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Detection and microsequencing of juvenile hormone-binding proteins of an insect by the use of an iodinated juvenile hormone analog

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An [^{125}I]iodinated juvenile hormone (JH) analog can be used as a sensitive and highly selective probe for the visualization of high-affinity, (JH)-specific binding proteins from insect hemolymph samples. The proteins can be detected in their native form using a two-dimensional (isoelectric focusing then native gradipore gel) separation of the crude protein mixture containing the ^{125}I-labeled iodinated JH analog. The proteins can be transferred to activated glass fiber paper by electroblotting, and the location of the bound γ-emitter can be found by exposure of the dried gel or the electroblot to X-ray film. The radiolabeled protein spot can be excised from the Coomassie-stained glass fiber paper and subjected directly to gas-phase N-terminal amino acid sequencing. This non-destructive, non-denaturing technique may have wide applicability in identifying and sequencing ligand-specific binding proteins in complex mixtures.

Electrophoresis; Electroblotting; Juvenile hormone; Iodinated hormone analog; Radioiodination; *(Manduca sexta)*

1. INTRODUCTION

The detection of high-affinity binding proteins in insect tissues is greatly facilitated by the use of high specific activity radioligands [1]. In particular, photoaffinity labeling with 10–60 Ci/mmol ^{3}H-labeled analogs of juvenile hormone (JH) have proven valuable during our studies of insect juvenile hormone-binding proteins (JHBPs) [2–4]. The covalently-modified JHBPs can be purified under denaturing electrophoretic or liquid chromatographic conditions, and can be used for amino acid sequencing [4]. However, the additional steps (impregnation with a scintillant in a hydrophobic solvent [5]) are required for fluorographic visualization of the weak ^{3}H emissions in the electrophoretic gel which are (i) not compatible with electroblotting and microsequencing procedures and (ii) not suitable for analysis of non-covalently bound JH radioligands.

One solution to this dilemma is the use of a radioiodinated JH analog, which would allow visualization of the native JH-binding proteins [6] and also permit electroblotting and sequencing to be performed using the same electrophoretic gel. We report here the use of 12-[^{125}I]iodo-JH I (fig. 1) to visualize specifically two electromorphs of the hemolymph JHBP of *Manduca sexta* larvae separated by native two-dimensional polyacrylamide gel electrophoresis, and the microsequencing of the N-terminal amino acid residues of these two proteins following electroblotting onto glass fiber paper.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Fine chemicals were obtained from the following sources: acrylamide, *N,N'*-methylenebisacrylamide, Tris, glycine and Coomassie brilliant blue R-250 from Amresco; pharmalyte pH 3–10 from Sigma; TEMED and ammonium persulfate from BioRad; reagent grade chemicals and solvents from Fisher. Nanopure water was used in the water-based solutions. The

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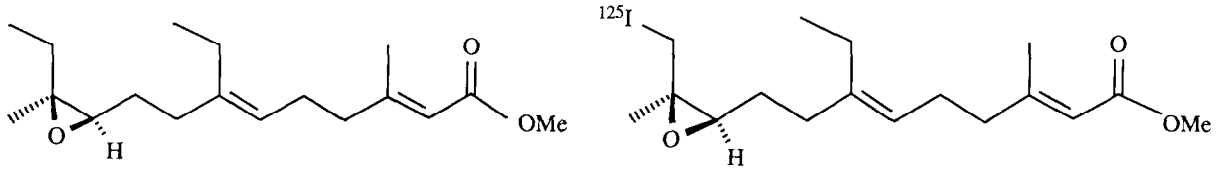

Fig.1. Structures of natural JH I and its radioiodinated analog.

radioiodinated juvenile hormone analog 12-[^{125}I]iodo-JH I was synthesized by W. Eng [6].

2.2. *Insect proteins*

M. sexta eggs were obtained from Dr J.S. Buckner (USDA, Fargo, ND) and larvae were reared on a commercial diet (BioServe). Chilled fifth instar larvae (L5D2) were bled from an anal proleg into a saturated solution of phenylthiourea in TK buffer (10 mM Tris-HCl, 10 mM KCl, pH 7.4). The crude hemolymph was fractionated by gel filtration on Ultragel AcA 54 according to [7].

2.3. *Binding of 12-[^{125}I]iodo JH I*

A 3 μl aliquot of a 0.5 μM solution of 12-[^{125}I]iodo-JH I (spec. act. 50 Ci/mmol) in ethanol was mixed with 100 μl of 10 mM Tris-HCl buffer, pH 7.50 [10 mM KCl, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM diisopropylfluorophosphate (DFP), 1 mM 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) and incubated for 4 h at 4°C to allow solubilization of the labeled hormone analog. Then, 50 μl of the gel-filtered *M. sexta* JHBP solution containing 100–300 μg protein was added, vortex-mixed, and incubated for 4 h at 4°C. Finally, solid sucrose was added (10%, w/v, final concentration) to the incubation mixture in preparation for electrophoretic separations.

2.4. *Native two-dimensional electrophoresis*

Glass tubes (0.4 × 15 cm) were filled with the IEF gel solution [5% *T* (total acrylamide), 2.7% *C* (crosslinking), 3% pharmalyte pH 3–10, 20 μl of 10% ammonium persulfate per 10 ml gel solution, and 10 μl TEMED per 10 ml gel solution] to 12 cm height and overlaid with water. After polymerization, the gels were washed and overlaid with 5% sucrose solution. The lower reservoir contained 50 mM NaOH and the upper reservoir 25 mM H_3PO_4. The protein solutions were mixed with pharmalyte pH 3–10 (1% ampholine). Isoelectric focusing in the tube gels was carried out at 850 V for 12 h at 5°C.

The tube gels were rinsed in water and then placed at the top of native gradient polyacrylamide slab gels. The second-dimensional slab gels (15 × 15 cm, thickness 0.75 mm) had been prepared in a gradient mixer with 5–15%, *T*, 4% *C*, using a discontinuous buffer system, and overlaid with a 3% *T* stacking gel. After 10 mA/gel running in the stacking gel, the separation was performed at 15–20 mA/gel.

2.5. *Autoradiography*

Following electrophoresis, gels were soaked without shaking in 10% glycerol at ambient temperature for 10 min. They were then dried at ambient temperature between hard cellophane sheets (Hoefer, San Francisco, CA) as described [6]. The dried gels were placed against pre-flashed Kodak X-Omat XAR-5 film [6] and exposed for 1–3 weeks at −80°C. The regions which showed radioactivity (see fig.2) were cut out from the dried gel, and soaked in 10% glycerol until they appeared totally rehydrated. The proteins associated with the radioiodinated ligands were electrophoretically transferred to a trifluoroacetic acid-activated glass fiber filter (Whatman GF/C) [8] in 1% acetic acid (4°C, 12 h, 250 mA, 60 V). The glass fiber filter was stained in 0.5% Coomassie brilliant blue R-250 in 30% 2-propanol, 10% acetic acid for 2–5 min, and then destained in 16% methanol, 8% acetic acid. The glass fiber paper was then lyophilized to dryness, and the blue spots corresponding to proteins binding the radioiodinated ligand were cut out and sequenced [8].

2.6. *Sequencing*

Gas-phase sequencing was carried out by Mr Thomas Fisher (Center for Analysis and Synthesis of Macromolecules, Stony Brook) using an Applied Biosystems model 470A gas-phase instrument.

3. RESULTS AND DISCUSSION

The [^{3}H]EFDA-labeled 32 kDa JHBP of *M. sexta* larval hemolymph has been purified (gel-filtration, ion-exchange and reverse-phase HPLC) and sequenced [4,7]. The [^{3}H]EFDA-labeled JHBP eluted as a single peak with ~ 21% CH_3CN from a C_8 reversed-phase HPLC column. The N-terminal amino acid sequence up to residue 35 is shown in fig.3, based on over 200 pmol protein, and is congruent with the 18 residues published for the *M. sexta* JH carrier protein which had been isolated using a binding assay to follow purification [9].

Using native PAGE, we found multiple electromorphs for the [^{3}H]EFDA-labeled JHBPs from *M. sexta*, *Lymantria dispar*, and *Heliothis virescens* larvae [1]. For *M. sexta*, we could demonstrate with one-dimensional (1D) native PAGE that [^{3}H]EFDA and 12-[^{125}I]iodo-JH I were associated with the same JH-specific binding protein [6]. The use of native 2D PAGE, i.e. isoelectric focusing followed by native gradient PAGE,

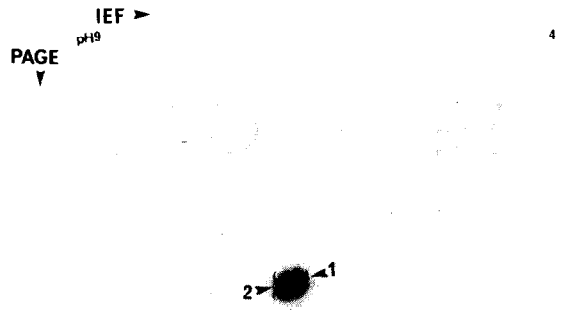

Fig.2. Autoradiogram of 12-[^{125}I]iodo JH I-binding proteins in electrophoretically separated *M. sexta* hemolymph proteins.

allowed separation of the native proteins still complexed to the iodinated JH analog. These proteins, which had the same molecular size but different isoelectric points, were readily detected in the dried gels by direct autoradiographic exposure of X-ray film by the weak γ emissions from the iodinated ligand (fig.2). Blotting of the proteins from the rehydrated gel segments onto activated glass fiber paper [8] gave several micrograms of each electromorph, which could be visualized by Coomassie blue staining and then excised for amino acid sequencing. The sequences were obtained at the 20 pmol level, close to the reported limit of sensitivity of the electroblotting-sequencing method [8]. It is noteworthy that both electromorphs have the same first 18 amino acid residues (fig.3), but that several differences (e.g. Arg-19 vs Thr-19) appeared in later sequencing cycles.

To our knowledge, this is the first report in which a biologically active iodinated ligand is used for a tandem procedure involving direct detection of electrophoretically separated native high-affinity binding proteins, followed by electroblotting and gas-phase sequencing. We anticipate that this methodology will be generally applicable for the detection and sequencing of picomolar quantities of scarce high-affinity binding proteins in crude or partially purified protein preparations.

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                                5                                  10
Asp - Gln - Gly - Ala - Leu - Phe - Glu - Pro - Cys - Ser -
Asp - Gln - Gly - Ala - Leu - Phe - Glu - Pro - Cys - Ser -
Asp - Gln - Gly - Ala - Leu - Phe - Glu - Pro - Cys - Ser -

                               15                                  20
Thr - Gln - Asp - Ile - Ala - Cys - Leu - Ser - Thr - Ala -
Thr - Gln - Asp - Ile - Ala - Cy? - Leu -  ?  -  ?  - Ala -
Thr - Gln - Asp - Ile - Ala - Cys - Leu - Ser - Arg - Ala -

                               25                                  30
Thr - Gln - Gln - Phe - Leu - Asp - Lys - Ala - Cys - Arg
  -   -   -   -   -   -   -   -   -   -
  ?   - Gln - Gln - Phe - Leu - Glu - Lys - Ala -  ?  -  ?

                               35
Gly - Val - Pro - Asn - Ile   [3H]-EFDA-labeled JHBP
  -   -   -   -   -   IodoJHI-detected JHBP 1
Gly -  ?  - Pro - Glu - Tyr   IodoJHI-detected JHBP 2
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Fig.3. N-terminal amino acid sequences of [^{3}H]EFDA-labeled [4] and 12-iodo-JH I-detected juvenile hormone-binding proteins isolated from *M. sexta* larval hemolymph.

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