Structural comparison of two esterases from *Drosophila mojavensis* isolated by immunoaffinity chromatography

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Antibodies raised against esterase-4 and esterase-5 from *Drosophila mojavensis* were coupled to Protein A–Sepharose CL-4B to prepare high-efficiency immunomatrices used for their purification. Final purification was achieved by anion-exchange h.p.l.c., in the case of esterase-5 followed by gel-filtration h.p.l.c. The resultant esterase preparations were homogeneous, as judged by gel-filtration h.p.l.c., SDS/polyacrylamide-gel electrophoresis and non-denaturing gel electrophoresis. Esterase-4 and esterase-5 are the products of a duplicated gene. They are differently localized in the insect's body and expressed in different periods during development. Although both enzymes exhibit little immunological cross-reactivity, their amino acid compositions show few significant differences and their *N*-terminal sequences are largely identical, which clearly show their common origin.

INTRODUCTION

The esterases of the genus *Drosophila* are highly polymorphic (Johnson, 1973, 1974). Although a few esterases have been purified (Narise & Hubby, 1966; Narise, 1973; Mane *et al.*, 1983), no structural work has been done to detect the nature of their variability. The esterases from *Drosophila mojavensis* have been studied by Zouros and co-workers (Zouros, 1973; Zouros & Van Delden, 1982; Zouros *et al.*, 1982), who demonstrated about six bands with esterase activity on non-denaturing gels. The bands were numbered consecutively according to increasing negative charge. Esterase-4 and esterase-5 are the most polymorphic esterases from *Drosophila mojavensis*. They are supposed to be the products of a duplicated gene, but they have diverged considerably after the duplication event. Localization in the insect differs, as well as the periods of expression during development (Zouros *et al.*, 1982). After immunodiffusion no cross-reactivity of esterase-5 with an antiserum directed against esterase-4 was observed (Pen *et al.*, 1984). Still, the enzymes form heterodimers *in vitro* (Zouros *et al.*, 1982). Alloenzymes of esterase-4 and esterase-5 differ in charge, i.e. they may be detected by their different mobilities during non-denaturing gel electrophoresis. Alloenzymes of esterase-4 with an altered substrate preference have been detected, whereas the alloenzymes of esterase-5 all exhibit the same preference (Zouros & Van Delden, 1982; Zouros *et al.*, 1982).

Purification of esterase-4 has been described previously (Pen *et al.*, 1984). Esterase-5 could be purified by a fairly similar procedure but less reproducibly. In the present paper we describe an improved, rapid and highly reproducible purification procedure for both esterases by the use of adsorption on high-efficiency immunomatrices followed by h.p.l.c. The amino acid compositions of the proteins and the *N*-terminal sequences, determined with

a gas-phase Sequenator because of the small amounts of pure material available, are compared.

MATERIALS AND METHODS

Drosophila stocks

The laboratory stocks A426-100 α and BI-86 β of *Drosophila mojavensis* were used. The stocks have been described by Zouros & D'Entremont (1980) and Zouros & Van Delden (1982). Stock A426-100 α is homozygous for the 100 α allele of esterase-4 and esterase-5¹⁰⁰. Stock BI-86 β is homozygous for the 86 β allele of esterase-4 and esterase-5⁹⁶. The α and β designate preferences for 1- and 2-naphthyl esters respectively. Purification of esterase-4 from the A426-100 α stock and of esterase-5 from both stocks is described here. The stocks were a gift from Dr. E. Zouros (Halifax, N.S., Canada). The collected larvae, predominantly in their last instar, were washed with demineralized water, dried on paper towels and stored at –20 °C until use.

Other materials

Fast Blue B salt (*o*-dianisidine, tetra-azotized), 1-naphthyl acetate, carbonic anhydrase, soya-bean trypsin inhibitor, bovine serum albumin, human serum albumin and α -lactalbumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Coomassie Brilliant Blue R-250 was from Serva Chemical Co. (Heidelberg, Germany), Na₂S₂O₄ was from J. T. Baker Chemicals (Deventer, The Netherlands), ovalbumin was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), Tris was from Janssen Chimica (Beerse, Belgium), Freund's complete adjuvant was from Difco (Detroit, MI, U.S.A.), SDS was from BDH Chemicals (Poole, Dorset, U.K.), Protein A–Sepharose CL-4B and Sepharose CL-4B were from Pharmacia Fine Chemicals (Uppsala, Sweden), and dimethyl pimelimidate dihydro-

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chloride was from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other reagents used in the purifications were analytical-grade products from Merck (Darmstadt, Germany). The reagents and solvents for sequence analysis were from Applied Biosystems (Foster City, CA, U.S.A.).

Assay

Determination of esterase activity with 1-naphthyl acetate as the substrate, essentially following the method of Van Asperen (1962), was as described previously (Pen *et al.*, 1984). One unit of activity is defined as 1 μ mol of naphthol produced/min at 37 °C, and specific activity as activity units/mg of protein. An indication of the protein content was obtained from the absorbances at 280 nm and 260 nm (Layne, 1957).

Identification of esterases

Non-denaturing gelelectrophoresis, with 6% polyacrylamide in the gel, was used to screen fractions for the presence of esterases and for identification of the type of esterase present. The gels were stained for esterase activity (Pen *et al.*, 1984).

H.p.l.c. system and columns

H.p.l.c. was performed in a system consisting of an LKB 2150 pump, a Rheodyne 7125 injector and a Pye-Unicam LC-UV detector connected to a recorder. Anion-exchange h.p.l.c. was performed on an Aquapore AX-300 column (100 mm $\times$ 4.6 mm; Brownlee Laboratories, Santa Clara, CA, U.S.A.). The gradients employed in ion-exchange h.p.l.c. were generated by an Acorn Atom computer interfaced with a three-way solenoid valve (Lee Co., Westbrook, CT, U.S.A.) (Van Der Zee & Welling, 1984). Gel-filtration h.p.l.c. was done on an Ultropac TSK-G 3000 SW column (600 mm $\times$ 7.5 mm; LKB, Bromma, Sweden). Fractions were collected manually, guided by the elution profile at 280 nm or 220 nm.

M_r determinations

The M_r values of esterase-4 and esterase-5 were determined under denaturing conditions by SDS/polyacrylamide-gel electrophoresis by the procedure of Laemmli (1970) with 10% polyacrylamide in the gel.

Esterases and antisera

Esterase-4 was purified, and antiserum against esterase-4 and an $(\text{NH}_4)_2\text{SO}_4$ fraction of this antiserum were prepared as described previously (Pen *et al.*, 1984). Esterase-5 was purified to about 95% homogeneity by a similar procedure as described previously for esterase-4 (Pen *et al.*, 1984). Antiserum against esterase-5 was raised in a rabbit by injecting esterase-5 (about 20 μ g in 0.3 ml of demineralized water) mixed with an equal volume of Freund's complete adjuvant subcutaneously three times at 2-week intervals. At 1 week after the last injection, blood was collected from the ear vein. $(\text{NH}_4)_2\text{SO}_4$ fractionation was omitted for the esterase-5 antiserum.

Preparation of immunomatrices

Antibodies from both antisera were covalently coupled with the use of dimethyl pimelimidate dihydrochloride to Protein A-Sepharose CL-4B by following the method described by Schneider *et al.* (1982). The antibodies

present in 7.5 ml of the $(\text{NH}_4)_2\text{SO}_4$ fraction of the esterase-4 antiserum, and the antibodies present in 2 ml of the esterase-5 antiserum, were cross-linked to 0.5 g portions of gel. Both immunomatrices were diluted 1:1 with Sepharose CL-4B and packed into small columns.

Purification of esterase-4 and esterase-5 by immunoaffinity chromatography

Larvae (25 g), predominantly in their last instar, were homogenized, in an Ultra-Turrax homogenizer (Janke und Kunkel), in 60 ml of phosphate-buffered saline (0.15 M-NaCl/0.02 M-sodium phosphate buffer, pH 7.6) containing 40 mg of $\text{Na}_2\text{S}_2\text{O}_4$. The homogenate was centrifuged at 48000 *g* for 20 min at 4 °C in a Sorvall SS34 centrifuge. The pellet was washed with 25 ml of phosphate-buffered saline containing 10 mg of $\text{Na}_2\text{S}_2\text{O}_4$, followed by centrifugation as above. The combined supernatants were filtered through glass-wool.

Routinely, this crude extract was applied to a Sepharose CL-4B column (25 ml) serving as a 'filter', connected to the esterase-4 immunoaffinity column, which in turn was connected to the esterase-5 immunoaffinity column. All three columns were pre-equilibrated with phosphate-buffered saline. After application of the sample, the columns were washed with 30 ml of phosphate-buffered saline. The 'filter' was disconnected and the two affinity columns, still in tandem, were washed with 20 ml of phosphate-buffered saline. Finally, the immunoaffinity columns were disconnected and separately washed with 20 ml of phosphate-buffered saline and 25 ml of 0.1 M-sodium carbonate buffer, pH 9.5, containing 0.5 M-NaCl. Both esterases were eluted with 25 ml of 0.1 M-sodium carbonate buffer, pH 11.2, containing 0.5 M-NaCl. Fractions were immediately neutralized with 0.5 M- NaH_2PO_4 . They were screened for esterase activity by non-denaturing gel electrophoresis and the absorbance at 280 nm was determined.

After dialysis against demineralized water, the combined esterase pools from three batches of extract purified on an immunoaffinity column were pumped on to the anion-exchange h.p.l.c. column and fractionated with a salt gradient. Esterase-5 had to be purified further by gel-filtration h.p.l.c. The esterase-5 pool obtained was concentrated by anion-exchange h.p.l.c., which did not further increase the specific activity. Both esterase pools were dialysed against demineralized water.

Amino acid analyses

Samples were hydrolysed, in evacuated sealed tubes, with constant-boiling HCl for 24 h at 110 °C. Amino acid analyses were performed on a Kontron Liquimat III analyser.

N-Terminal sequence determination

Automated Edman degradation was carried out in a gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.), with 25% (v/v) trifluoroacetic acid in water for the two esterase-4 samples and methanolic HCl for esterase-5 as the conversion reagents. The amino acid phenylthiohydantoin derivatives were quantitatively analysed off-line the sequencer by reversed-phase h.p.l.c. with the Waters Intellink System consisting of two 6000A pumps, the 720 System Controller, the 730 Data Module and the WISP 710B autoinjector, supplemented with a Waters 440 detector set at 254 and 313 nm. The separations were carried out on a 5 μ m-particle-size

cyanopropyl column (250 mm × 4.6 mm; IBM, Biotech, U.K.) thermostatically maintained at 37 °C. The solvent and gradient program used for the analysis of the esterase-4 amino acid phenylthiohydantoin samples was similar to the one described by Hunkapiller & Hood (1983), with the phenylthiohydantoin samples being methylated before injection on to the h.p.l.c. column. For the run on esterase-5 the adaptation of the protocol proposed by Touchstone was used (A.B.I., Users Bulletin no. 3), including the addition of tetrahydrofuran

to the sodium acetate buffer component of the gradient system. One of the major advantages over the earlier procedure used for esterase-4 was that diphenylthiourea was eluted baseline-separated from the methionine phenylthiohydantoin derivative.

RESULTS

A summary of the purification of esterase-4 is given in Fig. 1 and Table 1 and of esterase-5¹⁰⁰ in Fig. 2 and Table

Fig. 1. Purification of esterase-4 from *Drosophila mojavensis*

(a) Immunoaffinity chromatography. Crude extract of larvae (40 g) was applied to the esterase-4 immunoaffinity column via a Sepharose CL-4B 'filter'. The immunoaffinity column was washed with 20 ml of phosphate-buffered saline (a) and 25 ml of 0.1 M-sodium carbonate buffer, pH 9.5, containing 0.5 M-NaCl (b). Esterase-4 was eluted with 0.1 M-sodium carbonate buffer, pH 11.2, containing 0.5 M-NaCl (c). The flow rate was 60 ml/h and the fraction volume was 8 ml. (b) Non-denaturing gel electrophoresis. A schematic drawing is shown of a gel with 10 µl samples of the fractions from the immunoaffinity column stained for esterase activity; ● indicates the crude larval extract. An extra band of esterase activity is indicated by ★, and the heterodimer of esterase-4 and esterase-5 by 4/5. (c) Anion-exchange h.p.l.c. The esterase-4 pool from the immunoaffinity column was dialysed against demineralized water and pumped on to an Aquapore AX-300 column. Protein was eluted with a linear NaCl gradient (—) in 0.01 M-Tris/HCl buffer, pH 7.6. The flow rate was 0.5 ml/min. —, A_{280} . The esterase-4 pool is indicated by the thick horizontal bar. The positions of elution of the heterodimer of esterase-4 and esterase-5 and of esterase-5 are indicated with A and B respectively.

Table 1. Purification of esterase-4 from 25 g of *Drosophila mojavensis* larvae

For experimental details see the text.

	Protein (mg)	Activity (units)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Crude extract	2423	4.9	0.002	100	1
Immunoaffinity chromatography	3.2	0.12	0.04	2.5	20
Anion-exchange h.p.l.c.	0.13	0.05	0.4	1.0	200

Fig. 2. Purification of esterase-5¹⁰⁰ from *Drosophila mojavensis*

(a) Immunoaffinity chromatography. See the legend to Fig. 1(a). Crude extract of larvae (25 g) was applied to the esterase-5 immunoaffinity column via a Sepharose CL-4B 'filter' and the esterase-4 immunoaffinity column. (b) Non-denaturing gel electrophoresis. A schematic drawing is shown of a gel with 10 μ l samples of the fractions from the immunoaffinity column stained for esterase activity; ● indicates the crude larval extract. The heterodimer of esterase-4 and esterase-5 is indicated by 4/5. (c) Anion-exchange h.p.l.c. See the legend to Fig. 1(c). Esterase-5 pools from three purifications with immunoaffinity chromatography were combined before anion-exchange h.p.l.c. (d) Gel-filtration h.p.l.c. The esterase-5 pool from the anion-exchange column was applied to a TSK-G 3000 SW column in 200 μ l portions. The flow rate was 1 ml/min and the elution buffer was phosphate-buffered saline. —, A_{220} . The esterase-5 pool is indicated by the thick horizontal bar.

Table 2. Purification of esterase-5¹⁰⁰ from 25 g of *Drosophila mojavensis* larvae

For experimental details see the text.

	Protein (mg)	Activity (units)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Crude extract	1753	4.7	0.003	100	1
Immunoaffinity chromatography	2.6	0.04	0.02	0.9	7
Anion-exchange	0.030	0.01	0.3	0.2	100
Gel-filtration h.p.l.c.	0.015	0.006	0.4	0.1	133

2. These are two different purifications. The large fall in the recovery after immunoaffinity chromatography is mainly explained by the fact that other esterases than the one purified contribute to the activity in the crude extract. Esterase-5⁹⁶ may be purified in a similar way to that shown for esterase-5¹⁰⁰. Both enzymes were judged to be at least 95% pure by SDS/polyacrylamide-gel electrophoresis (Fig. 3).

Further evidence that the purified esterase-4 and esterase-5 are distinct homogeneous proteins is found in

the shapes of the peaks on gel-filtration h.p.l.c. (results not shown for esterase-4; for esterase-5 see Fig. 2d). The esterase activities coincide with the protein peaks. The best criterion for the homogeneity of the two esterases is from the determination of the *N*-terminal sequence (see below). Esterase-4 was also subjected to non-denaturing gel electrophoresis (Fig. 4). Following electrophoresis, one part was stained for protein and another part for esterase activity. Just one band of esterase activity (except for some aggregated material; see below) was

Fig. 3. Estimation of M_r values of esterase-4 and esterase-5 of *Drosophila mojavensis* by SDS/polyacrylamide-gel electrophoresis

Lane A, standard proteins: human serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and soya-bean trypsin inhibitor (M_r 20100). Lane B, purified esterase-4. Lane C, purified esterase-5⁹⁶. Lane D, purified esterase-5¹⁰⁰.

Fig. 4. Non-denaturing gel electrophoresis of esterase-4 of *Drosophila mojavensis*

Two samples of purified esterase-4 were electrophoresed on the same gel, which contained 6% polyacrylamide. Lane A, about 1.5 μ g of purified esterase-4, with staining for esterase activity; $\star$ indicates aggregated esterase-4 (see the text). Lane B, about 15 μ g of purified esterase-4, with staining for protein.

Fig. 5. *N*-Terminal sequence and comparison of esterase-4 and esterase-5* from *Drosophila mojavensis*

The data were obtained by micro sequence analysis on the Applied Biosystems gas-phase Sequenator. The proposal for the esterase-4 is the result of two sequence runs, and that for esterase-5 was obtained with one single run. The yields of the amino acid phenylthiohydantoin derivatives at each step are given in Table 4. The identification for the residues in parentheses is tentative. The alignment shows the high number of identical residues between the two proteins, those that differ being 'boxed'.

seen, which migrated with the same mobility as the only protein band seen on the gel. Therefore purified esterase-4 is indeed a distinct homogeneous protein. Purified esterase-5 also gives one band of esterase activity on a non-denaturing gel (results not shown). From the evidence described above and the resemblance with esterase-4 (see Fig. 5), it may be concluded that purified esterase-5 is also a distinct homogeneous protein.

The M_r of esterase-4 was between 62000 and 64000, as found previously (Pen *et al.*, 1984). The M_r of esterase-5 was slightly higher: between 64000 and 66000 (Fig. 3). The specific activities of both proteins were 0.4 unit/mg.

As indicated in Fig. 1(b), an extra band of esterase activity in the esterase-4 pool was detected on a non-denaturing gel after immunoaffinity chromatography. This band was still seen after ion-exchange h.p.l.c. As found by gel-filtration h.p.l.c., this band is accounted for by a very small amount of aggregated esterase-4, since a small peak in the high- M_r range contained both the normal band of esterase-4 and the extra band with low mobility indicated with a star in Fig. 1(b) (results not shown).

The amino acid compositions of both enzymes are very similar, and do not show peculiar features (Table 3). The most significant differences observed are the amount of isoleucine, which is higher, and the amount of phenylalanine, which is lower in esterase-5 than in esterase-4. The values for valine and isoleucine may be underestimated because no analyses after extended periods of hydrolysis were performed.

The *N*-terminal sequence of esterase-4 (Fig. 5) is the result of micro sequence analysis on two different preparations containing 0.64 and 2.9 nmol of protein (amounts calculated from the amino acid analysis data; see Table 3). The net increases of the amino acid phenylthiohydantoin derivatives at each Edman cycle are given in Table 4. Combining the results of both experiments, reliable assignments could be done for 32 out of the first 40 positions. At the first cycle three major amino acid phenylthiohydantoin derivatives were detected (those of alanine, serine and glycine), but for nearly all the other positions an increase of only one residue was noticed. Since serine and glycine are known to be possible free amino acid contaminants in many protein prepara-

tions, alanine is proposed as the *N*-terminal residue of esterase-4.

The *N*-terminal sequence of esterase-5 (Fig. 5) determined on 420 pmol was pursued for 38 cycles. Of these, 32 allowed unambiguous identification. No methionine residue seemed to occur within this region of the polypeptide chain, although the chromatographic conditions were such (see the Materials and methods section) that it could have been detected separately from

Table 3. Amino acid compositions of esterase-4 and esterase-5 of *Drosophila mojavensis*

The values for esterase-4 are the means $\pm$ s.d. for four determinations on approx. 0.2–0.8 nmol of protein. The values for esterase-5 are the means $\pm$ s.d. for three determinations on approx. 0.1–0.3 nmol of esterase-5¹⁰⁰ (two determinations) and esterase-5* (one determination). The data are not corrected for hydrolysis losses.

Amino acid	Composition (residues/100 residues)	
	Esterase-4	Esterase-5
Asx	12.4 $\pm$ 1.0	11.6 $\pm$ 0.5
Thr	3.7 $\pm$ 0.5	3.7 $\pm$ 0.7
Ser	7.2 $\pm$ 0.8	7.3 $\pm$ 0.3
Glx	12.1 $\pm$ 0.4	12.7 $\pm$ 0.8
Pro	6.6 $\pm$ 0.6	7.4 $\pm$ 3.6
Gly	9.0 $\pm$ 0.4	8.8 $\pm$ 1.5
Ala	8.1 $\pm$ 0.7	8.1 $\pm$ 0.5
Cys	1.3 $\pm$ 0.2*	0.7†
Val	4.9 $\pm$ 0.1	4.9 $\pm$ 0.1
Met	1.6 $\pm$ 0.5	1.4 $\pm$ 0.3
Ile	3.4 $\pm$ 0.4	4.6 $\pm$ 0.3
Leu	8.5 $\pm$ 0.2	8.7 $\pm$ 0.5
Tyr	3.5 $\pm$ 0.5	3.5 $\pm$ 0.2
Phe	5.9 $\pm$ 0.4	5.1 $\pm$ 0.2
Lys	5.0 $\pm$ 0.5	4.6 $\pm$ 1.4
His	2.2 $\pm$ 0.1	1.8 $\pm$ 0.2
Arg	5.2 $\pm$ 0.3	5.5 $\pm$ 0.5
Trp	N.D.‡	N.D.‡

* From three determinations.

† From one determination.

‡ Not determined.

Table 4. Gas-phase sequence analysis of esterase-4 and esterase-5 of *Drosophila mojavensis*

Two samples of esterase-4 were sequenced, the first one containing 640 pmol (A), the second one 2.9 nmol (B) of protein. The amount of esterase-5 sequenced (C) was 420 pmol. At each cycle, only the net increases of the amino acid phenylthiohydantoin (PTH) derivatives after correction for carry-over and background are given. Peak heights were normalized to correct for injection errors and other possible chromatographic variabilities. For the experiment on esterase-5, the peaks of glycine and proline phenylthiohydantoin derivatives were accompanied by an appreciable amount of unconverted phenylthiocarbamoyl derivatives (between 30 and 50%). Non-methylated glutamic acid and aspartic acid phenylthiohydantoin derivatives, resulting from incomplete esterification, could not be quantified because of their co-elution with acidic dithiothreitol by-products near the beginning of the chromatographic analyses. Because of the necessity to use conventional off-line instead of on-line analysis, several amino acid phenylthiohydantoin derivatives may have undergone partial decomposition in the period between their formation and their analysis.

Edman cycle	PTH derivative	Esterase-4		PTH derivative	Esterase-5	
		Amount (pmol)			Amount (pmol)	
		(A)	(B)		(C)	
1	Ala	496	3739	Ala	153	
	Ser	256	1307	Ser	156	
	Gly	494	3523	Gly	184	
2	Pro	222	651	Ala	280	
3	Leu	269	406	Ala	66	
4	Leu	183	593	Ala	—*	
5	Val	305	352	Asp	55	
6	Glu	160	247	Pro	97	
7	Leu	245	595	Leu	272	
8	Pro	198	356	Ile	66	
9	Asn	212	637	Val	103	
10	Gly	260	374	Glu	45	
11	Lys	61	182	Leu	117	
12	Leu	159	573	Pro	51	
13	Arg	146	204	Asn	117	
14	Gly	104	268	Gly	53	
15	Arg	42	N.Q.†	Lys	92	
16	Asp	53	109	Val	42	
17	Asn	66	202	Arg	43	
18	Glu	56	11	Gly	11	
19	Gly	104	37	Arg	N.Q.†	
20	Tyr	67	105	Asp	31	
21	Tyr	28	50	Asn	91	
22	Asp	11	—	Glu	16	
	Glu	—	20			
23	Ala	—	23	Gly	96	
24	Glu	22	38	Tyr	57	
25	Leu	15	25	Tyr	23	
26	Ile	28	99	Glu	8	
				Gly	9	
				Asp	8	
27	Pro	11	35	Ala	15	
28	Lys	—	32	Glu	15	
29	Ala	26	55	Gly	5	
30	Asp	—	43	Ile	14	
31	Pro	19	58	Pro	15	
32	Pro	16	28	Arg	N.Q.†	
33	Val	10	—	Ala	22	
34	Gly	19	—	Glu	6	
35	Asp	—	24	Pro	11	
36	Leu	14		Pro	5	
37	Ala	11		Val	5	
38	Phe	13		Gly	23	
				Ala	16	
39	Lys	14				
40	Asp	12				

* The total peak height was the same as for cycle 3.

† Not quantified.

diphenylthiourea if present. It should also be kept in mind that neither esterase-4 nor esterase-5 was carboxymethylated before sequence analysis. Therefore the possible presence of cysteine, known to be extremely labile during Edman degradation, could not be proven. For esterase-5 also three amino acid phenylthiohydantoin derivatives were found during the first cycle. Since for this protein also only a single amino acid was found at each of the subsequent steps, it is concluded that the protein sample contained only one molecular species and that two of the apparent residues at the *N*-terminus were free amino acid contaminants.

DISCUSSION

Immunoaffinity chromatography

The highly reproducible purification method described here permits recovery of esterase-4 activity comparable with that obtained by the method employed previously (Table 1; Pen *et al.*, 1984), but much more rapidly. The specific activity of esterase-4 purified with the new method is comparable as well (Table 1). The specific activities of esterase-4 and esterase-5 are about the same. However, the recovery of esterase-5 activity is only 10% of that of esterase-4 (Tables 1 and 2). This is partly due to the extra gel-filtration h.p.l.c. step involved in the purification of esterase-5.

Both immunoaffinity columns have been in use for over a year. The results remain reproducible, although the protein recoveries have decreased by about 50% during that period.

Since the immunological properties of the alloenzymes should be comparable, purification by immunoaffinity chromatography is expected to be more easily applicable for the purification of other alloenzymes than the one used to prepare the antiserum. Differences in charge between alloenzymes, by which property they are separated and identified on non-denaturing gels, may cause problems when ion-exchange chromatography would be used as the principal aid in separation. This problem is circumvented by the application of immunoaffinity chromatography.

This method is also suitable for the purification of related esterases from other *Drosophila* species. So far, conclusions about possible evolutionary relationships between esterases of related species have been based on tissue localization, presence during development and substrate preference (Johnson & Bealle, 1968; Johnson *et al.*, 1968; Kambyzellis *et al.*, 1968; Zouros *et al.*, 1982). However, the problems encountered in attempts to relate esterases from different species are manifold. Firstly, usually a number of, often unrelated, esterases are present in a crude extract; secondly, the substrates *in vivo* are generally unknown; thirdly, substrate specificities strongly overlap among the various classes of esterases (Masters & Holmes, 1972; Junge & Krisch, 1973; Walker & Mackness, 1983). Immunological cross-reactivity should give a more dependable indication of the degree of relatedness, since it takes structural resemblance into account as well.

Structural relationship between esterase-4 and esterase-5

Esterase-4 and esterase-5 are immunologically related, as inferred from the co-elution of small quantities of esterase-4 from the esterase-5 immunoaffinity column and of esterase-5 from the esterase-4 immunoaffinity

column at pH 11.2 (Figs. 1b and 2b). It is unlikely that the binding of the co-eluting esterase is non-specific because of the structural resemblance of the two esterases (heterodimer formation and *N*-terminal sequences). Moreover esterases-1, -2 and -3 were not found in the pH 11.2 eluate. The degree of cross-reactivity of esterase-4 and esterase-5 is very low, since only a trace of the cross-reacting esterase remained after immunoaffinity chromatography (Fig. 1c). That the esterases cross-react has been demonstrated by the use of double immuno-diffusion (J. Pen & J. J. Beintema, unpublished work). Consequently, the structural divergence of the enzymes at the surface must be considerable, although the amino acid compositions (Table 3) and the ability of the proteins to form heterodimers (Zouros *et al.*, 1982) do not indicate a large structural difference. The *N*-terminal sequences (Fig. 5) appear to be strongly different if they are aligned by their respective *N*-terminal amino acids. However, if the *N*-terminal residue of esterase-4 is aligned with aspartic acid at position 5 of esterase-5 a very high number of identical residues between the proteins is observed. In fact, within the region of 34 comparable residues only six amino acids are different, although some assignments are at present only tentative (Fig. 5). Four mutual replacements, at positions 4, 12, 28 and 30 (esterase-4 numbering), even involve chemically similar residues. It cannot be excluded that the remaining part of the sequences differs more. Also, for large proteins, which have a small surface-to-volume ratio, the structural divergence may be less than would be expected from the difference in immunological properties. This explanation is supported by the observation that the non-exposed contact areas between the subunits of esterase-4 and esterase-5 are similar enough to allow heterodimer formation (Zouros *et al.*, 1982). The *N*-terminal extension of esterase-5 as compared with esterase-4 may be due to different tissue-specific processing of the proteins.

For enzymes that exhibit considerable substrate ambiguity, a gene duplication followed by gradual modification of one of the copies may permit further specialization (Li, 1983). Since highly variable enzymes such as esterase-4 and esterase-5 are supposed to be multi-substrate enzymes (Gillespie & Langley, 1974), this model of specialization may apply.

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