Table 4. Effect of amino acids on the fungitoxicity of D,L-*m*-FPhe and L-*m*-FPhe-L-Ala-L-Ala

Amino acid	Concentration (mM)	MIC (μM) D,L-*m*-FPhe	L-*m*-FPhe-L-Ala-L-Ala
None		125	7.8
L-Phe	0.1	250	31.2
	1.0	2000	500
	10.0	>2000	>500
L-Met	0.1	250	7.8
	1.0	500	7.8
	10.0	1000	7.8
L-Ile	0.1	125	7.8
	1.0	250	7.8
	10.0	500	7.8
L-Leu	0.1	125	7.8
	1.0	250	7.8
	10.0	1000	7.8
L-Trp	0.1	250	7.8
	1.0	500	7.8
	10.0	1000	7.8
L-Val	0.1	125	7.8
	1.0	250	7.8
	10.0	500	7.8
L-Tyr	0.1	125	7.8
	1.0	250	7.8

Fungitoxicity was unaffected by L-Gly, L-Ala, L-Ser, L-Thr, L-Asp, L-Asn, L-Glu, L-Gln, L-Lys, L-His, L-Arg, L-Cys, L-Pro, L-Hyp, at 10 mM.

gitoxicity. Very similar results were obtained using the two methods, and the tripeptide L-Ala_3 was found to be the most effective antagonist. In amino acid antagonism experiments, L-Phe was the only amino acid which antagonized L-*m*-FPhe-L-Ala-L-Ala, whereas the fungitoxicity of *m*-FPhe was reduced by all 3 aromatic amino acids (L-Phe, L-Tyr and L-Trp) and the neutral amino acids L-Met, L-Ile, L-Leu and L-Val (table 4). Similar antagonism of L-*m*-FPhe-L-Ala-L-Ala by L-Phe was reported in *Candida albicans*[10], and was interpreted to indicate competition with L-*m*-FPhe at its intracellular site of action following hydrolysis of the tripeptide.

These results support the hypothesis that L-*m*-FPhe-L-Ala-L-Ala is transported into *P. ultimum* by a peptide permease, whereas *m*-FPhe is delivered by an amino acid transport system. Results of the amino acid antagonism experiments (table 4) suggest that *P. ultimum* has a very similar transport system for aromatic and neutral amino acids to that described for *Achlya*[13], another oomycete. The ineffectiveness of D-Ala_2 and D-Ala_3 as antagonists of L-*m*-FPhe-L-Ala-L-Ala and the lack of fungitoxicity of D-*m*-FPhe-L-Ala-L-Ala are consistent with the specificity of peptide permeases in general for L-amino acid containing peptides.

In conclusion, our results strongly suggest that *P. ultimum* has an operational tripeptide transport system, and that the concept of illicit transport can be applied in the design of fungicides active against oomycete fungi.

1 Ringrose, P. S., in: Microorganisms and nitrogen sources, p. 641. Ed. J. W. Payne. John Wiley and Sons, Ltd., Chichester, UK 1980.
2 Kenig, M., Vandamme, E., and Abraham, E. P., J. gen. Microbiol. *94* (1976) 46.
3 Endo, A., Kakiki, K., and Misato, T., J. Bact. *104* (1970) 189.
4 Hori, M., Kakiki, K., and Misato, T., J. Pestic. Sci. *2* (1977) 139.
5 Mehta, R. J., Kingsbury, W. D., Valenta, J., and Actor, P., Antimicrob. Agents Chemother. *25* (1984) 373.
6 Ames, B. N., Ames, G. F., Young, J. D., Tsuchiya, D., and Lecocq, J., Proc. natl Acad. Sci. USA *70* (1973) 456.
7 Fickel, T. E., and Gilvarg, C., Nature, New Biol. *241* (1973) 161.
8 Hammond, S. M., Claesson, A., Jansson, A. M., Larsson, L., Pring, B. G., Town, C. M., and Ekstrom, B., Nature *327* (1987) 730.
9 Andruskiewicz, R., Chmara, H., Milewski, S., and Borowski, E., J. med. Chem. *30* (1987) 1715.
10 Kingsbury, W. D., Boehm, J. C., Mehta, R. J., and Grappel, S. F., J. med. Chem. *26* (1983) 1725.
11 Mitani, M., and Inoue, Y., J. Antibiot. *21* (1968) 492.
12 Erwin, D. C., and Katznelson, K., Can. J. Microbiol. *7* (1961) 15.
13 Singh, D. P., and LeJohn, H. B., Can. J. Biochem. *53* (1975) 975.

0014-4754/89/040325-03$1.50 + 0.20/0

Carboxylesterases of high molecular weight in the hemolymph of *Locusta migratoria*

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Summary. The main carboxylesterase in the hemolymph of the migratory locust, *Locusta migratoria*, is a protein of high molecular weight; about 700–750 kDa. This esterase hydrolyzes juvenile hormone III, α-naphthylacetate and β-naphthylacetate. The carboxylesterase dissociates to give an esterase of molecular weight 148 kDa after treatment of the hemolymph with mercaptoethanol.
Key words. Carboxylesterase; juvenile hormone esterase; juvenile hormone binding protein; hemolymph proteins; migratory locust.

Hemolymph proteins of insects have many physiological functions. Analysis of the protein composition by either gel permeation chromatography or non-denaturing polyacrylamide gel electrophoresis (PAGE) revealed that many proteins in the hemolymph have high molecular weights. In the migratory locust, *Locusta migratoria*, for instance, almost 80% of the proteins from the hemolymph of adult males have molecular weights above

450 kDa [1]. We recently studied locust hemolymph electrophoretically and identified at least 4 proteins of high molecular weight (M_r); lipophorin (LP), $M_r \sim 675{,}000$; juvenile hormone binding protein (JHBP), $M_r \sim 566{,}000$; larval hemolymph protein (LHP), $M_r \sim 500{,}000$, and a biliverdin containing cyanoprotein (CP) with $M_r \sim 465{,}000$ [2-5]. Among these proteins, LP and CP are major proteins in the adults, whereas LHP and CP are major proteins in the last larval instar. LP functions as a vehicle for various lipid classes and LHP is probably a storage protein. The function of CP is not understood, except that it binds and transports biliverdin [6]. JHBP, which is JH-III-specific in the locust [5], is a minor protein in the hemolymph of larvae and adults, comprising about 1–2% of the protein content [4].

The minor proteins of the hemolymph also include enzymes. Carboxylesterases are particularly widespread among insects, and most species contain several types [7]. Some of those carboxylesterases also hydrolyze juvenile hormone (JH), but the exact physiological role of the carboxylesterase still remains to be established.

In the hemolymph of the migratory locust, Peter et al. [8, 9] reported two types of carboxylesterases, with M_r of 150,000 and 65,000, respectively. Both carboxylesterases hydrolyze α-naphthylacetate, JH-I and JH-III, and both are readily inhibited by diisopropylfluorophosphate (DFP). According to Peter et al. [9], the carboxylesterases could not be separated from JHBP by either gel permeation or ion exchange chromatography. Also in non-denaturing PAGE, the JHBP and carboxylesterase migrated together. Surprisingly, Peter et al. reported an M_r for JHBP of about 450,000.

We confirm that high-molecular weight proteins from locusts are difficult to separate by conventional non-denaturing PAGE. However, prolonged electrophoresis in gradient gels by the method of Levenbook [10] separated JHBP from some other hemolymph proteins of high molecular weight [2]. We used that system to separate hemolymph proteins from the locust and the Colorado potato beetle, *Leptinotarsa decemlineata*. After the run, the gels were stained with Coomassie brilliant blue for total protein and with α-naphthylacetate and Fast Blue RR for the presence of carboxylesterase activity.

Two sharp esterase bands are visible with locust hemolymph; one intense and one rather weak (fig. 1). Both esterases were found to have very large molecular weights ($M_r > 700{,}000$) when compared with high molecular weight markers stained with Coomasie brilliant blue. Isolated lipophorin, obtained by KBr gradient centrifuging [5], also showed weak esterase activity. In hemolymph from the Colorado potato beetle, the main carboxylesterases have lower molecular weights (fig. 1). In another electrophoretic system, agarose-gel electrophoresis, locust hemolymph also showed one intense and one faint band after staining for carboxylesterase, whereas hemolymph from the Colorado potato beetle showed several esterases (not shown).

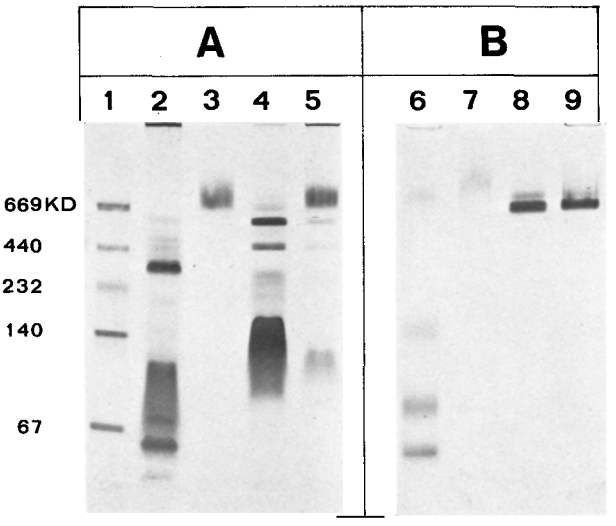

Figure 1. Separation of insect hemolymph proteins by non-denaturing PAGE. Electrophoresis was done as described previously [4, 5]. Gels were stained either with Coomassie Brilliant Blue *A* or with 12 ml of 0.03 M α-naphthylacetate in 0.04 M phosphate buffer for 5 min at 25 °C followed by Fast Blue RR salt (1 mg/ml H_2O) *B*. From left to right: 1) High-molecular weight protein markers (Pharmacia); 2) Whole hemolymph from the Colorado potato beetle (1 µl); 3) Isolated locust lipophorin (10 µg); 4) Locust hemolymph without lipophorin (80 µg); 5) Whole hemolymph from male locusts (1 µl); 6) Whole hemolymph from the Colorado potato beetle (5 µl); 7) Isolated lipophorin from locust (50 µg); 8) Locust hemolymph without lipophorin (130 µg); 9) Whole hemolymph from male locusts (5 µl).

Accurate determination of the M_r of locust carboxylesterase by comparison with high-molecular weight standard proteins is difficult, because of its very high molecular weight, and because gels shrink to different extent after staining by different procedures. However, shrinking of gels can be largely prevented by transferring gels immediately after carboxylesterase staining to the same ethanol/acetic acid/water mixture as that used for Coomassie staining (25:8.5:66.5 v/v). Calculation of the M_r on the basis of relative mobility, with the end of the gel as a reference point, results in an M_r of approximately 700–750 kDa for the main esterase of the locust. With the same calculation, we arrived at values of 837, 273, 148 and 56 kDa for the carboxylesterases of the Colorado potato beetle. Since the M_r of JHBP was 566,000 [4], these results suggest that the carboxylesterase and JHBP are distinct high-molecular weight proteins in the hemolymph of the locust. To get further evidence, we subjected locust hemolymph to electrophoresis on a preparative 4–10% gradient tube gel for 4000 Volthours, cut the part of the gel between the yellow lipophorin band and the blue cyanoprotein band into slices (this is the region of the gel which contains both the esterase and the JH binding activity), extracted the homogenized slices overnight and measured the carboxylesterase, the JH esterase and the JH binding activity in each fraction (fig. 2). The main carboxylesterase (fraction 5–7) in the hemolymph of the locust had an M_r larger than that of JHBP (fraction 7–8), which clearly shows that the two activities are due to

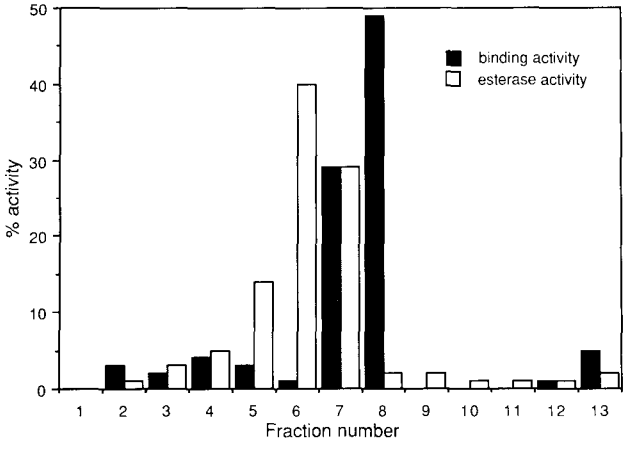

Figure 2. Distribution of JH esterase and JH binding activity in locust hemolymph, after separation of the proteins by non-denaturing PAGE. Hemolymph samples (500 µl) were separated for 4000 Volthours in a 11 × 1.2-cm tube containing a 4–10% gradient gel. After the run, gels were cut in 4-mm slices between the yellow lipophorin band (mobility: 30–40 mm; fractions 2 and 3) and the blue cyanoprotein band (mobility: 76–79 mm; fraction 13). Each fraction was pressed through a syringe fitted with a 18G1.5 needle. Fraction 1 is a gel slice without protein. The fractions were extracted overnight in the cold room with 1 ml 0.1 M phosphate buffer containing 0.15 M NaCl under constant shaking in an Eppendorf tube. After centrifuging, 500 µl from each fraction was withdrawn and used to determine JH esterase activity, general carboxylesterase activity with α-naphthylacetate and β-naphthylacetate as substrates, and JH binding activity. The assays are extensively described elsewhere[4, 10, 11]. The distribution of the general carboxylesterase activity with either of the two substrates was similar to that of JH esterase activity.

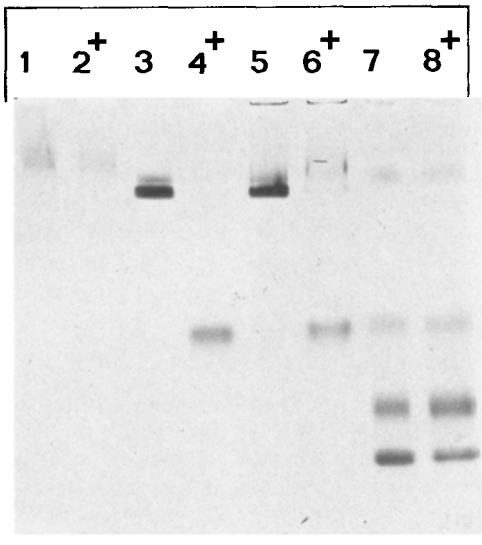

Figure 3. Separation of insect hemolymph esterases in non-denaturing PAGE in the presence or absence of 5% mercaptoethanol. Hemolymph samples were diluted 1:1 with 0.05 M phosphate buffer pH 7.5, containing 0.15 M NaCl; 10 mM EDTA and mercaptoethanol when indicated by (+). The gels were run and stained (fig. 1). From left to right: 1 and 2) *Isolated lipophorin (50 µg)*; 3 and 4) *Locust hemolymph without* lipophorin (130 µg); 5 and 6) Whole hemolymph from male locusts (5 µl); 7 and 8) Whole hemolymph from the Colorado potato beetle (5 µl).

different proteins. Moreover, the esterase from the locust hydrolyzes JH-III, α-naphthylacetate and β-naphthylacetate; this is shown by the fact that the distribution of the activity of the gel was similar with either of those three substrates. This confirms that the carboxylesterase in the locust is not JH-specific, but rather a general esterase which also degrades JH-III[8, 9].

In our trials with locust hemolymph, we never noticed the presence of a low-molecular weight carboxylesterase as described by Peter et al.[8, 9]. However, they indicated that treatment of hemolymph with mercaptoethanol resulted in dissociation of the high-molecular weight esterase to give a low-molecular weight carboxylesterase. So we separated hemolymph by PAGE after addition of mercaptoethanol to the sample buffer. The high-molecular weight carboxylesterase in the hemolymph of the locust dissociated into an esterase with much lower M_r, whereas the esterases in the hemolymph of the Colorado potato beetle were hardly affected by mercaptoethanol (fig. 3). The M_r of the low-molecular weight carboxylesterase was accurately determined in two different gradient gels. The plots of log molecular weight against relative mobility of standard proteins (standard proteins of high and low molecular weight, Pharmacia, Uppsala, Sweden) were straight lines, which were calculated by the method of least squares. The correlation coefficients of the two lines were − 0.9966 for gradient 4–20% polyacrylamide and − 0.9969 for the gradient 9–25%. From those lines an average M_r value of 148 ± 3 kDa (n = 3) was calculated for the carboxylesterase of low molecular weight from the locust.

We conclude that the molecular weight of the esterase in the hemolymph of the migratory locust is much higher than that reported by Peter et al.[8, 9], which explains why the esterase could not be separated from JHBP by gel permeation chromatography. The esterase in locust hemolymph is not JH-specific, since it hydrolyzes α- and β-naphthylacetate and JH-III, and because it is sensitive to the inhibitor DFP[8, 9]. The esterase of high molecular weight is the main carboxylesterase in the hemolymph of the locust, but the enzyme dissociates into an esterase of low molecular weight after reduction with mercaptoethanol. It remains to be established why a carboxylesterase of low molecular weight aggregates into a high molecular weight complex. However, after dissociation with mercaptoethanol, JHBP and the esterase activity can be separated, and the relative importance of these two proteins in the regulation of the JH titer can now be established.

1 van der Horst, D. J., van Doorn, J. M., and Beenakkers, A. M. Th., Insect Biochem. *9* (1979) 627.
2 de Bruyn, S. M., Koopmanschap, A. B., and de Kort, C. A. D., Physiol. Ent. *11* (1986) 7.
3 de Kort, C. A. D., and Koopmanschap, A. B., Archs Insect Biochem. Physiol. *4* (1987) 191.
4 Koopmanschap, A. B., and de Kort, C. A. D., Archs Insect Biochem. Physiol. *7* (1988) 105.

5 de Kort, C. A. D., and Koopmanschap, A. B., Archs Insect Biochem. Physiol. *5* (1987) 255.
6 Chino, H., Abe, Y., and Takahashi, K., Biochim. biophys. Acta *748* (1983) 109.
7 Hammock, B. D., in: Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 7, p. 431. Eds G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford 1985.
8 Peter, M. G., Gunawan, S., Gellissen, G., and Emmerich, H., Z. Naturforsch. *34c* (1979) 588.
9 Peter, M. G., Stupp, H. P., and Lentes, K. U., in: 2nd International CNRS Symposium Biosynthesis, Metabolism, Mode of Action of Invertebrate Hormones. Summaries, p. 145 (1983).
10 Levenbook, L., in: The Larval Serum Proteins of Insects. p 1. Ed. K. Scheller. Georg Thieme, Stuttgart 1983.
11 van Asperen, K., J. Insect Physiol. *8* (1962) 401.
12 Hammock, B. D., and Sparks, T. C., Analyt. Biochem. *82* (1977) 573.

0014-4754/89/040327-04$1.50 + 0.20/0

The presence of free D-amino acids in mouse tissues

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Summary. The presence of free D-amino acids in mouse kidney, liver, brain, heart, lung, thymus and serum has been shown with an enzymic microdetermination method. The D-amino acid levels were higher in the extracts of kidney and liver than in those from other organs.
Key words. D-amino acid; D-amino acid oxidase; mouse tissue.

It is generally believed that mammals neither synthesize nor possess D-amino acids (DAA) whereas microorganisms, some insects, marine invertebrates and higher plants contain DAA[1]. Only D-Asp is known to exist in metabolically stable proteins[1] and tissues[2]. We have observed considerable amounts of free DAA in human plasma from patients with renal diseases[3], using an enzymic method[4] which detects neutral DAA. In the present study, the investigation was extended to mouse tissues.

Materials and methods. a) Reagents. D-amino acid oxidase (DAO, EC 1.4.3.3) from porcine kidney was obtained from Sigma (St. Louis, MO, USA) as a crystalline suspension in ammonium sulfate. It was freed of ammonium ions by passage through a Sephadex G-25 column equilibrated with 0.019 M sodium pyrophosphate buffer, pH 8.3 (PPB) resulting in solution of 0.3 mg/ml protein concentration. Catalase (EC 1.11.1.6), as a purified powder (10,000–25,000 units/mg protein) from bovine liver, was also purchased from Sigma. All other chemicals were analytical-grade products.

b) Preparation of tissue extracts. Normal, healthy infant (4-week-old), adult (19-week-old) and old (11-month-old) BALB/cA female mice were used. Each group consisted of 5 litters. The mice were kept on a normal diet and fasted 16–19 h prior to sacrifice by bleeding from the axillary vessels under anesthesia with ether. After rinsing with phosphate-buffered saline, pH 7.4 (PBS), to remove blood, the liver, kidney, brain, heart, lung and thymus were homogenized as whole organs, each with 4–5 vol. of PBS in a glass homogenizer in an ice bucket, at 1,000 rpm for 1 min. The homogenate was centrifuged at 160,000 × g for 15 min at 4 °C, and the supernatant extracts passed through a 'Centricut' ultramembrane filter (Kurabo, Osaka, Japan) to remove substances larger than 10,000 dalton by centrifuging at 5,000 × g for 1 h at 4 °C. The filtrate was used for the DAA assay.

c) DAA determination. DAO catalyzes the oxidation of DAA to produce α-keto acids; $R-CH(NH_2)-COOH + O_2 + H_2O \rightarrow R-CO-COOH + NH_3 + H_2O_2$. Details of the assay method are described elsewhere[4]. Briefly, three reaction tubes were prepared as follows (table 1): tube 1 consisted of 40 µl of the tissue extract diluted to 1–5 mg of protein per ml, 40 µl of solution A (consisted of 1.2 ml of 0.19 M PPB, pH 8.3, 30 µl of 0.5 mg/ml FAD, and 120 µl of 6 mg/ml catalase), 10 µl of water, and 10 µl of 0.019 M PPB, pH 8.3. Tube 2 contained a similar mixture to tube 1 except that DAO in PPB substituted for the 10 µl-buffer, and tube 3 contained DAO in PPB and 1 mM D-Ala, substituted respectively for the buffer and water. D-Ala was employed as the standard substrate throughout the present experiments. All amino acids were measured as alanine equivalents although other amino acids led to different molar absorbances in the kidney extract: D-Ala, 100%; D-Ser, 28.4%; D-Thr, 4.3%; D-Phe, 92.2%; D-Met, 72.3%; D-Leu, 83.2%; D-Ile, 51.4%; D-Val, 55.3%; D-Trp, 53.1%.

The reaction was started by the addition of DAO. After a 10-min incubation at 37 °C, the reaction was stopped

Table 1. Composition of the reaction tubes for the DAA assay

	Tube 1	Tube 2	Tube 3
40 µl	Extract	Extract	Extract
40 µl	Solution A	Solution A	Solution A
10 µl	Distilled water	Distilled water	D-Ala
10 µl	PPB	DAO	DAO