Characterization of the glycosylated ecdysteroids in the hemolymph of baculovirus-infected gypsy moth larvae and cells in culture

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Baculovirus, Lymantria dispar, nuclear polyhedrosis virus, LdNPV, ecdysone, UDP-glucosyl transferase

Abstract. Fourth-instar gypsy moth (Lymantria dispar; Lepidoptera: Lymantriidae) larvae, infected with the gypsy moth baculovirus (LdNPV), show an elevated and prolonged extension of the hemolymph ecdysteroid titer peak associated with molting. The ecdysteroid immunoreactivity associated with this peak elutes as two peaks following HPLC on a C4 reverse-phase column. Both peaks elute in a region more polar than 20-hydroxyecdysone, but less polar than the highly polar ecdysteroid immunoreactivity associated with the apolysis peak of control animals. Glycosylated ecdysteroid standards, produced by in vitro incubation of UDP-glucose with ecdysone or 20-hydroxyecdysone and culture medium from LdNPV-infected gypsy moth cells, show elution times identical to the two immunoreactive peaks. Enzymatic hydrolysis studies verified this identity. The data suggest that the hemolymph of LdNPV-infected L. dispar larvae contains both glucose-conjugated ecdysone and glucose-conjugated 20-hydroxyecdysone which, by analogy with the literature, are presumably 22-O-β-D-glucopyranoside and 20-hydroxyecdysone 22-O-β-D-glucopyranoside.

INTRODUCTION

Baculoviruses constitute one of the largest groups of pathogenic organisms infectious to insects. Over 300 species of baculovirus have been described and some registered for biological control as alternatives to chemical pesticides (Wood et al., 1990; Kurstak, 1991; Leisy & van Beek, 1992). The nuclear polyhedrosis viruses (NPV) constitute the major subgroup of the family baculoviridae, with Autographa californica NPV (AcNPV) receiving the most attention on studies of NPV infection (Granados & Williams, 1986; Volkmann & Keddie, 1990). Recently, the Lymantria dispar NPV (LdNPV) has been investigated in detail as an agent for control of the gypsy moth, where LdNPV causes a polyhedrosis or wilt disease (Lewis, 1981). As a result of these studies, the LdNPV/gypsy moth system is currently the most thoroughly studied model for investigations on baculovirus/host interactions (Park, 1994).

The discovery by O’Reilly & Miller (1989) of a gene that encodes ecdysteroid UDP-glucosyl transferase (EGT) in AcNPV provides an explanation for the action of baculoviruses in controlling insect molting. Infected insects continue to feed but do not

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molt, indicating either a lack of the insect molting hormone, 20-hydroxyecdysone (20E), or an inability to respond to it. In non-infected insects, ecdysone (E) or its precursor, 3-dehydroecdysone, are produced by the prothoracic gland and converted in peripheral tissues to the active form, 20-hydroxyecdysone (20E) (Smith, 1985; Kirishi et al., 1990). In AcNPV-infected fall armyworms, Spodoptera frugiperda, EGT apparently catalyzes the galactose conjugation of hemolymph ecdysteroids, and the hemolymph ecdysteroids remain at low levels during virus replication due to apparent rapid clearing of the conjugated ecdysteroids from the hemolymph (O’Reilly et al., 1992). The structure of the conjugate formed by combination of E and UDP-glucose with virus-infected cell culture medium in vitro has been identified as ecdysone 22-O-β-D-glucopyranoside (O’Reilly et al., 1991). Using hemolymph from AcNPV-infected S. frugiperda larvae incubated in vitro with [3H]-ecdysone, O’Reilly et al. (1992) have identified the major product as an ecdysone galactose conjugate, presumably ecdysone 22-O-β-D-galactopyranoside, based on three criteria: susceptibility to cleavage by β-galactosidase, a high-performance liquid chromatography (HPLC) elution time identical to purified ecdysone 22-O-β-D-galactopyranoside, and a preference of EGT for UDP-galactose and/or UDP-glucose.

In L. dispar, LdNPV also affects the ability of the animal to molt (Burand & Park, 1992). As in S. frugiperda (O’Reilly & Miller, 1989; O’Reilly et al., 1992), virus infection of newly-ecdysed last (fifth)-instar L. dispar larvae results in inhibition of pupation, and the hemolymph ecdysteroid titers remain at low levels (Park et al., 1993). However, in contrast to virus-infected last-instar larvae, fourth-instar larvae of L. dispar infected with LdNPV show enhanced hemolymph ecdysteroid titers when examined by radioimmunoassay (RIA) (Park et al., 1993). The additional immunoreactive ecdysteroids consist of conjugated ecdysteroids that migrate on thin layer chromatography with an Rf identical to a standard E-glucose conjugate (Park et al., 1993). The E-glucose conjugate is detected because the RIA antiserum has an equal affinity for the E-glucose conjugate and E (Park et al., 1993). The enhanced hemolymph ecdysteroid titer and the inability to molt appear to be due to the expression of EGT activity by LdNPV (Park et al., 1993; Park, 1994).

The purpose of the present work was to further characterize the conjugated E produced in LdNPV-infected, fourth-instar L. dispar larvae using criteria similar to those used to characterize the conjugated ecdysteroid of AcNPV-infected S. frugiperda larvae (O’Reilly et al., 1992). Based on these criteria, we have identified the major conjugated ecdysteroid of L. dispar as an E-glucose conjugate, presumably ecdysone 22-O-β-D-glucoptyanoside.

MATERIALS AND METHODS

Animals

The New Jersey strain of L. dispar was obtained from the USDA Otis Methods Development Center, Otis ANGB, MA, U.S.A. and reared on artificial wheat germ diet according to Bell et al. (1981). Newly molted fourth-instar larvae were used in all experiments.

Cells, viruses, and preparation of conjugated ecdysteroid standards

The gypsy moth embryonic cell line, IPLB-Elta, and plaque-purified LdNPV isolate Ldg were obtained and maintained as described (Park et al., 1993). Ecdysteroid conjugates were produced by incubating E or 20E (5 μg) (Sigma Chemical Co., St. Louis, MO, U.S.A.) with UDP-galactose or UDP-glucose (10 μg) (Sigma) in the presence of viral-infected cell culture fluid (100 μl) that had been harvested at 48-h post-infection and centrifuged at 2000 g for 10 min to remove all debris. Incubation and termination conditions were as described previously (Park et al., 1993). The reaction was stopped by the addition of
two volumes of 100% ethanol, and the samples were vacuum dried. Dried samples were resolubilized in 75% aqueous methanol, and a few microliters were removed for RIA. The remaining material was vacuum dried, resolubilized in methanol/water (1/9), and fractionated on a C₈ SEP-PAK cartridge (Millipore Waters Chromatography Division, Milford, MA, U.S.A.) according to Weirich et al. (1986). Essentially, the resolubilized material was applied to a cartridge primed with 5.0 ml methanol and 5.0 ml water, and sequentially eluted with 4.0 ml methanol/water (1/9), 5.0 ml methanol/water (3/7), 5.0 ml methanol/water (6/4), and 5.0 ml 100% methanol. One milliliter fractions were collected and analyzed by RIA and HPLC (see below). Fractions 9, 11 and 12, (i.e., the next to last fraction in the 30% aqueous methanol eluate and the first two fractions in the 60% aqueous methanol eluate) gave high RIA activity and were free of contaminating UV absorbance as identified during HPLC. These fractions were combined and used as conjugated ecdysteroid standards. HPLC of these standards gave a single UV-absorbing peak. Since the position of ecdysteroid glycosylation has not been identified in the present paper, and the ecdysteroids have been glycosylated with both glucose and galactose, we have modified the abbreviated ecdysteroid nomenclature of Lafont et al. (1993) to represent the products as follows: ecdysone-glucose conjugate (EGlc), ecdysone-galactose conjugate (EGal), 20-hydroxyecdysone-glucose conjugate (20EGlc) and 20-hydroxyecdysone-galactose conjugate (20EGal).

Hemolymph collection and radioimmunoassay

Staging, viral infection and hemolymph collection was as described previously (Park et al., 1993). Newly eclosed animals were isolated, starved for 24 h, and fed on diet cubes inoculated with 8 x 10⁷ viral occlusion bodies, resulting in 100 percent mortality by 9 days post-infection (the wild-type LdNPV isolate, Gypchek, was provided by the U.S. Forest Service, Hamden, CT, U.S.A.). Control larvae were fed diet alone. After consuming all the diet (about 6 h), animals were transferred to fresh diet cups. Hemolymph was collected from virus-infected fourth-instar females at 4 days post-infection (dpi) and from controls about the time of apolysis (3.5 dpi). It was collected in 80% methanol, stored overnight at -20°C, vortexed, centrifuged and the supernatant vacuum dried. Samples were resolubilized in 1.0 ml of 75% methanol for assay and extraction. The RIA was performed as previously described (Kelly et al., 1992). The antiserum was a gift from W.E. Bollenbacher and was prepared against the 22-hemisuccinate derivative of E. The ratio of the mass of 20E required to displace 50% of the labelled E compared to the mass of E was 2.8. This antiserum recognizes E and the glucose conjugate of E with nearly equal affinity (Park et al., 1993) as is common for many conjugates formed by esterification of the 22-hydroxyl of E and 20E (Warren & Gilbert, 1988). [23,24-¹H(N)]Ecdysone (60-80 Ci mmol⁻¹) was obtained from NEN Research Products, Boston, MA, U.S.A.

HPLC and enzyme digestion studies

Samples were fractionated on a reverse-phase Suplecosil LC-18-DB column (4.6 mm i.d. x 25 cm, 5 µm particle size, Supleco, Inc. Bellefonte, PA, U.S.A.) in 41% methanol at 1 ml/min⁻¹ with a Spectro-Physics 8700 solvent delivery system. Absorbance was measured at 245 nm, and 600-µl fractions were collected. E, 20E, makisterone A (Sigma) and 26-hydroxyecdysone 22-glucoside (a gift from Jim Svoboda, Insect Neurobiology and Hormone Laboratory, which eluted in fraction #8) were used as standards.

For enzyme digestion studies, a mixture of glucuronidase/glucosidase enzymes, used by Thompson et al. (1987) to characterize 26-hydroxyecdysone-22-glucoside, was kindly supplied by Kenneth R. Wilzer, Jr (Insect Neurobiology and Hormone Laboratory). Their hydrolysis and extraction procedure was slightly modified. Essentially, HPLC-purified conjugate was dried and redissolved in 1 ml of 0.2 M sodium acetate/acetic acid buffer solution (pH 5) and added to a freshly prepared solution of 1 mg of each enzyme in 1 ml of 0.2 M NaCl. The mixture was incubated at 30°C for 48 h, followed by the addition of 2 ml 20% aqueous methanol and centrifugation at 12,000 g for 1 min. The supernatant was added to a methanol (5 ml) and H₂O (5 ml) primed SEP-PAK and eluted in aqueous methanol as described above (4 ml 10%, 5 ml 30%, 5 ml 60%, 5 ml 100%). The 30% and 60% eluates were collected, combined, vacuum dried and fractionated by HPLC (see above).

For β-galactosidase digestions, the procedure of O’Reilly et al. (1992) was modified. HPLC-purified conjugate was dried, resuspended in 50 µl of 25 mM sodium citrate, pH 3.5, and 0.33 units (50 µl) of jack bean β-galactosidase (Sigma) was added. The samples were incubated at 30°C overnight, followed by the addition of 100 µl 20% aqueous methanol, a 100 µl 10% aqueous methanol rinse, and centrifuging at
12,000 g for 1 min. SEP-PAK extraction was as previously described for the mixed enzyme preparation except that 4.7 ml 10% aqueous methanol was used for the initial elution.

RESULTS

HPLC/RIA analysis of control and virus-infected larvae

Initial HPLC/RIA analysis of the immunoreactive ecdysteroids present in the hemolymph of fourth-instar female *L. dispar* larvae during the apolysis-associated peak in ecdysteroid titer (Park et al., 1993; 3.5 dpi) revealed ca. 55% of the immunoreactivity co-eluting with a known standard of 20E, 5% co-eluting with E, and 40% present in the highly polar region (Fig. 1A). In contrast, HPLC/RIA analysis of hemolymph collected from LdNPV-infected fourth-instar female *L. dispar* larvae during the same period revealed immunoreactivity restricted to two peaks in a region more polar than 20E but less polar than the highly polar material present in control insect hemolymph (Fig. 1B). This was the only immunoreactive material present following examination of numerous individual or pooled samples.

Preparation of glycosylated standards

To characterize the immunoreactive material in virus-infected larvae, glycosylated ecdysteroid standards were prepared based on previous results (O’Reilly & Miller, 1989; O’Reilly et al., 1991, 1992; Park et al., 1993). Mixing of E or 20 E with UDP-glucose or UDP-galactose in combination with virus-infected cell culture medium generated the required standards. The mixture was eluted through a C18 SEP-PAK, and the glycosylated ecdysteroids were found in the 30% and 60% aqueous methanol eluates (see Materials and methods). HPLC analysis of the fractions from the latter part of the 30% aqueous
methanol eluate and the early part of the 60% aqueous methanol eluate gave single peaks when examined at 245 nm (data not shown). The elution time of the RIA activity shown in Figure 2 corresponded exactly with that of the peak of UV-absorbing material in these fractions. Furthermore, HPLC/RIA of crude, ethanol-extracted cell culture medium from ecysteroid- and sugar-supplemented cultures revealed that the percent conversion of the ecysteroids into glycosylated conjugates in response to EGT was greater than 90%, reaching nearly 100% conversion when UDP-glucose was used as a substrate (Table 1).

Table 1. Percent conversion of E and 20E into glycosylated ecysteroids by virus-infected cell culture medium in the presence of UDP-sugars.

<table>
<thead>
<tr>
<th>Glycosylated ecysteroid</th>
<th>Proportion of total ecysteroid equivalents (%)</th>
<th>Percent conversion (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycosylated</td>
<td>Free</td>
</tr>
<tr>
<td>EGlC</td>
<td>99.8, 99.9, 99.9</td>
<td>0.2, 0.1, 0.1</td>
</tr>
<tr>
<td>EGal</td>
<td>92.6, 98.5, 98.3</td>
<td>7.4, 1.5, 1.7</td>
</tr>
<tr>
<td>20EGLc</td>
<td>99.1, 99.4, 98.4</td>
<td>0.9, 0.6, 1.6</td>
</tr>
<tr>
<td>20EGal</td>
<td>89.9, 99.0, 87.6</td>
<td>10.1, 1.0, 12.4</td>
</tr>
</tbody>
</table>

1Glycosylated ecysteroids were prepared by incubating E or 20E with UDP-glucose or UDP-galactose in the presence of LDNPV-infected cell culture medium as described in the Materials and Methods. 2Ethanol extracts of the incubation mixture were resolubilized in aqueous methanol and analyzed by HPLC/RIA prior to SEP-PAK fractionation as described in the Materials and Methods. Picograms of ecysteroid were determined by RIA using E standards for EGlC and EGal and 20E standards for 20EGLc and 20EGal. Each sample was replicated 3 times.
Enzymatic hydrolysis of glycosylated standards

Enzymatic hydrolysis has been used previously in conjunction with HPLC to partially characterize glycosylated ecdysteroids, specifically a 26-hydroxyecdysone glucoside found in embryonated eggs of *Manduca sexta* (Warren et al., 1986; Thompson et al., 1987) and an EGal found in AcNPV-infected *S. frugiperda* larvae (O’Reilly et al., 1992). Following similar methods, we enzymatically hydrolyzed the glycosylated ecdysteroids described above. With the same enzyme mixture (β-glucuronidase/glucosidase) used by Thompson et al. (1987) (see Materials and methods), the hydrolyzed conjugates gave the products seen in Figure 3 following examination by HPLC/RIA. For EGlc and EGal hydrolysis, the digestion was incomplete, and the RIA activity was found co-eluting with both E and 20E standards. However, the RIA activity co-eluting with the 20E standard was due to contaminating RIA activity present in the β-glucuronidase/glucosidase mixture which co-eluted with 20E (unpublished results). With the 20EGlc and 20EGal standards, the hydrolysis was complete, and RIA activity was found co-eluting with the 20E standard, only.

β-galactosidase digestion was complete with the EGal and 20EGal standards (Fig. 4). As expected, digestion of EGal resulted in RIA activity co-eluting with the E standard, and 20EGal digestion gave RIA activity co-eluting with the 20E standard. No contaminating RIA activity was present in the β-galactosidase mixture at the concentrations used for this study (data not shown). Digestion of the EGlc and 20EGlc standards, on the other hand, showed less than 2% conversion, presumably due to contaminating β-glucosidase activity present in the mixture.
Analysis of the highly polar ecdysteroids in LdNPV-infected hemolymph

With the HPLC elution times of the ecdysteroid standards reproducibly identified and the activity and specificity of the enzymes verified, it was possible to examine the character of the highly polar, immunoreactive ecdysteroids presented in Fig. 1B. When the methanol-precipitated hemolymph was fractionated by HPLC and the immunoreactive fractions [i.e., fractions 10,11 (pooled) and fraction 13] collected, vacuum dried, enzyme digested, and the SEP-PAK-purified products analyzed by HPLC/RIA, the profiles presented in Figure 5 were obtained.

β-glucuronidase/glucosidase digestion of the major immunoreactive peak (fraction 13, Fig. 1B) resulted in an HPLC/RIA profile (Fig. 5A) identical to the profile resulting from the β-glucuronidase/glucosidase digestion of EGlc (Fig. 3A). On the other hand, β-glucuronidase/glucosidase digestion of fractions 10,11 (pooled) (Fig. 1B) did not result in a profile (Fig. 5B) identical to any presented in Figure 3. However, the immunoreactive material co-eluting with 20E in Figure 5B was 100% greater than the amount expected from the contaminating immunoreactivity present in the enzyme mixture. Thus, although the digestion was incomplete, part of fractions 10,11 (pooled) appeared to contain 20EGlc, comparable to the profile presented in Figure 3C. β-galactosidase digestion had no effect on fraction 13 or fractions 10,11 (pooled) (Fig. 5C,D), identical to its effect on the EGlc and 20EGlc standards (Fig. 4A,C), but in contrast to its complete digestion of the EGlc and 20EGlc standards (Fig. 4B,D).
Fig. 5. HPLC/RIA's of the HPLC-purified glycosylated ecdysteroids from the hemolymph of virus-infected larvae (see Fig. 1B) following digestion. A. B – β-glucuronidase/glucosidase digestion of fraction 13 and fractions 10, 11 (pooled), respectively; C, D – β-galactosidase digestion of fraction 13 and fractions 10, 11 (pooled), respectively.

DISCUSSION

The conjugation of various endogenous and exogenous substrates (e.g., drugs, carcinogens, steroids) by UDP-glucuronosyl transferases is a common reaction in mammals, and in insects it seems to involve glucose/UDP-glucosyl transferase rather than the glucuronic acid system of mammals (O’Reilly & Miller, 1989). Glucosylation of ecdysteroids in insects was previously reported and appears to act as an inactivation mechanism (Heinrich & Hoffmeister, 1970; Warren et al., 1986; Thompson et al., 1986, 1988). As expected, EGlc fails to show any feedback inhibition of E synthesis by fourth-instar L. dispar prothoracic glands in vitro in contrast to a 48 percent reduction in the presence of 20E (Park, 1994). Determination of whether or not glycosylated E or 20E have any function in molting will require analysis and comparison of their activity with their unglycosylated forms in vivo molting hormone assays, preferably using fourth-instar larvae of L. dispar.

The isolation of purified, glycosylated ecdysteroid standards in the present study was a fairly simple 2-step purification that involved elution through a $C_{18}$ SEP-PAK followed by HPLC fractionation on a $C_{18}$ reverse-phase column. Although the UV absorbance data (unpublished) suggested a single peak containing a quantity of ecdysteroid, based on extinction coefficient (Warren et al., 1986), consistent with the determined RIA activity, complete purification and chemical identity has not been verified by physicochemical means. Thus, their identification as glycosylated ecdysteroid conjugates is only presumed.
based on information and similar data provided by previous studies, summarized as follows. First, in vitro incubation of unlabelled E or 20E with radiolabelled UDP-glucose or UDP-galactose and culture medium from virus-infected cells results in radiolabelled ecdysteroids with polarities greater than E or 20E and migration rates on thin layer chromatography and HPLC identical to known glycosylated standards (O’Reilly & Miller, 1989; O’Reilly et al., 1991; O’Reilly et al., 1992; Park et al., 1993; Park, 1994). Second, the elution times obtained for the glycosylated ecdysteroids in the present study were expected, based on a previous HPLC study using similar fractionation techniques (O’Reilly et al., 1992). Third, EGT is highly specific for the conjugation of E and 20E with UDP-glucose and UDP-galactose (O’Reilly & Miller, 1989; O’Reilly et al., 1991; O’Reilly et al., 1992). Finally, the antibody is highly specific for ecdysteroids conjugated at the C22 or C26 hydroxyls (Warren et al., 1986; Warren & Gilbert, 1988). Although EGT was also shown to recognize makisterone A (O’Reilly & Miller, 1989) and 26-hydroxyecdysone (Warren et al., 1986; Thompson et al., 1987), no evidence for the existence of makisterone A or the conjugate of 26-hydroxyecdysone, 26-hydroxyecdysone 22-glucoside was found in the present study (unpublished results).

Based on the co-elution of the major immunoreactive ecdysteroid in the hemolymph of virus-infected, fourth-instar L. dispers larvae (Fig. 1B) with the EGlc standard (Fig. 2A) and from the information available from previous studies, it was likely that the major immunoreactive ecdysteroid in virus-infected, fourth-instar hemolymph was EGlc. However, even with the limited amount of material available, further verification was possible by enzymatic digestion studies. Furthermore, HPLC co-elution studies did not verify the identity of the other immunoreactive peak in virus-infected, fourth instar hemolymph (Fig. 1B; fractions 10,11). This material co-eluted with both the EGal and 20EGlc standards (Fig. 2B,C). Therefore, hydrolysis of the conjugates using the β-glucuronidase/glucosidase mixture of Thompson et al. (1987) was used to verify that fraction 13 (Fig. 1B) contained immunoreactive material that would co-elute with E following HPLC (Fig. 5A). The immunoreactivity co-eluting with 20E in this figure could be accounted for as contaminating RIA activity in the enzyme mixture, alone (see Results). Also, β-galactosidase digestion, comparable to the study of O’Reilly et al. (1992), was used to verify that the material in fraction 13 was not galactose conjugated (compare Fig. 4B,D with Fig. 5C).

Digestion studies were used similarly to verify whether or not the immunoreactive material in fraction 10,11 (Fig. 1B) contained EGal or 20EGlc. β-glucuronidase/glucosidase digestion of the EGal standard gave immunoreactivity co-eluting with E and 20E (Fig. 3B), where the immunoreactivity co-eluting with 20E could be accounted for as contaminating RIA activity in the enzyme mixture, alone (see Results). Thus, although the reaction was incomplete, E was the primary product. On the other hand, β-glucuronidase/glucosidase digestion of the 20EGlc standard gave only immunoreactivity co-eluting with 20E (Fig. 3C). β-glucuronidase/glucosidase digestion of virus-infected hemolymph fractions 10,11 gave only immunoreactivity co-eluting with 20E (Fig. 5B), although the reaction was again incomplete, consistent with the idea that at least some of the immunoreactivity in hemolymph fractions 10,11 was due to 20EGlc. The fact that hemolymph fractions 10,11 did not contain material that was galactose-conjugated was verified by the inability of β-galactosidase to digest it (compare Fig. 4B,D with Fig. 5D).
In summary, although enzyme digestion studies by themselves do not always provide a reliable method for confirming the identity of conjugated ecdysteroids because of contaminating enzyme activities (Weirich et al., 1986; Thompson et al., 1987), when used in combination with other methods such as HPLC and RIA, they can provide reliable information (Warren et al., 1986). The present study shows that the reactions using the β-galactosidase preparation progressed to completion under the current conditions (Fig. 4B,D) and that the preparation contained a small amount of glucosidase activity (Fig. 4A,C), as previously reported (O'Reilly et al., 1992). The reactions using the β-glucuronidase/glucosidase mixture also progressed to completion with 20EGlc and 20EGal as substrates (Fig. 3C,D), but ecdysteroids hydroxylated at C20 were probably preferred, since EGlc and EGal were only partially digested under the same conditions (Fig. 3A,B). Similarly, EGT from the cell culture medium of LdNPV-infected gypsy moth cells may prefer UDP-glucose to UDP-galactose since the conversion to EGlc or 20EGlc was 99 percent or greater versus 96 percent or less for EGal and 20EGal when reacted under identical conditions (Table 1). The digestion of fraction 13 from virus-infected, L. dispar fourth instar hemolymph was as complete as that of the standard EGlc, thus making this the first demonstration of a glucose-conjugated ecdysteroid as the major component of hemolymph ecdysteroids in baculovirus-infected larvae. The minor component (fractions 10,11) was not as completely digested as the 20EGlc standard (compare Fig. 3C with Fig. 5B), thus suggesting that it may contain immunoreactive ecdysteroids besides 20EGlc. In any event, as the infection of fourth-instar, L. dispar larvae with LdNPV progresses, EGlc becomes the only immunoreactive component of the hemolymph (Park, 1994).

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