aqueous phase. The amount of ammonium hydroxide utilized raises the pH of 0.1 M, pH 7.4 phosphate buffer to 10.2–10.3. At this pH the relatively stable conjugated JH ester is not hydrolyzed significantly during the course of an analysis.

The data in Table 1 indicate that the best partitioning values probably occurred with less than 17% methanol, but our routine assay consists of halting the enzymatic reaction (100 μl) with the addition of 50 μl of a solution consisting of 10 ml of methanol, 9 ml of distilled water, and 1 ml of concentrated ammonium hydroxide, followed by the addition of 250 μl of isooctane. The methanol stops the enzymatic reaction, disrupts protein binding, and probably reduces possible binding of JH acid to glass surfaces during transfer. Even when the phases appear to have separated, centrifugation usually reduces the amount of JH in the aqueous phase, possibly by removing suspended droplets of isooctane in the hypophase or eliminating an emulsion at the interface which complicates removal of an aqueous aliquot for counting.

This method resulted in 96.6 ± 0.3% of 96.6% pure (tlc analysis) JH appearing in the organic phase and 99.6 ± 0.1% of >99% pure (tlc analysis) JH acid appearing in the aqueous phase at five substrate concentrations of 1×10^{-9}–1×10^{-4} M and five protein concentrations of 0.005–10 mg/ml of protein. At protein concentrations greater than 1 mg/ml, extensive centrifugation was required to separate the two phases. The recovery of added counts for JH was 100.1 ± 0.1%, while for JH acid it was 97 ± 3%. The data in Table 1 and pH determinations not reported here indicate that there can be relatively large changes in the methanol:ammonium hydroxide: buffer ratios without causing highly significant errors in the assay.

Thin-layer chromatography analysis of the two phases after centrifugation revealed that >95% of the radioactivity in the hyperphase cochromatographed with JH and 3% chromatographed in the region of JH diepoxide or allylic alcohol (possibly spotting artifacts) with negligible counts in the region of JH acid, diol, or diol acid. The hypophase had less than 1% of its radioactivity with a higher R_f than JH acid, and at least 92% of the remaining counts cochromatographed with JH acid.

Ten analyses of JH esterase activity were performed on each of two different *T. ni* hemolymph samples containing known amounts of JH acid and JH by tlc and the partition method. The study was done by a student not previously familiar with the method. The contamination of the hypophase by JH as subtracted as background, and the percentage of JH acid was determined from a ratio of hyper- to hypophase counts. The average investigator time per assay was 1 min for the partition assay, 3 min for the tlc assay with one extraction, and 10 min for the tlc assay with three extractions. The values found by each method on the two assays were 87.6 ± 2% for the partition compared to 86.0 ± 6% for tlc, and 54.9 ± 5% for the partition compared to 55.5 ± 2% for the tlc method. The partition assay accounted for 91.1 ± 10% of the total counts, while the tlc assay using three extractions recovered 60.0 ± 15%. The partition and tlc methods for analyzing JH:JH acid ratios gave results identical at the 99 and 95% confidence level, respectively. Counting only the aqueous phase reduces time and expense still further, but it obviously results in an increase in the error of the assay giving 90.4 ± 6.1% and 64.2 ± 9.2% when only the hypophase is considered in the above assays. The errors increase as the percentage of JH acid present decreases.

The partition method indicates linear formation with time of JH acid from JH I by *T. ni* esterases and gives the same distribution of JH esterases eluting from a Sephadex column or during the larval–pupal development of *T. ni* as charcoal precipitation of JH III esterase activity or tlc analysis of JH I esterase activity. The specific activity of commercially available JH easily allows incubations to be run with substrate concentrations as low as 10^{-9} M. This laboratory routinely determines JH esterase titers in *T. ni* blood with 1 μl of actual hemolymph diluted to 100 μl with buffer

and a substrate concentration of 5×10^{-6} M and incubation times of 5–30 min (13).

DISCUSSION

The partition method for analysis of JH esterase activity is obviously inappropriate if reactions other than ester cleavage occur; however, for most routine assays it is faster, less expensive, and more precise than chromatographic assays. Ester hydrolysis is the only metabolic pathway so far described for JH in blood or the 100,000*g* soluble fractions of tissue homogenates. In microsomal fractions of tissue homogenates, minor oxidative pathways of JH metabolism can be eliminated by the exclusion of necessary cofactors, and epoxide hydrase activity, which so far appears to be entirely membrane-bound in insects for JH-like substrates, may possibly be eliminated by selective inhibitors allowing the partition method to be utilized for assaying JH esterase availability even in these fractions.

The partition method has other uses in studying insect endocrine biochemistry. It is applicable to the analysis of JH esterase as a replacement for charcoal precipitation when utilizing methyl-labeled JH. Partitioning was used in this study to remove some polar impurities which may form during the storage of commercially available labeled JH I. JH acid is often needed for endocrine experiments, and it is routinely prepared enzymatically (12). This procedure rapidly gives greater than 90% conversion to JH acid, but unless excessively long reaction times are used, a small percentage of unreacted JH usually remains which may complicate some experiments. Thin-layer chromatography purification of JH acid leads to substantial loss and degradation, while removal of unreacted JH by partitioning with isooctane is very mild. The partition assay is flexible enough to be adapted to specific applications, and it should greatly facilitate studies of JH esterase activity.

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