Pergamon

Insect Biochemistry and Molecular Biology 30 (2000) 443–453

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Differential glycosylation produces heterogeneity in elevated esterases associated with insecticide resistance in the brown planthopper *Nilaparvata lugens* Stål

Graham J. Small *, Janet Hemingway

Cardiff School of Biosciences, University of Wales Cardiff, P.O. Box 915, Cardiff CF1 3TL, UK

Received 9 April 1999; received in revised form 29 December 1999; accepted 4 January 2000

Abstract

The major insecticide resistance mechanism in the brown planthopper *Nilaparvata lugens* involves overproduction of esterases. Esterases purified from a resistant strain appeared as a ladder of bands on isoelectric focussing (IEF) gels from pI 4.7 to 5.0. Two-dimensional electrophoresis showed that isozymes ranged in size from 66 to 68 kDa with those of lower pI being apparently smaller. All isozymes detected by two-dimensional electrophoresis were glycosylated. N-glycosidase A reduced the number of isozymes on IEF to two, with increased pI and an increased molecular weight of 69 kDa. No O-linked glycans were detected. Deglycosylation had no effect on esterase activity, hence glycosylation is not involved in active site conformation. As N-glycosidase F completely deglycosylated the esterases, none of the glycans has an α1,3-bound core fucose. Reactivity with the lectins GNA, MAA and DSA, combined with differential cleavage of N-linked glycans with endoglycosidases F1 and F2, indicated that terminally linked mannose is present in high mannose and/or hybrid type glycans and that terminally linked sialic acid and galactose-β(1-4)-N-acetylglucosamine are present in biantennary complexes. Neuraminidase treatment had the same effect on pI of isozymes as complete deglycosylation. Therefore, the majority of the heterogeneity of elevated esterases on IEF is due to differential attachment of sialic acid to glycans of the two proteins.

Keywords: *Nilaparvata lugens*; Carboxylesterase; Insecticide; Resistance; Glycoprotein; Glycosylation

1. Introduction

Nilaparvata lugens, the brown planthopper (BPH), is a major pest of rice in many parts of Asia, with severe infestations causing hopperburn and a reduction in rice yields. As a consequence of intensive chemical control measures, resistance to organophosphorus (OP) and carbamate insecticides has been selected in populations from Japan, the Philippines, the Solomon Islands, Sri Lanka, Indonesia and Japan (Hemingway et al., 1999; Hasui and Ozaki, 1984; Tranter, 1983). The underlying mechanism of resistance to these insecticides, and possibly the pyrethoid permethrin, is an elevation of carboxylesterases (Karunaratne et al., 1999; Chen and Sun, 1994; Chung and Sun, 1983). Chen and Sun (1994) purified and characterised several of the BPH esterase isozymes from Japanese strains. They reported that the esterases had a dual role, hydrolyzing malathion and *trans*-permethrin and sequestering the oxon analogs of the OPs, the oxons acting as poor substrates for the esterases which bind them rapidly but then hydrolyse them slowly. However, a recent study of partially purified BPH esterases from a Sri Lankan strain, whilst confirming their role in sequestering the oxons of OPs, did not detect any interaction with malathion or permethrin (Karunaratne et al., 1999).

When crude homogenates of BPH strains are subjected to native polyacrylamide gel electrophoresis on homogeneous gels, the esterases appear as a single diffuse β-naphthyl acetate specific band (Karunaratne et al., 1999). However, on isoelectric focussing gels the esterases resolve into a number of isozymes (>10) varying in pI from 4.3 to 5.3 (Chen and Sun, 1994; Chang and Whalon, 1987). Characterisation of several of these isozymes suggested some slight variation in their activity towards model esterase substrates but little variation in

* Corresponding author for correspondence. Fax: +44-1222-876807.
E-mail address: smallg@cardiff.ac.uk (G.J. Small).

0965-1748/00/$ - see front matter
PII: S0965-1748(00)00007-2

their interaction with the oxon analogs of organophosphorus insecticides (Chen and Sun, 1994). A decrease in pI of the various isozymes was correlated with a slight increase in molecular weight (Chen and Sun, 1994). From these results it was suggested that all BPH esterase isozymes may represent the products of different post-translational modifications of the nascent protein (Chen and Sun, 1994). In the small brown planthopper *Laodelphax striatellus* Fallén, where a similar complex of esterase isozmes of differing pI is found and where the correlation with molecular weight also holds true, a study of the glycosylation of these isozymes suggested that these polymorphic characteristics were due to differential attachment of N-linked glycans (Sakata and Miyata, 1994). The purpose of this study was to investigate whether the BPH esterases are glycosylated and, if so, whether the nature of the attached glycans could account for the presence of the complex of BPH esterase isozymes and for the observed variation in their physical characteristics.

2. Materials and methods

2.1. Chemicals

Q-Sepharose, phenyl Sepharose, Superdex 75 prep grade, PD10 columns, PhastGels (IEF 4–6.5, Gradient 4–15 and Gradient 10–15) and PhastGel buffer strips (Native and SDS) were purchased from Pharmacia LKB (Uppsala, Sweden). Hydroxylapatite, protein assay reagent and broad range markers for SDS–PAGE were purchased from Bio-Rad Laboratories (Hemel Hempsted, UK). The DIG glycan detection kit; DIG glycan differentiation kit; peptide-N^4-(N-acetyl-β-glucosaminyl) asparigine amidase (N-glycosidase A) from sweet almonds; peptide-N^4-(acetyl-β-glucosaminyl) asparigine amidase (N-glycosidase F) cloned from *Flavobacterium meningosepticum* and expressed in *E. coli*; endo-β-N-acetylglucosaminidase F1 (endoglycosidase F1) cloned from *F. meningosepticum* and expressed in *E. coli*; endo-β-N-acetylglucosaminidase F2 (endoglycosidase F2) cloned from *F. meningosepticum* and expressed in *E. coli* and neuraminidase (neuraminidase) from *Arthobacter ureafaciens* were purchased from Boehringer Mannheim (Mannheim, Germany). Hybond ECL membrane was purchased from Amersham Life Science (Little Chalfont, UK). Amicon Centriprep 10 and Centricon 10 protein concentrator units were purchased from Amicon (Stonehouse, UK). All other reagents were purchased from Sigma Chemical Co. (Poole, UK).

2.2. Insects

Two strains of *N. lugens* were used, both of which were derived from a heterogeneous population sampled from rice plants in Batalagoda, Sri Lanka in 1980. Adult malathion selection at the LT_{80} level for five generations resulted in Sri Lanka-R, a strain homogeneous for an elevated esterase-based organophosphorus insecticide resistance mechanism. A strain homozygous for low esterase activity, Sri Lanka-S, was derived by single family selection of the parental strain for five generations. Sri Lanka-R was 8.5-fold more resistant to malathion than Sri Lanka-S at the LT_{50} level.

2.3. Purification of elevated esterases

The BPH elevated esterases were purified from the Sri Lanka-R strain by a further development of the method of Karunaratne et al. (1999). 4–5 g of adult Sri Lanka-R BPH was used as the starting material for purifications. During purification esterase activity was followed by incubating 10 μl aliqouts of fractions with 200 μl of 1 mM *p*-nitrophenyl acetate (*p*-NPA) in 50 mM sodium phosphate buffer (pH 7.4) in a microtitre plate well. The increase in absorbance at 405 nm was measured continuously for 2 min in a UV*max* microtitire plate reader (Molecular Devices, USA) at 22°C. An extinction coefficient of 6.35 mM^{-1} (taking into account the pH of 7.4 and the path length of 0.6 cm) was used to convert absorbance to millimoles of product in the volume of the reaction. Protein concentrations of the fractions were determined by the method of Bradford (1976) using Bio-Rad protein assay reagent with bovine serum albumin as the standard protein. 10 μl aliquots of fractions were mixed with 300 μl of working solution (prepared according to manufacturer's instructions) and the absorbance measured at 570 nm after incubation for 5 min at 22°C. After *Q*-Sepharose, phenyl Sepharose and hydroxylapatite column chromatography, fractions with esterase activity were pooled and concentrated in an Amicon Centriprep 10 unit to a volume of 2.5 ml. Buffer was exchanged with 25 mM Bistris propane, 0.15 M NaCl pH 7.4 with 10 mM dithiothreitol using a PD10 column according to manufacturer's instructions and the sample further concentrated to a volume of 0.5–1 ml using an Amicon Centricon 10 unit. This was applied to a 2.5×50 cm Superdex 75 column at 0.5 ml/min equilibrated with the BisTris propane buffer. Elution was at 1 ml/min and the eluate was collected in 3 ml fractions. Active fractions were concentrated using Centriprep 10 and Centricon 10 units. An equal volume of glycerol was added, the concentration of dithiothreitol made up to 25 mM and the sample stored at −20°C. Under these conditions, BPH esterases were stable for several months.

2.4. Electrophoresis

To check the purity of samples after purification, an aliquot of BPH esterases was diluted in SDS–PAGE sample buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

To this diluted sample was added SDS to 2.5% and β-mercaptoethanol to 5%. The sample was heated at 100°C for 5 min and bromophenol blue added to 0.01%. After a brief centrifugation to remove any insoluble material, 3 μg of protein was loaded onto a PhastGel gradient 10–15 together with SDS–PAGE standard markers. Following electrophoresis, proteins were visualised using Coomassie Blue R250.

For isoelectric focussing, PhastGel IEF 4–6.5 gels were used. Aliquots of BPH esterase were diluted in distilled water and applied to gels together with IEF standard protein markers. To ensure that none of the bands seen in the purified BPH esterases were artifacts introduced during purification and to study the banding pattern of non-elevated esterases, aliquots of crude homogenates of individual Sri Lanka-R and Sri Lanka-S adults were also applied to IEF 4–6.5 gels. Activities of the purified BPH esterases and of the Sri Lanka-R crude homogenate were adjusted to that of the Sri Lanka-S crude homogenate to achieve similar rates of staining of all samples. Following isoelectric focussing, gels were cut so that the BPH esterases and IEF standards could be stained separately, the former with an esterase activity stain and the latter with a Coomassie Blue R 250 protein stain. The esterase activity stain was 0.04% (w/v) α- and β-naphthyl acetate, 0.1% (w/v) Fast Blue B in 100 mM sodium phosphate buffer, pH 7.4.

Native PAGE was carried out on PhastGel 4–15 gels. Samples of BPH were prepared as for isoelectric focussing. After electrophoresis, esterases were visualised by activity staining as for IEF.

For two-dimensional (2-D) electrophoresis, samples were prepared as for isoelectric focussing and applied to a PhastGel IEF 4–6.5. The PhastSystem was programmed with a sample application step (200 V, 2.5 mA, 3.5 W, 15°C, 30 Vh) (2-D electrophoresis was found to work best without a pre-run step) and a separation step (2000 V, 2.5 mA, 3.5 W, 15°C, 770 Vh). After separation of esterases on the IEF gel, the parts of the gel that the electrodes rested upon were removed and a strip of gel cut corresponding to the lane to which the sample had been applied. This strip was equilibrated for 2 min in 0.112 M Tris/HAc, 1% dithiothreitol and 2.5% SDS followed by 2 min in 0.112 M Tris/HAc, 2.5% SDS, 0.26 M iodoacetamide and 0.001% bromophenol blue adjusted to pH 6.4. The equilibrated strip was placed, gel side down, onto the stacking gel zone of a PhastGel gradient 10–15 gel that had been pre-run (250 V, 2.5 mA, 3.0 W, 15°C, 5 Vh). Standard proteins for SDS–PAGE and a sample of BPH esterases (prepared as for SDS–PAGE) were also applied. Following a sample application step (250 V, 5 mA, 3.0 W, 15°C, 5 Vh), the gel strip was removed and the proteins separated (250 V, 10 mA, 3.0 W, 15°C, 60 Vh). Proteins were then either visualised with Coomassie Blue R250 or were electrophoretically transferred to Hybond ECL membrane.

Semi-dry electrophoretic transfer of proteins was achieved by use of a Bio-Rad Trans-blot SD Semi-Dry Transfer Cell. Ten pieces of Whatman 3 MM filter paper and a Hybond ECL membrane were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). After separating the 2-D PAGE gel from its plastic backing, the gel was equilibrated in transfer buffer and then placed into a blotting sandwich consisting of five pieces of filter paper, the Hybond ECL membrane, the gel and another five pieces of filter paper. Proteins were transferred for 1 h at 20 V. The membrane-immobilised proteins were then ready for the DIG glycan detection procedure.

2.5. Glycan detection using digoxigenin labelling

After washing the membrane in 50 ml phosphate buffered saline (PBS, 50 mM potassium phosphate, 150 mM NaCl, pH 6.5), glycans were visualised using the Boehringer Mannheim DIG Glycan Detection Kit and following manufacturer's instructions. Incubation of the membrane in blocking solution was extended to 2 h as this was found to increase blocking efficiency.

2.6. Glycan differentiation using digoxigenin-labelled lectins

Glycans were differentiated using the Boehringer Mannheim DIG Glycan Differentiation Kit. 1 μg of BPH esterases was dotted onto Hybond ECL membrane together with 1 μg of each of four control glycoproteins: carboxypeptidase Y, having a N-linked 'high mannose' glycan; transferrin, having sialic acid terminally linked α(2-6) to galactose; fetuin, having sialic acid terminally linked α(2-6) and α(2-3) to galactose; and an N-linked galactose-β(1-4)-N-acetylglucosamine; and asialofetuin, having an N-linked glycan with galactose-β(1-4)-N-acetylglucosamine and an O-linked glycan with galactose-β(1-3)-N-acetylgalactosamine. After immobilisation of these proteins on the membrane it was incubated for 2 h at room temperature in blocking solution. After two 10 min washes in 50 ml TBS the membrane was equilibrated in lectin incubation buffer (TBS; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 1 mM $CaCl_2$; pH 7.5). Five digoxigenin-labelled lectins were used for glycan differentiation: *Galanthus nivalis* agglutinin (GNA), which recognises a terminal mannose α(1-3), α(1-6), and α(1-2) linked to mannose in N- and O-linked glycans; *Sambucus nigra* agglutinin (SNA), which recognises sialic acid linked α(2-6) to galactose in N- and O-linked glycans; *Maackia amurensis* agglutinin (MAA), which recognises sialic acid linked α(2-3) to galactose in complex N-linked glycans and the same linkage in O-linked glycans; Peanut agglutinin (PNA), which recognises the O-linked core

disaccharide galactose-β(1-3)-N-acetylgalactosamine; and *Datura stramonium* agglutinin (DSA), which recognises galactose-β(1-4)-N-acetylglucosamine in complex and hybrid N-linked glycans and N-acetylglucosamine in O-linked glycans. Each lectin was supplied as a 1 mg/ml stock in 50 mM Tris/HCl pH 7.0 containing 0.05% sodium azide (w/v). The required amount of lectin stock (for GNA, SNA and DSA 10 μl each, for MAA 50 μl and for PNA 100 μl) was added to 10 ml of the lectin incubation buffer and the membrane incubated in this solution for 1 h. Thereafter, the incubation with anti-digoxigenin-AP and staining of the membrane was as described in the manufacturer's instructions.

2.7. Enzymatic cleavage of N-linked glycans and of sialic acids

The different specificities of N-glycosidase A, N-glycosidase F, endoglycosidase F1, endoglycosidase F2 and neuraminidase were exploited in conjunction with glycan detection, glycan differentiation and electrophoresis to characterise the N-linked glycans of BPH esterases. Whilst N-glycosidase A has the ability to cleave all types of N-linked glycans from proteins, N-glycosidase F can only cleave those N-linked glycans lacking α1,3-bound core fucose residues (Tretter et al., 1991). Endoglycosidase F1 cleaves high mannose type and biantennary hybrid type glycans but not biantennary complex type glycans. N-glycosidase F2 cleaves only biantennary and to some extent high mannose type and biantennary hybrid type glycans (Trimble and Tarentino, 1991). Neuraminidase cleaves terminal N- (or O-) sialic acids which are α2-3, α2-6 or α2-8 linked to glycans (Uchida et al., 1979).

N-glycosidase A was used to cleave N-linked glycans from BPH esterases in both their native form and denatured form. For cleavage of N-linked glycans from the native esterases, 12 μl of esterases (1 μg/μl) in incubation buffer (100 mM citrate/phosphate buffer, pH 5.0) was mixed with 24 μl (0.6 mU) N-glycosidase A (in incubation buffer, glycerol 50% w/v) and 8 μl incubation buffer at 37°C. As a control, the same amount of esterases was incubated in the same buffer minus the glycosidase. Aliquots were withdrawn at 0, 12, 24, 48 and 120 h and frozen at −20°C prior to electrophoresis. 1 μl of each aliquot was run on a PhastGel IEF 4–6.5 and stained for esterase activity as previously described. To detect the presence of glycans attached to the glycosidase-treated BPH esterases, 1 μl of the aliquots withdrawn at 0 and 120 h of glycosidase cleavage and 1 μl of the control incubation (120 h) were dotted onto Hybond ECL membrane and glycan detection performed as previously described. The effect of deglycosylation on esterase activity was investigated by assaying the aliquots from each of the time points of the glycosidase-treated and control esterases for activity as previously described.

The effect of deglycosylation on BPH esterase molecular weights was studied by cleaving N-linked glycans with N-glycosidase A under denaturing conditions. 0.5 μl (0.5 μg) of purified esterases was incubated with 1 μl (50 μU) of N-glycosidase A in 8.5 μl of denaturing incubation buffer (10 mM sodium actetate buffer, 0.5 M sodium thiocyanate, 0.1 M β-mercaptoethanol, pH 5.1) for 24 h at 37°C. As a control, the same incubation was set-up without glycosidase. 200 μg of both deglycosylated and control incubations of BPH esterases were electrophoresed on two SDS–PAGE gels as previously described. One gel was Coomassie stained for proteins and the other transferred to Hybond ECL membrane and glycan detection performed as previously described.

To determine if N-linked glycans had an α1,3-bound core fucose residue, 0.5 μl (0.5 μg) of purified esterases was boiled for 5 min in 8.5 μl of denaturing incubation buffer (20 mM sodium phosphate buffer, 10 mM EDTA, 0.5% (v/v) Nonidet P-40, 0.2% SDS, 1% (v/v) β-mercaptoethanol, pH 7.2), cooled to 37°C, 1 μl (0.2 U) of N-glycosidase F added and the whole incubated at 37°C for 24 h. The control had the same incubation mixture without glycosidase. Both deglycosylated and control incubations of BPH esterases were dotted onto Hybond ECL membrane and glycan detection performed as previously described.

To characterise the structure of N-linked glycans of the BPH esterases, 0.5 μl of a 1 μg/μl stock of purified esterases was boiled for 5 min in 8.5 μl of denaturing incubation buffer (20 mM sodium citrate phosphate buffer, 10 mM EDTA, 0.5% (v/v) Nonidet P-40, 0.2% SDS, 1% (v/v) β-mercaptoethanol, pH 4.75), cooled, 1 μl (0.2 U) of either endoglycosidase F1 or endoglycosidase F2 added and the whole incubated at 37°C for 24 h. Each of the posistive control proteins used in glycan differentiation were treated in the same way to confirm results obtained with the BPH esterases. All incubations were dotted onto Hybond ECL membrane and glycan differentiation performed as previously described.

The effect of removal of terminal sialic acid residues on the pI of BPH esterases was investigated by removal of these residues with neuraminidase. 4 μl (4 μg) of BPH esterases was incubated with 6 μl (60 mU) of neuraminidase and 10 μl 100 mM sodium acetate buffer, pH 5.0 at 37°C. Aliquots were removed at 0, 4, 8 and 24 h and frozen at −20°C. From each aliquot 0.5 μl was electrophoresed on PhastGel IEF 4–6.5 and activity stained as previously described.

3. Results

3.1. Purification of BPH esterases

Esterase purification was achieved using sequential column chromotography on *Q*-Sepharose, phenyl-

Sepharose, hydroxylapatite and Superdex 75 prep grade. An overloaded SDS–PAGE PhastGel 10–15 (3 μg) showed only a broad single band of 67.6±1.0 kDa (SE; *n*=4) (Fig. 1). The specific activity of the purified esterases with *p*-NPA was 11.85 μmol/mg/min giving a purification factor of 21.2 over the crude homogenate. Recovery of activity after purification was 25.2%. This low recovery was at least partly due to the sacrifice of esterase activity in the tails of peaks eluted from columns to ensure purity at the end of the purification procedure.

3.2. Physical charcteristics of BPH esterases

The purified esterases responsible for the elevation of activity appeared as two β-naphthyl acetate specific bands on native gradient PAGE (Fig. 2). One was intensely staining and diffuse, the other, of slightly lower mobility, less intense but tighter. On IEF gels these same esterases appeared as a ladder of five bands ranging in pI from 4.7 to 5.0 (Fig. 3). The same banding pattern on IEF gels was seen for the BPH esterases in crude homogenates of individual Sri Lanka-R and Sri Lanka-S BPH. Therefore, despite the heterogeneity in the physical characteristics of these esterases, they purified together throughout. On 2-D electrophoresis the purified BPH esterases ranged in size from 66 to 68 kDa [Fig. 4(a)], with the esterases having a lower pI being apparently smaller in size.

3.3. Glycans of BPH esterases and the effect of their removal on physical characteristics and enzymatic activity

Glycan detection of Western-blotted, 2-D separated esterases showed that all detectable isozymes were glycosylated [Fig. 4(b)]. Cleavage of N-linked glycans from

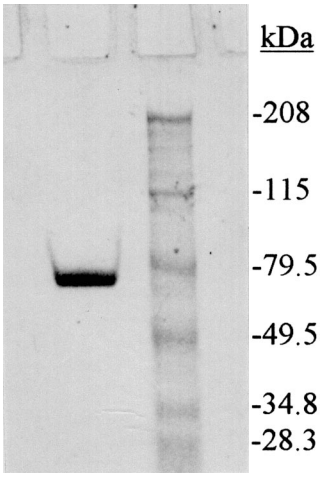

Fig. 1. SDS–PAGE (10–15% gradient gel) of purified brown planthopper esterases. SDS–PAGE standard markers were run in the adjacent lane. Proteins were visualised by Coomassie Blue staining.

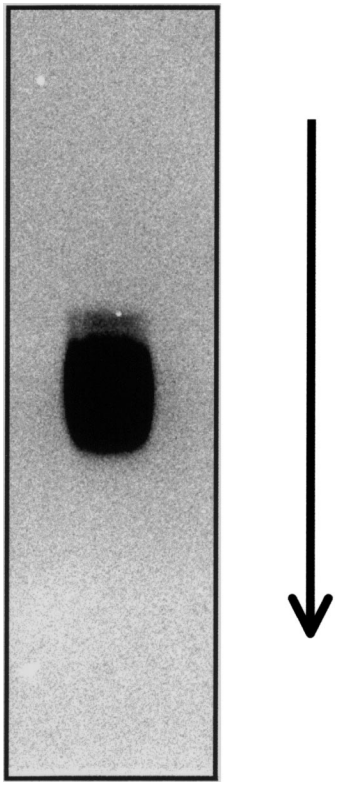

Fig. 2. Native PAGE (4–15% gradient gel) of purified brown planthopper esterases. Esterases were visualised histochemically using 0.04% α- and β-naphthyl acetate as substrates and Fast Blue B stain. The arrow indicates the direction of migration on the gel.

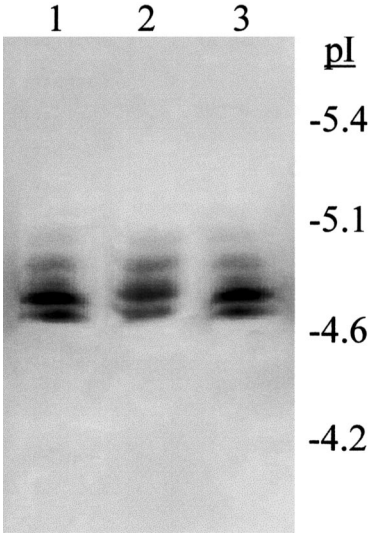

Fig. 3. Isoelectric focussing (pH 4–6.5) of purified Sri Lanka-R brown planthopper esterases (lane 1), crude homogenate of an individual Sri Lanka-R adult (lane 3) and of an individual Sri Lanka-S adult (lane 2).The enzyme activities of purified esterases and the Sri Lanka-R homogenate were adjusted down to that of the Sri Lanka-S homogenate to give equal staining in all lanes. IEF standard markers were run on the same gel for pI estimation. Esterases were visualised histochemically using 0.04% α- and β-naphthyl acetate as substrates and Fast Blue B stain.

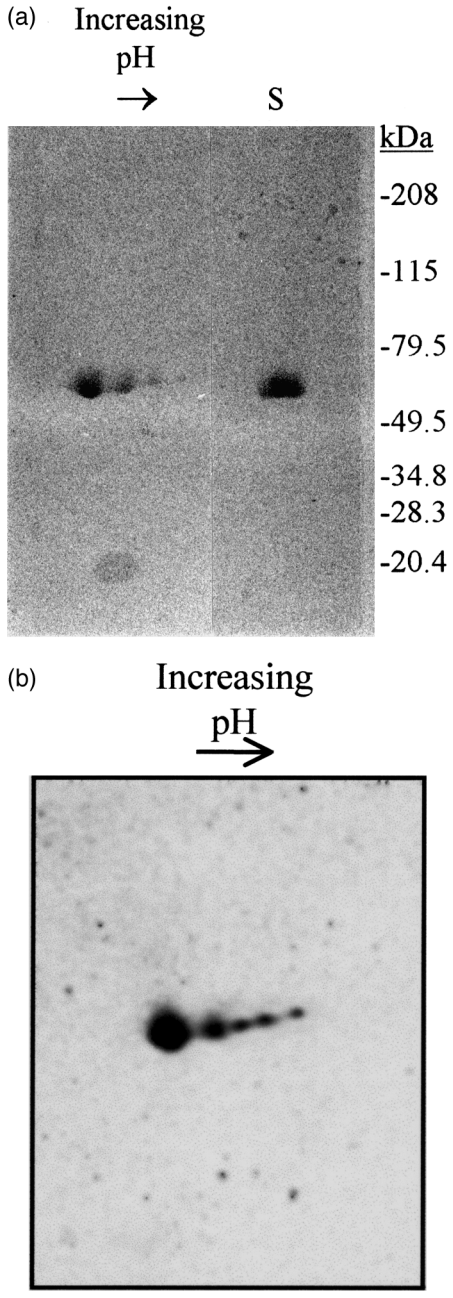

Fig. 4. (a) Two-dimensional electrophoresis [isoelectric focussing (pH 4–6.5) followed by SDS–PAGE (10–15% gradient gel)] of purified brown planthopper esterases, together with the same esterases applied directly to the SDS–PAGE gel (lane S). Proteins were visualised by Coomassie Blue staining. (b) Glycan detection of Western-blotted, two-dimensionally separated purified brown planthopper esterases. Glycans were detected using the Boehringer Mannheim glycan detection kit following manufacturer's instructions. The arrow on each figure shows the orientation of the isoelectric focussing gel strip when applied to the SDS–PAGE gel.

the native form of the esterases with N-glycosidase A reduced the number of esterase bands seen on IEF gels to two and increased their pIs (Fig. 5). Cleavage of N-linked glycans from the denatured form of the esterases increased the apparent molecular weight, as seen on SDS gradient PAGE to 69 kDa [Fig. 6(a)]. Western-blotting of N-glycosidase A treated esterases after separation on SDS gradient PAGE showed that they had been completely deglycosylated [Fig. 6(b)]. Therefore, there are no O-linked glycans attached to the BPH esterases. The percentage remaining *p*-NPA activities of esterases incubated with and without N-glycosidase A were equivalent throughout the 120 h incubation, both glycosidase-treated and control incubations of esterases losing activity at similar rates [Fig. 7(a)]. Hence progressive deglycosylation had no effect on active site conformation. No glycans could be detected in the N-glycosidase A digested BPH esterase following incubation for 120 h [Fig. 7(b)]. Cleavage of N-linked glycans using N-glycosidase F again completely deglycosylated the BPH esterases (Fig. 8). Therefore, none of the N-linked glycans had an α1,3-bound core fucose residue.

3.4. Differentiation of BPH esterase glycans

Posistive reactions were obtained with the digoxigenin-labelled GNA, MAA and DSA [Fig. 9(a–c)] indicating that the N-linked glycans of BPH esterases have terminally linked mannose, sialic acid terminally linked α(2-3) to galactose and terminally linked galactose-β(1-4)-N-acetylglucosamine. The lectins SNA and PNA both gave negative results [Fig. 9(d–e)] indicating that there are no sialic acid residues α(2-6) linked to galactose and confirming that there are no O-linked glycans, galactose-β(1-3)-N-acetylgalactosamine commonly forming the core unit of such glycans. Reactivity with GNA was removed on incubation of BPH esterases with endoglycosidase F1 but not with endoglycosidase F2 indicating that the mannose residues are terminally linked to high mannose and/or hybrid type glycans [Fig. 10(a)]. Reactivity with MAA and DSA was removed by incubation with endoglycosidase F2 but not with endoglycosidase F1 indicating that both the sialic acid α(2-3) linked to galactose and the galactose-β(1-4)-N-acetylglucosamine are terminally linked to biantennary complexes [Fig. 10(b,c)].

3.5. The effect of cleavage of terminal sialic acid residues on the pI of BPH esterases

Removal of terminal sialic acid residues of N-linked glycans by incubation with neuraminidase, as with incubation with N-glycosidase A reduced the number of esterase bands seen on IEF gels to two and increased their pIs (Fig. 11). Therefore, the heterogeneity of BPH esterase isozymes on IEF is due to the presence or absence of sialic acid terminally linked α(2-3) to galactose in biantennary complex glycans. It is not possible to determine the exact number of sialic acid-containing glycans in the two proteins by neuraminidase treatment and the change in pattern on IEF without first purifying

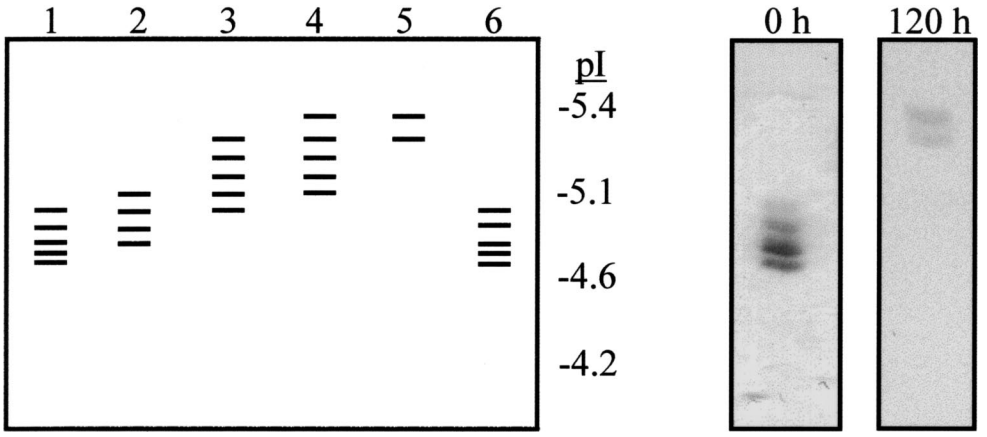

Fig. 5. Diagrammatic representation of the banding pattern of brown planthopper esterases applied to an isoelectric focussing gel (pH 4–6.5) following incubation with N-glycosidase A under native conditions for 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 48 h (lane 4) and 120 h (lane 5) at 37°C. Lane 6 is the pattern of brown planthopper esterases following incubation under the same conditions without glycosidase for 120 h. The inset boxes show the actual banding pattern of these esterases on the isoelectric focussing gel at 0 h and 24 h. Esterases were visualised histochemically using 0.04% α- and β-naphthyl acetate as substrates and Fast Blue B stain.

them away from each other. However, the change in pI of the proteins during such treatment suggests that each protein must contain three or four such glycans.

4. Discussion

Native gradient PAGE of BPH esterases in this study gave two distinct bands whereas a previous study using native PAGE found only one broad band (Karunaratne et al., 1999). This difference in banding pattern is probably due to the different acrylamide gels and buffer systems used. Both the molecular weights (62–64 kDa) and pIs (4.7–4.9) of the BPH esterases reported by Chen and Sun (1994) are slightly lower than those found in this study. However, in both studies the esterases of lower pI had higher apparent molecular weights. Estimated molecular weights for other insect esterases are in the same range as the BPH esterases e.g. esterases E4 and FE4 in the aphid *Myzus persicae* (Devonshire et al., 1986), esterase-C in the fruitfly *Drosophila melanogaster* (Holwerda and Morton, 1983) and esterases E_1, E_2 and E_3 in the brown planthopper *Nilaparvata lugens* (Chen and Sun, 1994) have M_rs of 62 000–66 000. The five esterase isozymes resolved by IEF in this study were fewer in number than the >10 reported by Chen and Sun (1994). This discrepancy may be due to a difference in the resolution of the esterase isozymes by IEF in the two studies as the pH 4 to 6.5 gels used in this study would not have resolved the esterase isozymes to the same extent as the pH 4.5 to 5.4 gels used in the study of Chen and Sun (1994). The pIs of the BPH esterases are all within the range of pH 4.7–6.5 typical of carboxylesterases (Heymann, 1980).

A pattern of isoforms similar to the BPH esterases on IEF gels is seen in the esterases of the small brown planthopper *Laodelphax striatellus* but in this species there is no clear relationship between pI and molecular weight (Sakata and Miyata, 1994). The change in apparent molecular weights of esterases on deglycosylation also differs in the two species, the *L. striatellus* esterases showing a marked decrease (from 70 to 66 kDa) (Sakata and Miyata, 1994) whilst the BPH esterases showed an increase. No analysis of glycosylation has been carried out for the esterases of *L. striatellus*, but these differences suggest that the nature of glycosylation of isozymes is different in the two species.

Glycosylation has been noted in the E4 esterase in the peach-potato aphid *Myzus persicae* (Devonshire et al., 1986), EST6 in *Drosophila melanogaster* (Myers et al., 1996) and the juvenile hormone esterases (JHEs) from *Trichoplusia ni* (Jones et al., 1993; Wozniak and Jones, 1990) and *Heliothis virescens* (Eldridge et al., 1992). In the E4 esterase of *Myzus persicae* (Takada and Murakami, 1988) and both the lepidopteran JHEs several different isoforms exist (Eldridge et al., 1992; Wozniak and Jones, 1990). As in the BPH esterases, the presence of these isoforms in both the JHEs is due to differential glycosylation (Eldridge et al., 1992; Wozniak and Jones, 1990) and the same has been suggested for the E4 esterase (Devonshire et al., 1986). No detailed analysis has been carried out of the glycans attached to these esterases, but digestion of the *Drosophila* EST6 with specific glycosidases indicated that one of the attached glycans was of the N-linked high mannose type whilst three others were more complex N-linked glycans (Myers et al., 1996). The function of these attached glycans has been studied only in the *Drosophila* EST6 esterase in which abolition of some glycosylation sites reduced thermostability but had no effect on secretion, transfer, translocation or activity (Myers et al., 1996). However, activity did not persist for as long in the hemolymph as

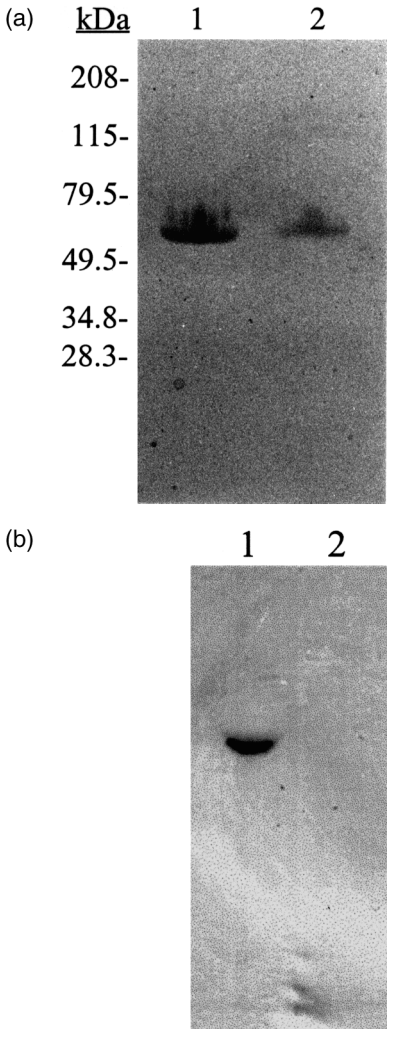

Fig. 6. (a) SDS–PAGE (10–15% gradient gel) of denatured brown planthopper esterases incubated with (lane 2) and without (lane 1) N-glycosidase A for 24 h at 37°C. Proteins were visualised by Coomassie Blue staining. (b) Glycan detection of denatured brown planthopper esterases incubated with (lane 2) and without (lane 1) N-glycosidase A for 24 h at 37°C, run on SDS–PAGE (10–15% gradient gel) and Western-blotted. Glycans were detected using the Boehringer Mannheim glycan detection kit following manufacturer's instructions.

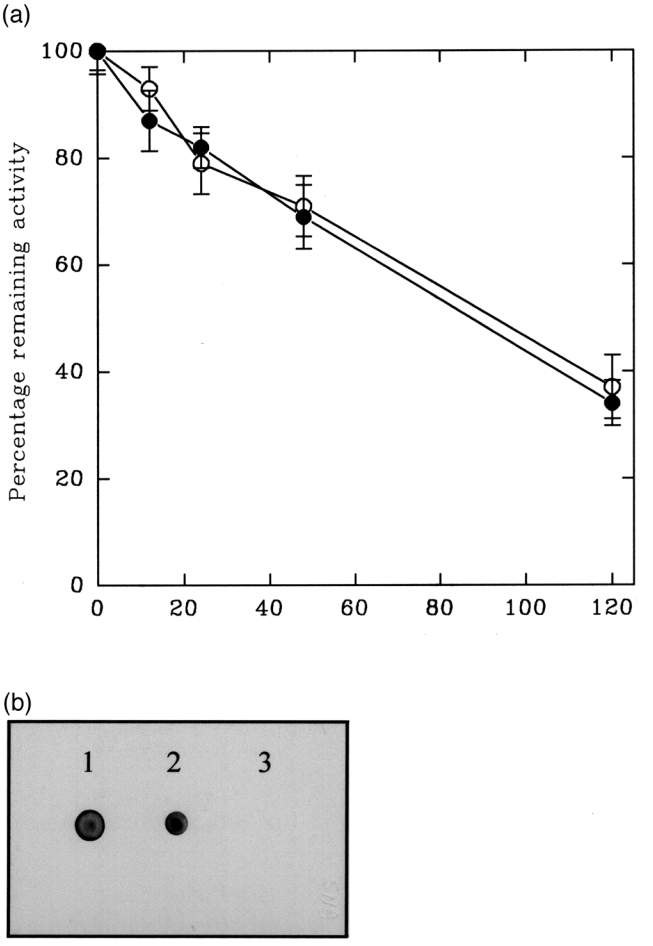

Fig. 7. (a) Mean percentage remaining *p*-nitrophenyl acetate activity of brown planthopper esterases (± standard deviations) incubated with (●) and without (○) N-glycosidase A under native conditions for up to 120 h at 37°C (n=4). 10 μl aliquots of incubations were withdrawn at intervals, mixed with 200 μl of 1 mM *p*-nitrophenyl acetate (*p*-NPA) in 50 mM sodium phosphate buffer (pH 7.4) and the increase in absorbance at 405 nm was measured continuously for 2 min at 22°C. (b) Dot blot of aliquots of the same N-glycosidase A incubations withdrawn at 0 h (sample 1) and 120 h (sample 3). Sample 2 is brown planthopper esterases incubated in the same buffer without glycosidase for 120 h. Glycans were detected using the Boehringer Mannheim glycan detection kit following manufacturer's instructions.

in the fully glycosylated enzyme (Myers et al., 1996). Glycosylation plays a role in protein folding, secretion, activity, thermostability and persistence in other serine hydrolases (Robbi et al., 1996; Sheriff et al., 1995; Morlockfitzpatrick and Fisher, 1995; Benzeev et al., 1994; Kronman et al., 1995; Velan et al., 1993; Abouakil et al., 1993). However, unglycosylated human acetylcholinesterase and human milk bile salt-stimulated lipase, like the deglycosylated BPH esterases, had the same activity as the glycosylated forms (Hernell and Blackberg, 1994; Velan et al., 1993).

In common with other insect glycoproteins, the BPH esterases had high mannose and complex glycans containing sialic acid attached (Altmann, 1996; Davis and Wood, 1995). However, the BPH esterases lacked the α(1,3) fucosylation of the core asparigine-bound galactose-N-acetylglucosamine and O-linked glycans found in some (Cociancich et al., 1994; Bulet et al. 1995, 1993). The effect of differential attachment or removal of sialic acid residues on the physical charactersitics of these insect glycoproteins has not been studied. However, in other glycoproteins, sialic acid-induced heterogeneity is well documented (Poly, 1997; Cousin et al., 1996; Veiga et al., 1995; Isaksson and Hultberg, 1995; Canonne et al., 1995; Yasuda et al., 1992), as is the decrease in heterogeneity following neuraminidase treatment (Poly, 1997; Cousin et al., 1996; Yasuda et al., 1992). For example, neuraminidase treatment of the human serum beta-hexosaminidase, recombinant thyroid-stimulating

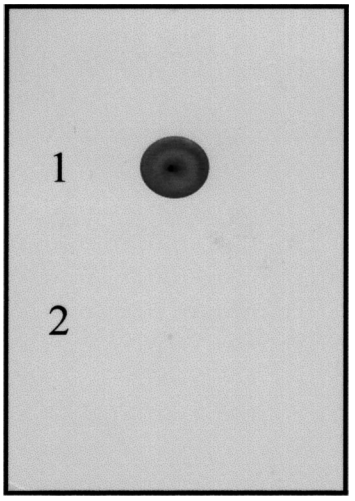

Fig. 8. Dot blot of 0.5 μg of brown planthopper esterases incubated with (sample 2) and without (sample 1) N-glycosidase F at 37°C for 24 h. Glycans were detected using a Boehringer Mannheim glycan detection kit following manufacturer's instructions.

with the presence of two species after complete deglycosylation, suggests there are two distinct BPH elevated esterase proteins. In the small brown planthopper, two proteins were seen after deglycosylation, but in contrast to the BPH where the esterases have the same molecular weight, these deglycosylated forms differed in molecular weight (Sakata and Miyata, 1994). As with the BPH esterases, removal of sialic acids from two porcine intestinal adhesion receptors caused them to migrate more slowly on SDS–PAGE. This suggests that the sialic acids are contributing to the migration of these proteins on SDS–PAGE and that their removal results in a reduced migration (higher apparent molecular weight).

A key step in the linkage of glycans to asparigine residues is the transfer of the oligosacharide from the precursor to the polypeptide. It is commonly found that for each potential glycosylation site transfer may or may not take place. This leads to heterogeneity in the observed physical characteristics of a protein (Shelikoff et al., 1997). Differential glycosylation has been noted in several examples of over-expressed recombinant genes in

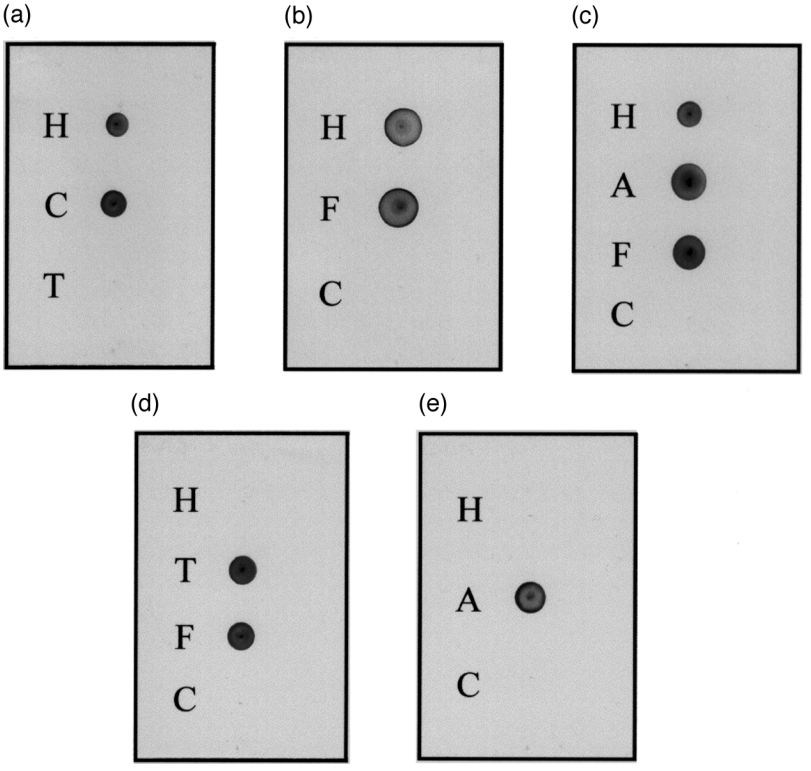

Fig. 9. Dot blots of 1 μg of brown planthopper esterases probed with *Galanthus nivalis* agglutinin (a), *Maackia amurensis* agglutinin (b), *Datura stramonium* agglutinin (c), *Sambucus nigra* agglutinin (d) and Peanut agglutinin (e). 1 μg of the glycoproteins carboxypeptidase Y (C), transferrin (T), fetuin (F) and asiaolofetuin (A) were applied as positive and negative controls. Glycans were differentiated using a Boehringer Mannheim glycan differentiation kit following manufacturer's instructions.

hormone and beta-1 intergrins from mouse melanoma cells converted the several isoforms into a single species of a higher pI indicating a single protein (Veiga et al., 1995; Isaksson and Hultberg, 1995; Canonne et al., 1995). The BPH esterase isoforms were converted into two species after neuraminidase treatment. This, together

insect cell and yeast systems (Sato et al., 1986; Kuga et al., 1986; Geisse and Kocher, 1999), possibly due to a shortage in processing enzymes (Geisse and Kocher, 1999). However, this is not the cause of heterogeneity in the over-expressed esterases in insecticide resistant BPH as the same heterogeneity was observed in the

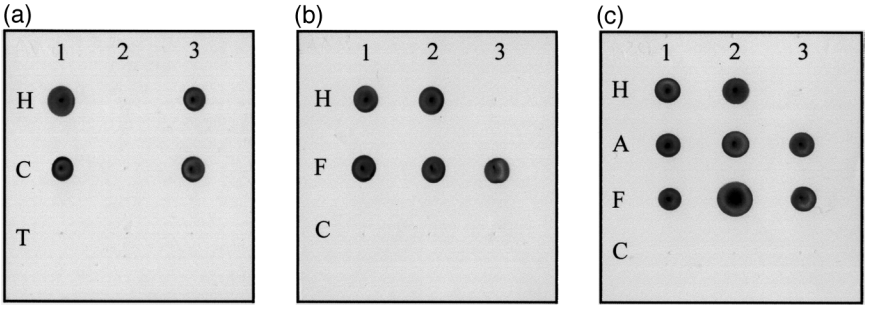

Fig. 10. Dot blots of 0.5 μg of brown planthopper esterases (H) incubated with endoglycosidase F1 (column 2), endoglycosidase F2 (column 3) or without endoglycosidases (column 1) at 37°C for 24 h and probed with *Galanthus nivalis* agglutinin (a), *Maackia amurensis* agglutinin (b), *Datura stramonium* agglutinin (c). 0.5 μg of the control glycoproteins carboxypeptidase Y (C), transferrin (T), fetuin (F) and asiaolofetuin (A) were incubated under the same conditions and applied to the dot blots. Glycans were differentiated using a Boehringer Mannheim glycan differentiation kit following manufacturer's instructions.

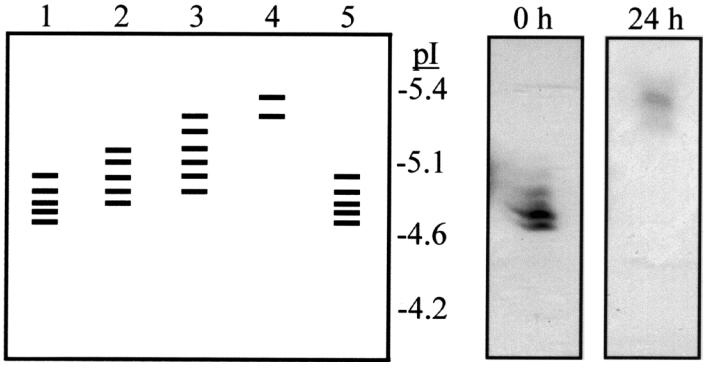

Fig. 11. Diagrammatic representation of the banding pattern of brown planthopper esterases applied to an isoelectric focussing gel (pH 4–6.5) following incubation with neuraminidase under native conditions for 0 h (lane 1), 4 h (lane 2), 8 h (lane 3) and 24 h (lane 4) at 37°C. Lane 5 is the pattern of brown planthopper esterases following incubation under the same conditions without neuraminidase for 24 h. The inset boxes show the actual banding pattern of these esterases on the isoelectric focussing gel at 0 h and 24 h. Esterases were visualised histochemically using 0.04% α- and β-naphthyl acetate as substrates and Fast Blue B stain.

esterases of susceptible insects. Heterogeneity may also be due to major variations in the structure of attached glycans (Rudd and Dwek, 1997). The functional significance of the individual carbohydrate variants is becoming ever more apparent (Rudd and Dwek, 1997). This investigation is part of an on-going project to study the nature of esterase-based insecticide resistance in the BPH. By mutational analysis of N-linked glycosylation of these esterases, the effect of differential glycosylation, and glycosylation in general, on the insecticide binding efficiency and localisation of the BPH esterases will now be investigated.

Acknowledgements

We thank J. Morgan for technical assistance. This work was funded by a BBSRC ROPA award to J.H.

References

Abouakil, N., Mas, E., Bruneau, N., Benajiba, A., Lombardo, D., 1993. Bile salt-dependent lipase biosynthesis in rat pancreatic AR2 J-cells—essential requirement of N-linked oligosaccharide for secretion and expression of a fully active enzyme. J. Biol. Chem. 268, 25755–25763.

Altmann, F., 1996. N-glycosylation in insects revisited. Trends Glycosci. Glyc. 8, 101–114.

Benzeev, O., Stahnke, G., Liu, G.Q., Davis, R.C., Doolittle, M.H., 1994. Lipoprotein-lipase and hepatic lipase—the role of asparigine-linked glycosylation in the expression of a functional enzyme. J. Lipid Res. 35, 1511–1523.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., Hoffmann, J.A., 1993. A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. J. Biol. Chem. 268, 14893–14897.

Bulet, P., Hegy, G., Lambert, J., Van Dorsselaer, A., Hoffmann, J.A., Hetru, C., 1995. Insect immunity—the inducible antibacterial peptide diptericin carries 2 O-glycans necessary for biological activity. Biochemistry 34, 7394–7400.

Canonne, C., Papandreou, M.J., Medri, G., Verrier, B., Ronin, C., 1995. Biological and immunochemical characterization of recombinant human thyrotrophin. Glycobiology 5, 473–481.

Chang, C.K., Whalon, M.E., 1987. Substrate specificities and multiple forms of esterase in the brown planthopper *Nilaparvata lugens* (Stal). Pest. Biochem. Physiol. 27, 30–35.

Chen, W.-L., Sun, C.-N., 1994. Purification and characterization of carboxylesterases of a rice brown planthopper *Nilaparvata lugens* Stål. Ins. Biochem. Mol. Biol. 24, 347–355.

Chung, T.C., Sun, C.N., 1983. Malathion and MIPC resistance in *Nilaparvata lugens* (Homoptera: Delphacidae). J. Econ. Ent. 76, 1–5.

Cociancich, S., DuPont, A., Hegy, G., Lanot, R., Holder, F., Hetru, C., Hoffmann, J.A., Bulet, P., 1994. Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug *Pyrrhocoris apterus*. Biochem. J. 300, 567–575.

Cousin, X., Creminon, C., Grassi, J., Meflah, K., Cornu, G., Saliou, B., Bon, S., Massoulie, J., Bon, C., 1996. Acetylcholinesterase from *Bungarus* venom: a monomeric species. FEBS Lett. 387, 196–200.

Davis, T.R., Wood, H.A., 1995. Intrinsic glycosylation potentials of insect cell cultures and insect larvae. In Vitro Cell. Dev. Biol. 31, 659–663.

Devonshire, A.L., Searle, L.M., Moores, G.D., 1986. Quantitative and qualitative variation in the messenger-RNA for carboxylesterases in insecticide-susceptible and resistant *Myzus persicae* (Sulz). Ins. Biochem. 16, 659–665.

Eldridge, R., O'Reilly, D.R., Hammock, B.D., Miller, L.K., 1992. Insecticidal properties of genetically engineered baculoviruses expressing an insect juvenile-hormone esterase gene. Appl. Envir. Micro. 58, 1583–1591.

Geisse, S., Kocher, H.P., 1999. Protein expression in mammalian and insect cell systems. Meth. Enzymol. 306, 19–42.

Hasui, H., Ozaki, K., 1984. Electrophoretic esterase patterns in the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) which developed resistance to insecticides. Appl. Ent. Zool. 19, 52–58.

Hemingway, J., Karunaratne, S.H.P.P., Claridge, M.F., 1999. Insecticide resistance spectrum and underlying resistance mechanisms in tropical populations of the brown planthopper (*Nilaparvata lugens*) collected from rice and the wild grass *Leersia hexandra*. Int. J. Pest. Manage. 45, 215–223.

Hernell, O., Blackberg, L., 1994. Human milk bile salt-stimulated lipase: functional and molecular aspects. J. Pediatr. 125, S56–61.

Heymann, E., 1980. Carboxylases and amidases. In: Jakoby, W.B. (Ed.), Enzymatic basis of Detoxication, vol. II. Academic Press, New York, p. 291.

Holwerda, B.C., Morton, R.A., 1983. The in vitro degradation of [C^{14}]malathion by enzymatic extracts from resistant and susceptible strains of *Drosophila melanogaster*. Pest. Biochem. Physiol. 20, 151–160.

Isaksson, A., Hultberg, B., 1995. Serum betahexosaminidase isoenzymes are precursor froms. Scand. J. Clin. Lab. Invest. 55, 433–440.

Jones, G., Manczak, M., Wozniak, M., Korrati, R., 1993. Characterization of 2 major isoforms of juvenile-hormone esterase from *Trichoplusia ni* (Lepidoptera). Biochim. Biophys. Acta 1161, 235–243.

Karunaratne, S.H.P.P., Small, G.J., Hemingway, J., 1999. Characterization of the elevated esterase-associated insecticide resistance mechanism in *Nilaparvata lugens* Stal and other planthopper species. Int. J. Pest. Manage. 45, 225–230.

Kronman, C., Velan, B., Marcus, D., Ordentlich, A., Reuveny, S., Shafferman, A., 1995. Involvement of oligomerization, N-glycosylation and sialylation in the clearance of cholinesterase from the circulation. Biochem. J. 311, 959–967.

Kuga, T., Hattori, S., Yoshida, M., Taniguchi, T., 1986. Expression of human T-cell leukemia virus type I envelope protein in *Saccharomyces cerevisiae*. Gene 44, 337–340.

Morlockfitzpatrick, K.R., Fisher, E.A., 1995. The effects of O-linked and N-linked glycosylation on the secretion and bile-salt stimulation of pancreatic carboxyl ester lipase activity. Proc. Soc. Exp. Biol. Med. 208, 186–190.

Myers, M.A., Healy, M.J., Oakeshott, J.G., 1996. Mutational analysis of N-linked glycosylation of esterase-6 in *Drosophila melanogaster*. Biochem. Genet. 34, 201–218.

Poly, W.J., 1997. Nongenetic variation, genetic–environmental interactions and altered gene expression. 3. Posttranslational modifications. Comp. Biochem. Physiol. 118, 551–572.

Robbi, M., Schaftingen, E.V., Beaufay, H., 1996. Cloning and sequencing of rat liver carboxylesterase ES-4 (microsomal palmitoyl-CoA hydrolase). Biochem. J. 313, 821–826.

Rudd, P.M., Dwek, R.A., 1997. Glycosylation: heterogeneity and the 3D structure of proteins. Crit. Rev. Biochem. Mol. 32, 1–100.

Sakata, K., Miyata, T., 1994. Biochemical characterization of carboxylesterase in the small brown planthopper *Laodelphax striatellus* (Fallen). Pest. Biochem. Physiol. 50, 247–256.

Sato, T., Tsunasawa, S., Nikamura, Y., Emi, M., Sakiyama, F., Matsubara, K., 1986. Expression of the human salivary α-amylase in yeast and characterization of the secreted protein. Gene 50, 247–257.

Shelikoff, M., Sinskey, A.J., Stephanopoulos, G., 1997. A modelling framework for the study of protein glycosylation. Biotechnol. Bioeng. 50, 73–90.

Sheriff, S., Du, H., Grabowski, G.A., 1995. Characterization of lysosomal acid lipase by site-directed mutagenesis and heterologous expression. J. Biol. Chem. 270, 27766–27772.

Takada, H., Murakami, Y., 1988. Esterase variation and insecticide resistance in japanese aphis-gossypii. Ent. Exp. Applic. 48, 37–41.

Tranter, B.C., 1983. Elucidation of insecticide resistance mechanisms in the brown planthopper *Nilaparvata lugens*. PhD thesis, University of Reading.

Tretter, V., Altmann, F., Marz, L., 1991. Peptide-N^4-(N-acetyl-b-glucosaminyl) asparigine amidase F cannot release glycans with fucose attached a-3 to the asparigine-linked N-acetylglucosamine residue. Eur. J. Biochem. 199, 647–652.

Trimble, R.B., Tarentino, A.L., 1991. Identification of distinct endoglycosidase (endo) activities in *Flavobacterium meningosepticum*: Endo F1, Endo F2 and Endo F3. J. Biol. Chem. 266, 1646–1651.

Uchida, Y., Tsukada, Y., Sugimori, T., 1979. Enzymatic neuraminidases from *Arthrobacter ureafaciens*. J. Biochem. 86, 1573–1585.

Veiga, S.S., Chammas, R., Cella, N., Brentani, R.R., 1995. Glycosylation of beta-1 integrins in B16-F10 mouse melanoma cells as determinant of differential binding and acquisition of biological activity. Int. J. Cancer 61, 420–424.

Velan, B., Kronman, C., Ordentlich, A., Flashner, Y., Leitner, M., Cohen, E., Shafferman, A., 1993. N-glycosylation of human acetylcholinesterase: effects on activity, stability and biosynthesis. Biochem. J. 296, 649–656.

Wozniak, M., Jones, G., 1990. Glycosylation and isoform variation of juvenile-hormone esterase in the fat-body and hemolymph during metamorphosis of *Trichoplusia ni* (lepidoptera). Mol. Cell. Endocrin. 70, 255–262.

Yasuda, T., Nadano, D., Awazu, S., Kishi, K., 1992. Human urine deoxyribonuclease II (DNase II) isoenzymes: a novel immunoaffinity purification, biochemical multiplicity, genetic heterogeneity and broad distribution among tissues and body fluids. Biochim. Biophys. Acta 1119, 185–193.