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ARTICLE *in* INSECT BIOCHEMISTRY AND MOLECULAR BIOLOGY · JANUARY 2002

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Purification of juvenile hormone esterase and molecular cloning of the cDNA from *Manduca sexta*

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Received 9 October 2000; received in revised form 26 March 2001; accepted 28 March 2001

Abstract

Juvenile hormone esterase (JHE) is a highly specific enzyme important for regulating the onset of metamorphosis in lepidopteran insects. After affinity chromatography of the hemolymph proteins of *Manduca sexta*, the pure JHE protein was digested with Lys-C and the resultant peptides were purified by microbore HPLC. Two peptides were selected for sequencing. Based upon these amino acid sequences, degenerate RT-PCR was performed in order to amplify a partial cDNA sequence from mRNA from the fat body of *M. sexta*. A 1512 bp partial cDNA was generated and found to be highly homologous to the JHE from *Heliothis virescens*. 5′ and 3′ RACE were performed to obtain the full length cDNA sequence. The cDNA has a total length of 2220 bp, with a 1749 bp coding region. The deduced protein sequence contains 573 amino acids.

Keywords: *Manduca sexta*; Juvenile hormone; Esterase; cDNA; Affinity purification; PCR

1. Introduction

In holometabolous insects, juvenile hormone (JH) is of crucial importance in the regulation of larval development. In the final larval instar, the methyl ester form of JH must be cleared effectively if successful pupation is to occur (Riddiford, 1980). In addition to a decrease in JH synthesis, there is also a dramatic increase in JH hydrolysis. In lepidopterans (moths and butterflies), juvenile hormone esterase (JHE) has been demonstrated to be the principal enzyme responsible for the inactivation of JH at this point in development (reviews: Hammock, 1985; Roe and Venkatesh, 1990). Most of the biological activity attributed to JH appears due to the parent methyl ester. However, there is increasing evidence that JH acid may have specific regulatory roles at key times in development (Ismail et al., 1998). In this case, JHE can be considered as a biosynthetic enzyme. Because of the profound role which this enzyme plays both physiologically and endocrinologically, its usefulness as a biopesticide has been explored. JHE from *Heliothis virescens* (Lepidoptera: Noctuidae) has been cloned (Hanzlik et al., 1989) and expressed in a nuclear polyhedrosis virus (Hammock et al., 1990). Recombinant nuclear polyhedrosis viruses expressing modified forms of JHE (AcJHE) have been shown to increase the rate at which the virus kills its larval host as compared to wild type AcNPV (Bonning et al., 1995).

One limiting factor in the effectiveness of the AcJHE virus is that the enzyme is unstable in vivo. When injected into larvae, or expressed in vivo, JHE is actively taken up by pericardial cells via receptor mediated endocytosis, thus leading to a very short half life in vivo (Ichinose et al., 1992a,b; Booth et al., 1992; Bonning et al., 1997). It has been observed that recombinant JHE from *H. virescens* displays this specific recognition and uptake phenomenon when it is injected into the larvae of *Manduca sexta* (Lepidoptera: Sphingidae) (Ichinose et al., 1992a). This implies that there are factors important for recognition present on the JHE protein from both species, though they are from different families within the lepidopteran order. It was our interest to purify and clone the JHE from *M. sexta* with the goal in mind that these two JHE proteins could be studied together in order to ascertain the motifs that are conserved within the proteins' sequences. JHE has been previously purified from *M. sexta* as shown in several

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0965-1748/01/$ - see front matter
PII: S0965-1748(01)00079-0

reports (Coudron et al., 1981; Abdel-Aal and Hammock 1985, 1986; Hammock et al., 1988; Venkatesh et al., 1990) but not cloned. Other cDNA clones have been reported within the Noctuid and Tortricuid families (Hanzlik et al., 1989; Jones et al., 1994; Feng et al., 1999) of lepidoptera but not from Sphingidae. We report here the sequencing of peptides from homogeneous JHE purified from final larval instar *M. sexta*, and the subsequent cloning of the full length cDNA corresponding to this protein. The sequences of JHE from the lepidopteran species are compared.

2. Materials and methods

2.1. Manduca sexta rearing and dissection

Eggs of *M. sexta* were obtained from Carolina Biological Supply. Hatched eggs were reared on an artificial diet from Bioserv (Frenchtown, NJ). The larvae were maintained at 26°C on a 14L:10D photoperiod. Individuals were selected by weight from 5th instar, day 3 and treated by topical application of 100 μg methoprene dissolved in ethanol. Topical application of juvenoid analogs at this stage has shown an increase in JHE activity (Sparks et al., 1983). Treated individuals were then dissected 12 h later for fat body extraction or 24 h later for hemolymph extraction.

2.2. JHE assay

JH hydrolysis was assayed by the method of Hammock and Sparks (1977). This assay uses tritiated JH III (New England Nuclear Research Products, Boston, MA: 17 Ci/mmol), the metabolite of which is partitioned into an aqueous phase after organic extraction and then detected in a scintillation counter. The labeled JH III is diluted in cold JH III (Sigma) and brought to a final concentration of 5 mM in ethanol. One microliter of this JH solution is added to a 100 μl solution of diluted enzyme in purification buffer, and then incubated at 30°C for 15 min. Enzyme assays were performed in duplicate or triplicate.

2.3. JHE protein purification

JHE was purified by affinity chromatography, with modifications to the technique previously described by Abdel-Aal and Hammock (1986). This technique utilizes a transition state analog of JH, 3-(4-mercaptobutylthio)-1,1,1-trifluoropropan-2-one (MBTFP, $HSC_4H_8SCH_2C(O)CF_3$), which is linked to Sepharose beads. The enzyme is purified by binding to the MBTFP gel, and then eluted with another strong JHE inhibitor, 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP, $C_8H_{17}SCH_2C(O)CF_3$). The affinity ligand, MBTFP, and the eluting inhibitor, OTFP, were synthesized in this laboratory as previously described by Abdel-Aal and Hammock (1986), and by Hammock et al. (1984), respectively.

Hemolymph was collected from each insect by clipping the dorsal horn and holding the insect over a collection tube containing some purification buffer. Purification buffer consisted of 100 mM $NaPO_4$ (Fisher), pH 7.4, 0.05% Triton X-100 (Sigma), 0.02% sodium azide (Sigma), 5% sucrose (Fisher), 1 mM mercaptoethanol (Aldrich), with a few crystals of phenylthiourea (Aldrich). Each hemolymph sample was diluted 1:5 with purification buffer, centrifuged at 1000*g* for 5 min, filtered through glass wool and stored at −85°C until enough samples were ready for large scale purification. Samples were later thawed and pooled. Affinity purification was performed in a glass Econocolumn (ID=2.5 cm, *l*=10 cm) from BioRad. Five hundred microliters of affinity gel was placed into 50 ml of ethanol, and then after the ethanol was removed the gel was washed successively with 75% ethanol, 50% ethanol, 25% ethanol, pure water, and then with purification buffer.

The thawed hemolymph sample was preincubated with 2×10^{-4} M diisopropylfluorophosphate (DFP, Sigma) for 30 min at room temperature to inhibit general esterase activity. Comparison of JHE activity before and after DFP incubation showed that it is not significantly inhibited by DFP. This sample was then loaded onto the column and the enzyme bound to the affinity gel as the column was rotated at 4°C for several hours. Every 2 h, a small aliquot was removed and assayed for JHE activity to determine binding. After 8 h, greater than 95% of the JHE activity was bound to the affinity gel. The gel was transferred to a small glass vial containing purification buffer with 10^{-3} M OTFP. This sample was placed on a rotary shaker for 12 h at 4°C to remove enzyme from the affinity gel. Eluted enzyme sample was removed by letting the affinity gel settle in the vial and removing the eluate. This process was repeated five times until no detectable protein was eluted from the affinity gel. An aliquot of the enzyme bound to the inhibitor OTFP was reactivated by dialyzing in the presence of 100 μg/ml bovine serum albumin (BSA, Sigma) at 4°C against 1 liter of purification buffer. The buffer was replaced daily for 6 days.

2.4. Protein analysis

Total protein in each sample was determined by the Bradford assay using a kit from BioRad. Protein samples were analyzed by either SDS-PAGE or isoelectric focusing. For SDS-PAGE, precast 8–16% gradient polyacrylamide gels (Novex) were used. Isoelectric focusing was performed on precast gels from Novex. Replicate samples were loaded on each gel such that one lane could be stained with Coomassie Brilliant Blue (or by silver stain) and the other analyzed for JHE activity. To

recover sample for the JHE assay, the lane was sliced into approximately 5 mm samples and placed into 100 μl of purification buffer and allowed to elute at 4°C for several hours. Enzyme samples were loaded in offset lanes to prevent artifacts from diffusing. PAGE gels were stained with Coomassie Brilliant Blue G-250, by the methods described in Neuhoff et al. (1988). Silver staining was performed using the GelCode Silver Stain SNAP Stain Kit from Pierce. Molecular weight markers for SDS-PAGE were purchased from Novex (Mark 12 Wide Range Protein Standard). Purified proteins with documented p*I*s (carbonic anhydrase (bovine erythrocytes), myoglobin (equine heart) and trypsin inhibitor) were purchased from Sigma and used as IEF standards.

2.5. Protein digestion and peptide sequencing

Ten micrograms of pure JHE protein was loaded onto an SDS-PAGE gel which was stained and destained with Coomassie Brilliant Blue as described above. From the gel, the JHE band was excised with a razor blade and washed five times with 15 ml water. The band was then cut into small 1–2 mm sections and vacuum dried. Rehydration was done in two gel volumes (200 μl) of 100 mM Tris–HCl, pH 7.9, and 0.01% SDS containing 0.1 μg of Lys-C (Boehringer Mannheim) at 37°C for 16 h. The peptides were then removed from the gel by extracting with 100 μl water four times followed by an extraction in acetonitrile. These combined extracts were dried down in a Speed-vac. The pellet was resuspended in 100 μl water and then 5 μl of trifluoroacetic acid (Applied Biosciences) was added. The solution was extracted twice with an equal volume of organic solvent (20% isoamyl alcohol (Sigma), 80% heptane (Applied Biosciences)). Acetonitrile (100 μl) was added to the aqueous layer and it was then dried down. The dry pellet was resuspended in 25 μl of a 8 M urea, 400 mM Tris, pH 7.9 solution, 20 mM dithiothreitol. After heating for 15 min at 55°C, iodoacetamide was added to a final concentration of 40 mM and incubated for 15 min at room temperature. Water was then added to bring the volume to 100 μl, 1 μg of Lys-C was added and incubated at 37°C for 16 h. The resultant peptides were fractionated on a 1 mm by 10 cm reverse phase C-18 microbore HPLC column (ABI 172 Model 140B). Using a two solvent system, the chromatography began with 92% solvent A (0.1% TFA), 8% solvent B (0.075% TFA, 70% acetonitrile) with the percentage gradually altered to 100% solvent B by 105 min. Two peptides from the HPLC fractions were selected for sequencing. Sequencing was done at the UC, Davis Protein Structure Laboratory using an Applied Biosystems 470A Sequencer.

2.6. Synthesis of cDNA and partial cDNA cloning

Total RNA was isolated from the fat bodies of larvae of *M. sexta* using TriReagent (Molecular Research Center) as per manufacturer's instructions. From total RNA, mRNA was subsequently purified using PolyA Tract mRNA isolation kit (Promega). Fat bodies were dissected from L5D3 larvae which had been treated with 100 μg methoprene 12 h prior to dissection. From approximately 500 mg of wet tissue, the yield was 6.2 μg mRNA. Some mRNA from this sample (500 ng) was used to make a first strand cDNA using JHE specific oligonucleotide primer (P23RT) for a reverse transcriptase reaction. This was followed by two rounds of PCR using degenerate oligonucleotide primers. A nested PCR strategy was used based upon the amino acid sequences shown in Table 2 and also indicated in Fig. 3. In this strategy, the second set of PCR primers are aligned slightly to the interior of the first set of PCR primers (according to the proximal amino acids within the same peptide) so that the correct product from the first amplification could be reamplified with increased specificity. P23RT was based upon the C-terminal fragment (VYQNVK) of peptide 23. The sequence in the antisense orientation was 5′-NACG/ATTC/TTGA/GTAI ACA/GTC-3′. Five hundred nanograms of mRNA was hybridized with 10 pmol of the P23RT oligonucleotide in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) in a total volume of 5 μl. The mixture was incubated at 70°C for 5 min, cooled slowly to 46°C and then placed on ice. The RNA:oligonucleotide hybrid mixture was then mixed in a 20 μl reaction containing 20 U/μl Superscript Reverse Transcriptase II (Gibco Life Technologies), 2 U/μl of RNasin ribonuclease inhibitor (Promega), and Reverse Transcriptase buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 2 μM DTT) (Gibco Life Technologies) and 0.25 mM dNTP (TaKaRa). The reverse transcriptase reaction was carried out at 42°C for 50 min followed by 50°C for 20 min. A 0.5 μl aliquot of this first strand reaction was used for the first round of PCR amplification in a 100 μl reaction which contained 100 pmol each of primer P23-1 and PNT-1, 2.5 U of Taq DNA Polymerase (TaKaRa), 0.2 mM of each dNTP (TaKaRa), and Taq polymerase buffer (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) (TaKaRa).

P23-1 (5′-TCNGGIGTG/ATCT/G/AATG/ATCT/CTG-3′) is based upon the middle of peptide 23 (QDIDTPD) and PNT-1 (5′-GAA/GGTNGTIGTIA/CGI ACIG-3′) is based upon an N-terminal fragment of the N-terminal sequence (EVVVRTE). Thermocycling parameters were 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min. The thermocycling was performed in a PTC-100 thermocycler from MJ Research, Inc. The second round of PCR was run under the same conditions as the first round except that 2 μl of the first round was

used as template and the primers were replaced with oligos 23-2 (5′-G/ATCC/TTGA/GTAICT/GIAG/AG/ATTG/ATC-3′), based upon the peptide fragment DNLRYQD, and NT-2 (5′-A/CGIACNIAA/GA/TG/CIGGIUGG-3′), based upon the peptide fragment RTESGW. The products from this amplification were resolved on an agarose gel and individual bands were cut out and cloned into pPCR-Script Amp SK(+) plasmid according to the manufacturer's instructions using the PCR-Script Amp Cloning Kit (Stratagene). The recombinant plasmid, p23-1, was amplified in *Epicurian coli* XL1Blue MRF′ Kan cells. The p23-1 DNA was purified from the recombinant bacteria by the protocol of Sambrook et al. (1989). The subcloned PCR product was sequenced from p23-1 DNA using an ABI sequencing machine (Perkin–Elmer). The deduced protein sequence from this partial cDNA showed a high level of identity to JHE from *H. virescens* (Hanzlik et al., 1989). This partial cDNA sequence was then used for obtaining the full length sequence by the following Rapid Amplification of cDNA End (RACE) methods.

2.7. 3′RACE (rapid amplification of cDNA end)

All reagents used for 3′RACE cDNA synthesis and amplification were supplied by TaKaRa Biomedical, with the exception of the primer MsXho1S (Gibco Life Technologies). Prior to amplification, a reverse transcription reaction was performed using an oligo-dT primer with adapter sequence at its 5′ end. The 20 μl reaction contained 548 ng of mRNA, 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 5 mM $MgCl_2$, 5 mM each dNTP, 0.25 U of AMV reverse trancriptase, 1 U/μl RNase inhibitor, 0.125 μM oligo-dT primer (5′-CTGATCTAGAGGTACCGGATCC-polyT-3′). The reaction was performed at 30°C for 10 min, 50°C for 30 min, and 95°C for 5 min. Following the first strand cDNA synthesis, amplification was performed using a sense primer (MsXho1S: 5′-CCGTTCTCTTCTCGAGTGCTCC-3′) corresponding to the 3′ region of the partial clone (p23-1) and the adapter primer (5′-CTGATCTAGAGGTACCGGATCC-3′) corresponding to the 5′ end of the oligo-dT primer used in the first step. The 100 μl reaction contained 10 μl of the first reaction as template, with 0.025 U/μl Ex-Taq DNA polymerase, 1X Ex-Taq PCR buffer (TaKaRa), 0.4 mM each dNTP, 0.2 μM MsXho1S, 0.2 μM adapter primer. Thermocycling parameters were 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The products from this PCR reaction were analyzed as described above.

2.8. 5′RACE (rapid amplification of cDNA end)

Double stranded cDNA was used for this experiment, which was synthesized using CLONTECH Capfinder cDNA synthesis kit, but 200 U Superscript II RNase H-Reverse Transcriptase (Gibco Life Technologies) was substituted as the reverse transcriptase enzyme. The mRNA (50 ng) from *M. sexta* fat body was used as the template for cDNA synthesis as per manufacturer's instruction. The cDNA from this kit has a priming site included at the 5′ end of each cDNA after a PCR amplification by virtue of the use of the Capfinder oligonucleotide which is used during the initial cDNA synthesis step (see CLONTECH manual). The cDNA from this reaction was then used for two rounds of DNA synthesis using specific primers as follows. The first reaction was a single primer DNA polymerase reaction in which an antisense primer (Ms1285AS), corresponding to the 5′ region of p23-1, was extended for 36 cycles in order to enrich the following PCR reaction with the JHE sequence for template, since the PCR reaction would have one primer (5′PCR primer) as a non-specific primer opposing a nested antisense primer (Ms1083AS). JHE primers were synthesized by Gibco Life Technologies. The first single primer reaction used 1.5 μl of the amplified Capfinder cDNA as template. A 100 μl reaction contained 0.8 μM Ms1285AS, 2.5 U of rTaq DNA polymerase (TaKaRa), 0.2 mM each dNTP (TaKaRa), 10 mM Tris–HCl (pH 8.3) (TaKaRa), 50 mM KCl (TaKaRa), 1.5 mM $MgCl_2$ (TaKaRa). The reaction was run for 36 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min. Then 3 μl of this JHE enrichment reaction was used in the final RACE PCR reaction. The 100 μl reaction contained the same contents as the previous reaction except for the template and primers: 0.4 μM Ms1083AS, 0.4 μM 5′PCR primer. The reaction was run for 31 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 2 min. The PCR product was analyzed as described above.

3. Results

The results of affinity purification are shown in Table 1. The starting material was 50 ml of hemolymph, containing 1056 U of JHE activity (nmol JH III hydrolyzed min^{-1}) with specific activity at 1.08 U/mg protein. The OTFP eluate contained 665 U of JHE activity, and the final specific activity was 567 U/mg protein. Thus, the purification factor was 525 with 63% recovery of enzyme activity. SDS-PAGE analysis of crude hemolymph and pure JHE protein is shown in Fig. 1. A single band appears in the OTFP eluate at an approximate MW of 63,000 Da. The purified material was also run on an IEF gel. One major band appears at wide range IEF after staining with Coomassie Brilliant Blue at approximately 6.0. More sensitive protein detection by silver staining reveals a few minor bands at around 5.8–5.6. A replicate lane which was not stained was sliced and assayed for JHE activity in each slice. One lane was loaded with pure JHE dialyzed in purification buffer in the presence of BSA, and another lane was loaded with hemolymph

Table 1
Affinity purification of JHE from the hemolymph of *M. sexta*

	Volume (ml)	Total protein (mg)	Total activity (nmol JH III min^{-1})	Specific activity (U/mg protein)	Yield activity (%)	Purification factor
Hemolymph	50	978	1056	1.08	100	1
OTFP eluate	24	1.174	665.3	566.9	63	525

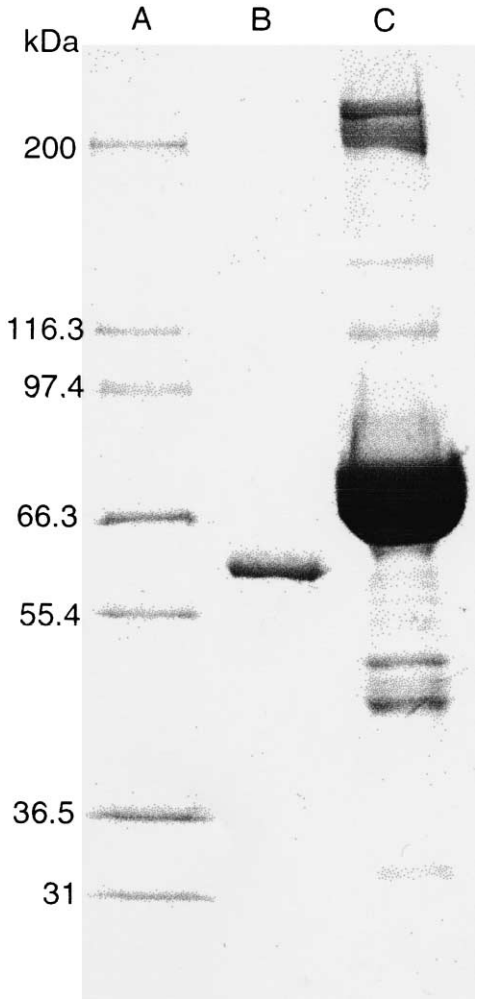

Fig. 1. SDS-PAGE analysis of affinity purified JHE and hemolymph following 1000*g* spin. Samples were run on a Novex precast SDS-PAGE gel (8–16% gradient), and stained with Coomassie Brilliant Blue G-250 (Neuhoff et al., 1988). Lane A contains 10 µl of Novex Mark 12 Wide Range Protein Standard. The molecular weights of standard proteins are indicated next to lane A. Lane B contains 4 µg JHE protein (OTFP eluate). Lane C contains 20 µg hemolymph protein.

from which the JHE protein was purified. From the affinity eluate and hemolymph, 84 and 77%, respectively, of the loaded JHE activity was recovered from the lanes. Peaks in JHE activity from both samples coincide with the same p*I* as the protein bands that are stained by silver stain (Fig. 2).

The 63,000 Da protein band on the SDS-PAGE was sliced and digested with Lys-C. The resultant peptides were chromatographed by microbore HPLC. Three peaks were selected from the HPLC chromatograph and submitted to the UC, Davis Protein Structure Facility for protein sequence analysis by Edman degradation. One of the peptides (peptide 27) was identical to the previously published N-terminal sequence (Hammock et al., 1988), and so was sequenced only to the 10th amino acid residue. The other two amino acid sequences were from peptide 23 and peptide 31. The sequence of each peptide is shown in Table 2. In addition, these sequences are underlined in the deduced protein sequence of the resultant cDNA clone shown in Fig. 3.

Degenerate oligonucleotides were designed corresponding to the peptide 23 sequence, and also to the N-terminal protein sequence (Hammock et al., 1988). Using mRNA purified from the larvae of *M. sexta* as a template, a first antisense strand of cDNA was synthesized using primer P23RT as a primer for reverse transcriptase. This single strand cDNA was then used as the template for two rounds of PCR. The first round was done using primers PNT-1 and P23-1. The products from this reaction comprised several bands on an agarose gel stained with ethidium bromide (data not shown). To increase specificity, a second round of PCR was performed with nested primers PNT-2 and P23-2. This reaction resulted in a single band which was also present in the product from the first PCR reaction. This band was purified from an agarose gel and cloned into the pPCR-Script vector (Stratagene). The sequence of the insert gave a 1512 bp DNA sequence. The deduced amino acid sequence of this clone indicated successful partial cloning and corresponded to the purified JHE protein, evidenced by the presence of peptide 31 and amino acid residues within the N-terminal peptide and peptide 23 which were downstream and upstream (respectively) of the primer sequences used for PCR (see Fig. 3). The deduced protein sequence also shows high homology to the JHE from *H. virescens* (Fig. 4), as discussed later in this report.

In order to obtain the full length cDNA sequence, the sequence from this partial cDNA clone was used to design primers in order to perform RACE. One 5′RACE product was obtained which overlapped with the partial clone by 1083 bp, and a 3′RACE clone was obtained which overlapped the partial clone by 464 bp. After the sequencing of these clones, primers were designed based upon the 5′ and the 3′ ends so that the cDNA could be reamplified by RT-PCR and resequenced.

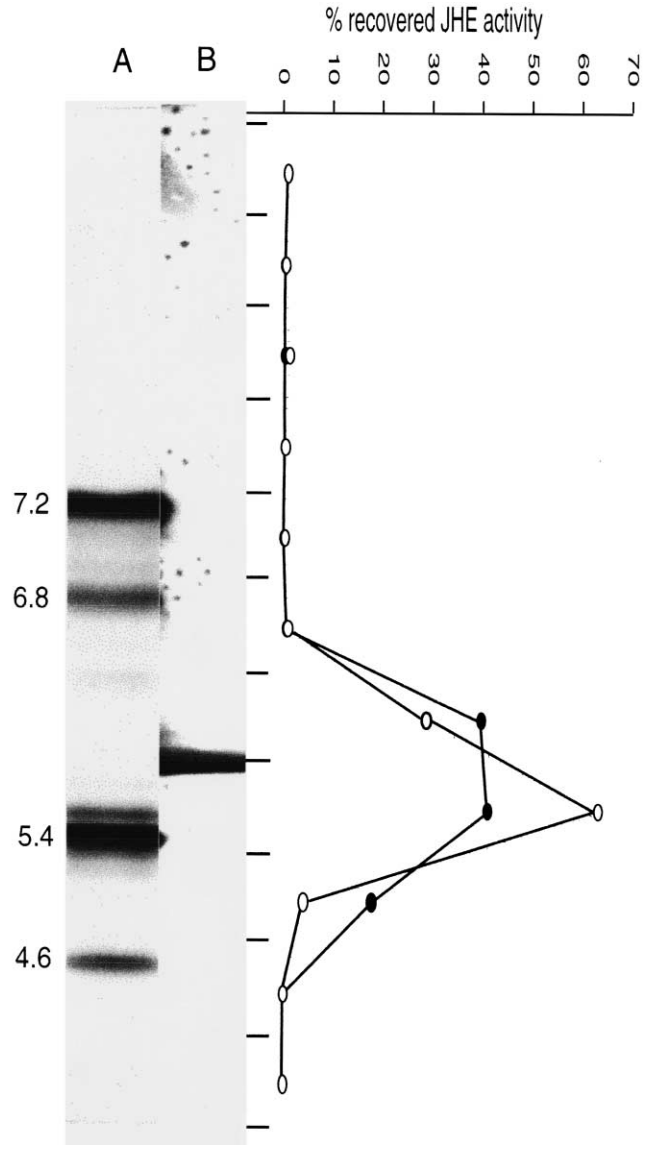

Fig. 2. IEF analysis of affinity purified JHE. Samples were run on a Novex precast isoelectric focusing gel, wide range (pH 3–10). The resultant gel was stained with the GelCode Silver Stain SNAP Stain Kit from Pierce. IEF markers purchased individually from Sigma were run in lane A. The p*I* of each marker is indicated next to the respective band. Lane B contains 3 μg of OTFP eluate. In two additional lanes, dialyzed JHE protein (OTFP eluate) and hemolymph were individually loaded onto offset lanes in the same gel, and then 7 mm gel slices were assayed for JHE activity. Markers next to lane B mark the spots which separate slices in the gel. The graph indicates the JHE activity recovered from each slice. Solid circles represent activity from purified JHE and hollow circles indicate activity from the hemolymph. Similar experiments comparing JHE activity in the hemolymph with the purified enzyme were performed on Pharmacia flat bed gels using both narrow and wide range gels. In each case, activity of the purified protein focused at a p*I* indistinguishable from the hemolymph JHE activity.

Table 2
Sequences from purified peptides after Lys-C digestion of *M. sexta* JHE. Parentheses indicate some level of uncertainty about the identity of amino acid due to instrumentation error. An X indicates the inability to identify the corresponding residue

P27	RIPSTEEVVV
P23	LWPEVXADNLRYQDIDTPDVYQNV(K)
P31	(S)VQRNAXFFGG(G)PDDV

The full length cDNA sequence is shown in Fig. 3 with the deduced amino acid sequence shown below the nucleotide sequence. The NCBI accession numbers for the cDNA sequence and protein sequence are AF327882 and AAG42021, respectively. Based upon the deduced protein sequence from the cDNA clone, the calculated molecular weight of the protein is 62127.53 Da, and the theoretical p*I* would be 5.72. The start codon is at 27 and the stop codon at 1676, giving a coding region of 1649 bp (543 amino acids). The protein sequence shows 54–52% identity to two other lepidopteran JHEs (*H. virescens* and *Choristoneura fumiferana*).

4. Discussion

JHE was purified to apparent homogeneity according to a single protein band on SDS-PAGE. The one step affinity purification resulted in a 63% yield of JHE activity, with a 525-fold increase in specific activity. This is comparable to the yields of previous reports (Abdel-Aal and Hammock, 1985; Venkatesh and Roe, 1990). The purified protein yields a single band on SDS-PAGE, but gives one major band and two minor bands when electrophoresed on an IEF gel. It is possible that the additional bands on IEF represent isoforms of the same protein. Since more than one individual insect was used as the biological source for the protein, it is not possible to say whether different forms exist in one individual due to multiple isoforms from different genes, or whether there is genetic variability between individual insects. It is also possible that there are differential post-translational modifications from a single gene. Previous studies on JHE from the hemolymph of *M. sexta* have also reported more than one isoelectric point, ranging from 5.5 to 6.1 (Abdel-Aal and Hammock, 1986; Jesudason et al., 1992). The first study reported only one band by SDS-PAGE which then separated into two p*I*s (Abdel-Aal and Hammock, 1986). The latter study showed that various tissues showed only one peak at a MW of 65,000 from gel filtration, though there was some variability in isoelectric points not only within the hemolymph but also between different tissues (Jesudason et al., 1992).

The single protein band on the SDS-PAGE gel was used in order to digest the protein into smaller peptides

Manduca sexta JHE

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 1   GAGTGTAAGAGCGGGCGCCAGTAGACATGGACGCGCGGCGACAAGTGTGCTACTACGTACTGGCTTTGCTCTGCGTGAGC
 1                          M  D  A  R  R  Q  V  C  Y  Y  V  L  A  L  L  C  V  S

 81  GAGCTAAGCGCACGCATCCCTAGCACGGAGGAGGTGGTGGTGAGGACCGAGTCGGGATGGATCCGCGGTCTCAAGCGCAG
 19  E  L  S  A  R  I  P  S  T  E  E  V  V  V  R  T  E  S  G  W  I  R  G  L  K  R  R

161  AGCGGAGGGCAACAAGTCTTACGCCAGCTTCCGCGGCGTGCCTTACGCGAAACAACCTCTTGGAGAACTTAGGTTTAAGG
 46    A  E  G  N  K  S  Y  A  S  F  R  G  V  P  Y  A  K  Q  P  L  G  E  L  R  F  K

241  AGCTTCAACCGTTAGAGCCGTGGCAAGACGAGCTGGACGCGACGCAGGAGGGCCCCGTGTGCCAGCAGACGGACGTGCTG
 72 E  L  Q  P  L  E  P  W  Q  D  E  L  D  A  T  Q  E  G  P  V  L  Q  Q  T  D  V  L

321  TACGGACGCATCATGAGACCTCGGGGCATGAGTGAGGCGTGTATCCACGCAAACATACATGTGCCGTATTACGCGCTGCC
 99  Y  G  R  I  M  R  P  R  G  M  S  E  A  L  C  I  H  A  N  I  H  V  P  Y  A  L  P

401  CAGGGACGCGGCAGATAAGAACCGGTTCGCCGGCTTGCCCGTGCTGGTGTTCATCCACGGAGGCGGCTTCGCGTTCGGCT
 126   R  D  A  A  N  K  N  R  F  A  G  L  P  V  L  V  F  I  H  G  G  G  F  A  F  G

481  CCGGCGACAGTGATCTGCACGGGCCCGAGTATCTCGTCAGCAAGGATGTCATTGTCATCACGTTTAACTACAGGCTGAAC
 152 S  G  D  S  D  L  H  G  P  E  Y  L  V  S  K  D  V  I  V  I  T  F  N  Y  R  L  N

561  GTGTACGGTTTCCTGTCGCTGAACTCTACAAGCGTGCCGGGCAATGCCGGCCTGCGCGACATGGTGACGCTCCTCAAGTG
 179  V  Y  G  F  L  S  L  N  S  T  S  V  P  G  N  A  G  L  R  D  M  V  T  L  L  K  W

641  GGTGCAGCGTAACGCGCATTTCTTCGGAGGGAGACCCGACGACGTCACCCTCATGGGCCAGAGCGCCGGCGCTGCTGCAA
 206   V  Q  R  N  A  H  F  F  G  G  R  P  D  D  V  T  L  M  G  Q  S  A  G  A  A  A

721  CTCACATACTCTCTCTGTCCAAGGCGGCTGACGGATTGTTTAGGAGAGCAATTTTAATGAGTGGTACCAGTTCATCTGCT
 232 T  H  I  L  S  L  S  K  A  A  D  G  L  F  R  R  A  I  L  M  S  G  T  S  S  S  A

801  TTCTTTACCACGAACCCGGTATTCGCTCAATATATAAATAAACTCTTCGTTACGAATATAGGCATCACAGCCACAGACCC
 259  F  F  T  T  N  P  V  F  A  Q  Y  I  N  K  L  F  V  T  N  I  G  I  T  A  T  N  P

881  TGAGGAAATCCACCAGAAACTCATAGAAATGCCAGCCGAAAAACTCAACGAAGCCAATCGCTTCCTGCTGGAACAGTTTG
 286   E  E  I  H  Q  K  L  I  E  M  P  A  E  K  L  N  E  A  N  R  F  L  L  E  Q  F

961  GACTCACCACTTTCTTCCCAGTGGTAGAATCTCCAATAAACGGTGTCACAACTATTTTAGATGAAGATCCAGAGCAGTTG
 312 G  L  T  T  F  F  P  V  V  E  S  P  I  N  G  V  T  T  I  L  D  E  D  P  E  Q  L

1041 ATAGCCAAAGGTCGAGGGAAACACATACCCTTGATAATTGGGTTTACTGACGCCGAGTGCGAGATATTCAGAAGGCAATT
 339  I  A  L  G  R  G  K  H  I  P  L  I  I  G  F  T  D  A  E  C  E  I  F  R  R  Q  F

1121 TGAGCAGATAGATATAGTCAGCAAAATAAAGGAGAACCCTGGAATACTCGTGCCTCTTTCCGTTCTCTTCTCGAGTGCTC
 366   E  Q  I  D  I  V  S  K  I  K  E  N  P  G  I  L  V  P  L  S  V  L  F  S  S  A

1201 CCGACACGGTTGCTGAAATAACNAAAGCGATGCACGAGAAGTACTTTAAAAAATCAGTAGACATGGAAGGATATATAGAA
 392 P  D  T  V  A  E  I  T  K  A  M  H  E  K  Y  F  K  K  S  V  D  M  E  G  Y  I  E

1281 CTTTGCACCGACAGTTATTTCATGTATCCAGCGATTAGTTTGGCAATTAAGAGAGCCCGCAGTAACGGAGCGCCGGTGTA
 419  L  C  T  D  S  Y  F  M  Y  P  A  I  S  L  A  I  K  R  A  R  S  N  G  A  P  V  Y

1361 CCTCTACCAATTTTCATTCGACGGGGACTACAGTGTCTTTAGGGAAGTGAACCACCTGAATTTCGAAGGAGCTGGCCATA
 446   L  Y  Q  F  S  F  D  G  D  Y  S  V  F  R  E  V  N  H  L  N  F  E  G  A  G  H

1441 TAGAAGACTTGACGTACGTGTTCAGAACCAACTCGATGTTGGGTGGCCACGCATCGTTCCCACCGCATGACAAAGACGAC
 472 I  E  D  L  T  Y  V  F  R  T  N  S  M  L  G  G  H  A  S  F  P  P  H  D  K  D  D

1521 CATATGAAGTACTGGATGACGTCCTTCATCACAAATTTTATGAAATACAGCAACCCAGTGACCGATGCCAAGCTATGGCC
 499  H  M  K  Y  W  M  T  S  F  I  T  N  F  M  K  Y  S  N  P  V  T  D  A  K  L  W  P

1601 CGAAGTCCGAGCAGACAATCTGAGGTATCAGGACATCGACACGCCCGACGTGTACCAGAACGTGAAGCCACACAGTGAA
 526   E  V  R  A  D  N  L  R  Y  Q  D  I  D  T  P  D  V  Y  Q  N  V  K  P  H  S  E

1681 CAGAGAGACATGTTGGATTTCTTCGACAGCATTTACAACTGGAACGGCACTTCGTATTGCATCAAATAGACGGTTGTGGA
 552  Q  R  D  M  L  D  F  F  D  S  I  Y  N  W  N  G  T  S  Y  C  I  K

1761 CTTACGATGTGTGGGTTTAAAATGCCGTGGATGTGTGGAGCGCGTTGGCATCATTGTATGAGTTGACGGTTGCAGCTATG
1841 GCAAACAGGTATCGGTTGACTAAGTTAATGCGTGATATTTTAAAATTTATTTCGCGTGTTGTATAATGTAGCGTCATGCA
1921 TACTTATTACACCTTTCAATGGTGTGACTCTGTAGCAGCTTCGGTCCGTTGCTTAAGGGTCAAGACTTTTGAGTGTATAT
2001 ACGGGAGCTTTTTAAAGCTTTTCAATTGTTTCCGGTAATGCCGGTACCAATGGCGTTATTCATTGTAACATAGACTTTAA
2081 TATGACATAGAACGCCTTCAAACCAAGCTGCAACTATGTAGATGTACCTTTTTAGTGTGTAATTATTAGGTTAAAATATT
2161 TTTCGGACTGATTAGGTAACGGTGTATGAAAATGTATTATAATTTTGTACATAAATGCTAAAAAAAAAAAAAAA
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Fig. 3. DNA sequence for the JHE cDNA clone is given with deduced protein sequence underneath the coding region. The putative signal peptide is underlined and in bold. Peptide sequences from the purified JHE protein are underlined. Individual amino acids underlined and in bold indicate putative residues involved in the catalytic tetrad, based upon studies with the homologous JHE protein sequence from *H. virescens* (Hanzlik et al., 1989; Ward et al., 1992; Thomas et al., 1999).

and obtain the amino acid sequences of internal peptides. The sequences from two peptides (Table 2) within this protein were used to design degenerate oligonucleotide primers for cDNA cloning. Fat body was used as the source of mRNA because there is strong evidence that the fat body contributes significant levels of JHE to the hemolymph in lepidopterans (Jones et al., 1987; Newitt and Hammock, 1989; Wroblewski et al., 1990).

The cDNA sequence reported here is likely to represent the major JHE protein that is in the hemolymph during the final larval instar. The presence of the internal peptides and N-terminal peptide sequence of the purified protein within the deduced protein sequence of the cDNA clone implies at least that the cDNA represents the protein that was purified (see Fig. 3). The deduced protein sequence shows a putative signal peptide which consists of 22 amino acids (Fig. 3). The signal peptide spans from the first potential start codon to the arginine residue which was reported at the N-terminus of the mature protein in two previous reports (Hammock et al.,

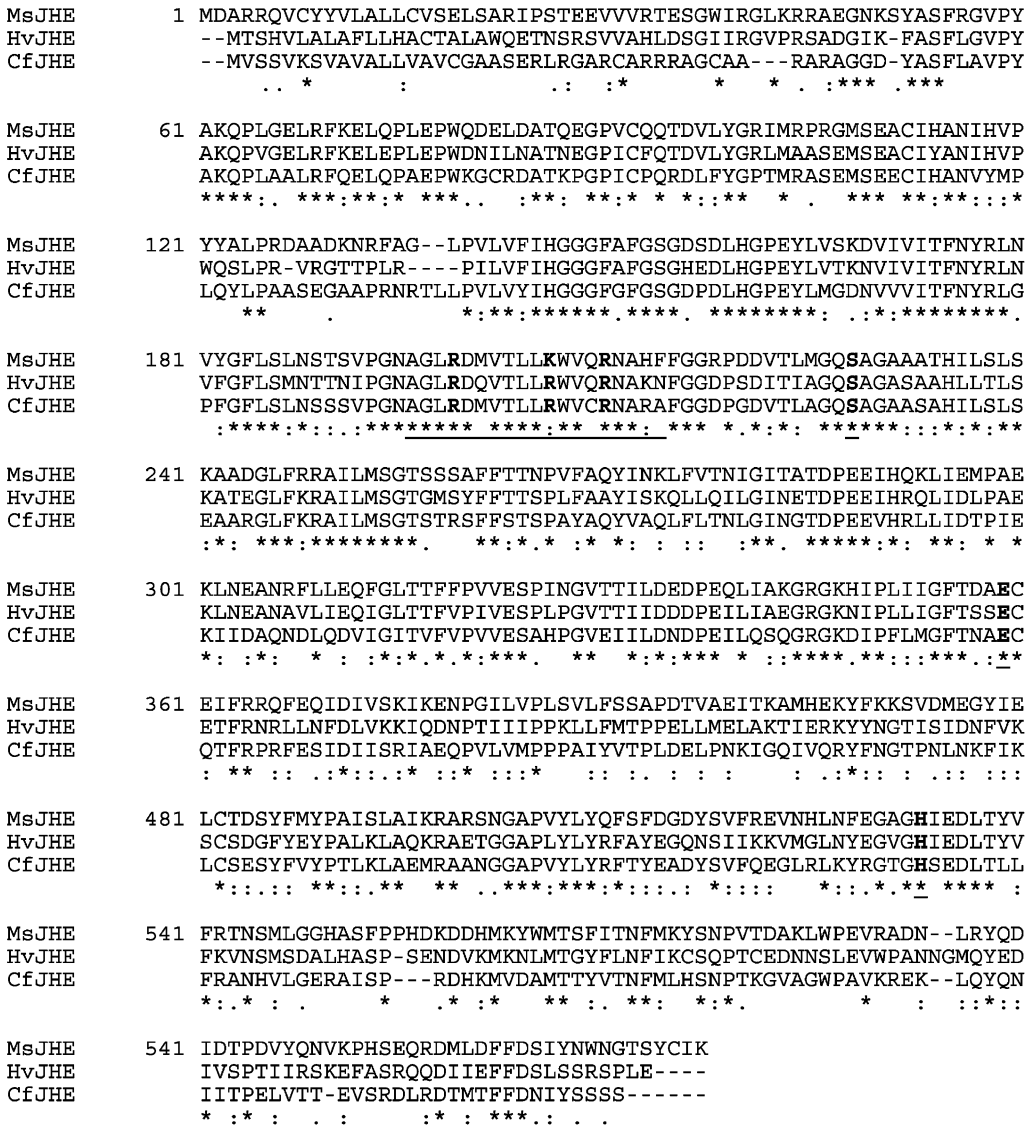

Fig. 4. Alignment of the deduced protein sequence of JHE from *M. sexta*, *H. virescens* and *C. fumiferana*. Amino acids that are conserved in identity are indicated underneath by a star. The underlined sequence indicates a putative amphipathic helix with conserved positive charges indicated in bold, implicated by Thomas et al. (1999) in receptor recognition. Also indicated in bold and underlined are conserved individual amino acids implicated as members of the catalytic tetrad (Ward et al., 1992; Thomas et al., 1999).

1988; Venkatesh et al., 1990). This is consistent with an expected signal peptide which would be clipped from the protein during secretion from the fat body cells into the hemolymph. This protein was purified from the hemolymph and is therefore likely to be a translation product from this cDNA. JHE activity has also been reported to be closely associated with other tissues in *M. sexta* (Jesudason et al., 1992), though it is not known whether this JHE activity is cytosolic or retained nearby in the extracellular medium. However, studies by Wing et al. (1981) in *Trichoplusia ni* argue against the extracellular JHE being trapped.

Furthermore, since JHE activity is recovered from the purified protein after dialysis of the eluting inhibitor, and the fact that this is the only protein present in the OTFP eluate on SDS-PAGE, this is likely to be the JHE protein which is prevalent in the larval hemolymph. Although there are two proteins present on IEF, they both migrate near each other in an area which corresponds to the peak in JHE activity on the same IEF (Fig. 2), and are likely to be isoforms of the same protein.

Further evidence of the identity of this cDNA comes from the alignment of the deduced protein sequence with that of JHEs cloned previously from *H. virescens* (Hanzlik et al., 1989) and *C. fumiferana* (Feng et al., 1999). The identity of the deduced amino acid sequence to the JHEs of *H. virescens* and *C. fumifera* is 54 and 52%, respectively. An alignment of the deduced JHE protein sequences from *M. sexta*, *H. virescens*, and *C. fumiferana* is shown in Fig. 4. Identical residues are indi-

cated by a star underneath the aligned amino acids. Specific motifs that are underlined include the catalytic residues at serine 226, glutamate 357 and histidine 471 (aligned with S220, E351, and H465, respectively, in *H. virescens*, and S224, E355 and H469, respectively in *C. fumiferana*). The aligned residues were implicated as a catalytic triad in the *H. virescens* JHE by site directed mutagenesis (Ward et al., 1992). Thomas et al. (1999) reported a highly conserved second serine in the JHE of *H. virescens*. Since the second serine is present in most catalytically active esterases and lipases, and also corresponds positionally to a second serine in the evolutionarily distinct serine protease family, a catalytic tetrad was proposed for serine proteases and lipases (Wallace et al., 1996; Thomas et al., 1999). This second serine is present in the JHE of *M. sexta* as well at position 252. Also indicated by a box are the basic residues (R197, K204 and R208) that lie in a putative amphipathic helix from residues 194–210. Thomas et al. (1999) suggested this amphipathic α-helix in their three-dimensional model of the *H. virescens* JHE, and pointed out the similarity between this motif and that which is reported by Wilson et al. (1991) for apo-lipoprotein E. This motif has been established as the structural feature which is important for receptor mediated recognition and the uptake of apolipoprotein E in humans (Wilson et al., 1991). It is possible that this motif has a similar function in the JHE protein. Though in *M. sexta* there exists a lysine residue at position 204 instead of an arginine (R198 in *H. virescens* JHE), the basic charge is conserved. JHEs were defined biochemically as enzymes with a high k_{cat}/K_m ratio for JH substrates, and physiologically as having a distribution and titer consistent with a role in JH regulation (Hammock, 1985). There are not sufficient data to define JHE as a group of enzymes with both a common function and a common evolutionary history as other enzymes such as acetylcholinesterase. However, the high identity of lepidopteran JHEs suggests a common evolutionary origin. Possibly, specific motifs such as the amphipathic helix will define JHEs as a group of enzymes. Mutagenesis studies are being performed in order to assess the possible role of this positively charged, amphipathic helix.

Other JHE sequences exist which also show high identity but are not included in the alignment. Partial cDNA clones from *Tenebrio molitor* (Thomas et al., 2000) and *Lymantria dispar* (Nussbaumer, unpublished) show even higher identity by alignment. However, since available sequences are limited to the middle regions of the protein (where the highest homology occurs among the full length clones) it is likely that the overall homology would decrease with the inclusion of the full length clone. Jones reported a full length clone of a JHE related protein in *T. ni* (Jones et al., 1994), which shows 50% identity to the JHE reported here. It is not clear how this JHE related protein differs from the actual JHE from *T. ni* since the latter was neither published nor submitted to the Genbank database.

Homology searches using the BLAST search program (Altschul et al., 1990) illustrate that the JHEs in lepidopterans seem to be set apart from other esterase enzymes. The nearest esterase in homology was determined by BLAST search using the JHE of *M. sexta* sequence and searching the nr database. Compared to an expectation value range of e-172 to e-140 for homology between this cDNA and JHEs from *H. virescens*, *C. fumifera*, and *T. ni*, the nearest esterase in homology (carboxylesterase from *Anisopteromalus calandrae* (Hymenoptera: Apocrita), sequence submitted to database but not published) scored as high as 2e-61 (an expectation value indicates the probability that two proteins would align by random chance). This same esterase had the highest score of homology (from a non-JHE esterase) to each of the JHE sequences when an independent BLAST search of the nr database was performed. Percent identities of this esterase to either of the JHE proteins was 32–33%.

Ultimate proof of the identity of the cDNA would lie with the demonstration of enzymatic activity in an in vitro expression system. This work is in progress. With active, recombinant JHE protein, and additional sequences from other species we can gain more insight into the structure/function relationship of the JHE protein. This work will also provide additional tools to probe JHE regulation and its utility as a biopesticidal agent.

Acknowledgements

The authors would like to thank Dr Christa Nussbaumer for her inspiration and technical assistance in everything, Tonya Severson for her helpful comments on the manuscript, and Young Moo Lee for his expertise in peptide digestion and sequencing. This work was funded in part by a grant from USDA (97-3502-4406).

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