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Purification and characterization of a β-glucosidase (linamarase) from the haemolymph of *Zygaena trifolii* Esper, 1783 (Insecta, Lepidoptera)

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Summary. A β-glucosidase (linamarase) was purified 52-fold with a recovery of 27% from the haemolymph of the larvae of *Zygaena trifolii*, ESPER, 1783 (Lepidoptera, Zygaenidae). The final enzyme preparation was found to be nearly homogeneous on both disc polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. The molecular weight of the enzyme was determined to be about 130 kDa; it consisted of two subunits of about 66 kDa. The enzyme showed an optimum between pH 4.5 and 5 with linamarin and a broad optimum between pH 3.5 and 6.5 for p-nitrophenyl-β-D-glucoside; the temperature optimum was 40 °C. The β-glucosidase showed a high specificity for its endogenous substrates linamarin and lotaustralin. Among the other natural and artificial substrates tested, only prunasin and p-nitrophenyl-β-D-glucoside were hydrolyzed by the enzyme, whereas linustatin, salicin, cellobiose and trehalose were not. The enzyme is strongly inhibited by β-glucosylpiperidine.
Key words. Cyanogenesis; β-glucosidase; Lepidoptera; linamarin; linamarase; lotaustralin; repellent; Zygaenidae.

Cyanogenic glycosides have long been accepted as defensive compounds for organisms because of their ability to release hydrogen cyanide (HCN) upon hydrolysis; this is the phenomenon of cyanogenesis [1, 2]. For plants as well as for arthropods, the protective function of HCN has been discussed [3, 4]. Cyanogenic glucosides are hydrolyzed by β-glucosidases to give corresponding hydroxynitriles which dissociate spontaneously above pH 6. At lower pH values, the reaction is catalyzed by hydroxynitrile lyases [5].

Several reports have shown that in plants cyanogenic glucosides and their catabolic enzymes are localized in different compartments (e.g. in the vacuole, the cytoplasmatic or apoplasmatic space) of cells belonging to either the same or different tissues. It is only after disruption of the tissue that HCN is liberated in major amounts [6–9].

β-Glucosidases often exhibit a rather high specificity towards the aglycone moiety[10].

It has been shown recently that cyanogenic glucosides occur in several insect orders (Coleoptera, Heteroptera, Hymenoptera and Lepidoptera; for review see Davis and Nahrstedt[11] and Nahrstedt[12]). These insects often liberate HCN after violation thereby indicating the presence of a β-glucosidase activity. Until now, however, no such enzyme has been localized and characterized.

One of the best investigated lepidopteran species is *Zygaena trifolii* whose larvae contain the cyanogenic glucosides linamarin and lotaustralin, one third of which is stored in the haemolymph and two thirds in the cuticle[11,13]. Upon irritation by a predator the larvae release, as a defensive response, fluid droplets out of cuticular cavities which contain the same cyanoglucosides[14].

In the present paper we report on the localization, purification and characterization of a β-glucosidase of *Zygaena trifolii* larvae which is responsible for liberation of hydrogen cyanide from the endogenous substrates linamarin and lotaustralin.

Materials and methods

Experimental animals. Specimens of *Zygaena trifolii* were reared as described previously[15]. The larvae are oligophagous feeders on *Lotus corniculatus* and *Lotus uliginosus* (Fabaceae). Larvae of the last instar were used for experiments. They were reared in containers, placed in incubators at 21 °C under long-day conditions (L/D = 16/8).

Distribution of enzyme activity. Two last-instar larvae of *Zygaena trifolii* were completely dissected and the organs were homogenized in ice-cold McIlvaine buffer (pH 5; 150 mM). After centrifugation at 10 000 × g for 5 min the enzyme activity was determined against linamarin and 4-nitrophenyl-β-D-glucoside (4NPG) using the standard enzyme assay (see below).

Enzyme purification. All steps were carried out at 4 °C. All buffer solutions contained 20% ethylene glycol. The haemolymph was collected by puncturing one of the abdominal prolegs and diluted 2–3-fold with McIlvaine buffer (pH 6.5; 17 mM). Between 400 and 700 µl of haemolymph were used for one purification procedure. The diluted haemolymph was centrifuged at 10 000 × g for 2 min and the supernatant was purified stepwise according to the following scheme:

A) Sephadex G-25 (5 × 1.5 cm) column (Pharmacia, Freiburg, FRG) equilibrated with McIlvaine buffer (pH 6.5, 17 mM) and eluted with the same buffer.

B) DEAE-Sephacel (13 × 1.8 cm) column (Pharmacia, Freiburg, FRG) washed with the above buffer after applying the active fractions obtained from A). A linear gradient of 0–250 mM sodium chloride in the same McIlvaine buffer was used for elution.

C) Cation exchanger CM-Biogel (16 × 2.2 cm) column (Biorad, Witten, FRG) equilibrated with McIlvaine buffer (pH 5, 15 mM) and washed with the same buffer after applying the active fractions obtained from B). Elution was performed with a linear gradient (15–500 mM) of McIlvaine buffer (pH 5).

D) Sephacryl S-200 (60 × 1.6 cm) column (Pharmacia, Freiburg, FRG) equilibrated and eluted with a 165 mM solution of sodium chloride in McIlvaine buffer (pH 6.5, 17 mM).

Between B and C and C and D, the protein fractions containing the linamarase activity were pooled, concentrated by ultrafiltration through an Amicon PM 30 filter (Richmond, California) and the pH was changed by gel filtration on Sephadex G-25 (compare to A).

Protein concentrations were monitored spectrophotometrically at 280 nm or determined by the method of Bradford[16].

Enzyme assay. The β-glucosidase activity against different substrates was determined by estimating HCN, 4-nitrophenol or glucose released:

Nitrophenyl-glycosides. An appropriate amount of enzyme fraction was diluted with substrate solution in McIlvaine buffer (pH 5; 150 mM) to a volume of 0.5 ml. In the standard enzyme assay, a concentration of 2 mM 4-nitrophenyl-β-D-glucoside (4NPG) and 2% ethylene glycol were used in the final mixture. After incubation for 10 min at 30 °C, 0.5 ml of 1 M sodium carbonate was added to stop the reaction. The amount of 4-nitrophenol liberated was determined spectrophotometrically at 400 nm.

Cyanogenic glucosides. In Thunberg vessels[17] an appropriate amount of enzyme solution was mixed with McIlvaine buffer (pH 5; 150 mM) in a final volume of 1 ml. The vessels were then immediately closed. A 2 mM concentration of linamarin containing 2% ethylene glycol was used in the final mixture. After incubation at 30 °C, the reaction was stopped by adding 1 ml of 1n NaOH from the side-arm without opening the vessel. The amount of HCN was determined using the Merck Spectroquant kit, described in data sheet 130 259 8 Do dt/5r of Merck (Darmstadt, FRG). The hydrolysis of some substrates (salicin, cellobiose, trehalose), which could not be measured by the above methods, were estimated by determining the glucose liberated. In these cases the reaction was stopped by quickly heating the incubation mixture to boiling after an appropriate time of incubation. The concentration of free glucose was measured by means of the hexokinase/glucose-6-P dehydrogenase assay system[18] as modified by Boehringer (Mannheim, FRG). Time course studies with the purified protein fraction showed that the enzyme reactions with linamarin and 4NPG were linear for at least 10 min at 30 °C under the conditions used (comp. standard enzyme assays). Therefore all standard enzyme assays were carried out for 10 min.

The inhibitors nojirimycin, glucosylpiperidine and δ-gluconolactone were tested at concentrations of 0.017, 0.02 and 1 mM respectively using the standard enzyme assay with 4NPG as the substrate.

Determination of molecular weight. The molecular weight of the purified linamarase was determined by filtration on Sephacryl S-200 gel according to the procedures of Andrews[19]. The column (60 × 1.6 cm) was equilibrated with McIlvaine buffer (pH 6.5; 17 mM) containing 20% ethylene glycol and 165 mM sodium chloride. Fractions of 1 ml were collected and linamarase activity was detected using 4NPG as a substrate. The following molecular weight standards (Low-molecular-weight calibration kit, Pharmacia, Freiburg, FRG) were used for calibrating the column (fig. 5): aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chrymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa).

Electrophoretic procedures. SDS-polyacrylamide gel electrophoresis was carried out in a flat bed apparatus with a 10% separation gel according to Laemmli[20]. Alkaline PAGE under non-denaturating conditions was performed as described by Maurer[21] with a 6.5% separation gel. For analysis of linamarase activity, the gel was immediately sliced into 5-mm pieces and incubated for 1 h with 1 ml of McIlvaine buffer (pH 5; 150 mM); 300 µl of the solution obtained were tested against linamarin with the standard enzyme assay. The molecular weight standards used are listed in figure 4.

Results

Linamarase activity in different larval organs. Table 1 shows the distribution of β-glucosidase activity within a larva of *Zygaena trifolii*. About 85% of the linamarase activity was found in the haemolymph, whereas the integument only contained about 11%. Gut epithelium, fat body, silk glands and malpighian tubules showed less than 2% enzyme activity. It is interesting to note that the defensive fluid does not contain a β-glucosidase capable of hydrolyzing linamarin. Compared to the activity against linamarin, the relative distribution of enzyme activity against 4NPG differs slightly for the gut epithelium, the integument and its secretion. This may indicate that these tissues are endowed with different (additional) β-glucosidases.

Table 1. Percentage distribution of β-glucosidase activity in different larval organs. The enzyme activity was measured against linamarin and 4NPG. The range of the values obtained from two larvae is shown assuming total dissection and recovery of enzyme activity.

Tissue	% of linamarin hydrolysis	% of 4NPG hydrolysis
Haemolymph (50 µl)	84–85	84–86
Gut epithelium	0.3–1	4–6
Gut content	1–3	3–4
Fat body	1–3	1–2
Silk glands	0	0
Malpighian tubules	0	0
Integument	9–13	4–6
Defense secretion (5 µl)	0	0.5

As the major part of the linamarase activity is stored in the haemolymph, this larval body fluid was used for further purification. Preliminary experiments on larvae of different ages during the last larval instar did not show major differences in the amount of linamarase activity in the haemolymph; therefore last-instar larvae were used regardless of their age.

Purification of linamarase. The specific activities observed with linamarin and 4NPG as substrates during the purification procedure are summarized in table 2. During centrifugation and Sephadex G-25 gel filtration the specific activity remained fairly constant. On the anion exchanger DEAE-Sephacel, the greater part of the activity was eluted with 45 mmolar NaCl. The small amount

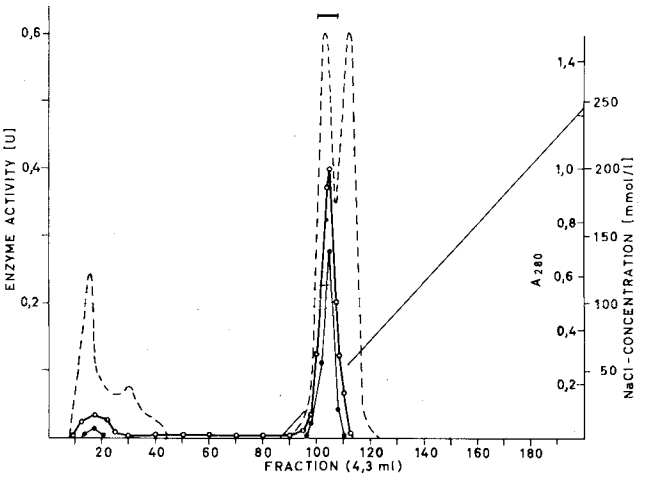

Figure 1. Separation of haemolymph proteins of *Z. trifolii* on a DEAE-Sephacel column. For details see 'Materials and methods'. The gradient starts at fraction 87. --- protein (A_{280}); ●—● linamarin hydrolysis; ○—○ 4NPG hydrolysis; ├─┤ fractions combined and further purified.

Table 2. Purification of the linamarase from the haemolymph of *Z. trifolii* larvae.

Procedure	4NPG Total activity (U)	Specific activity (U/mg)	Purification -fold	Protein (mg)	Linamarin Total activity (U)	Specific activity (U/mg)	Purification -fold
Crude extract	9.1	0.16		57.6	8.2	0.14	
Centrifugation	7.8	0.15	1	53.3	6.7	0.12	1
Sephadex G-25	6.4	0.15	1	42.7	6	0.14	1
DEAE-Sephacel	4.4	0.41	3	10.7	4.1	0.38	3
CM-Biogel	3.8	7.9	49	0.48	3	6.2	44
Sephacryl S-200	2.5	8.3	52	0.3	2.2	7.4	53

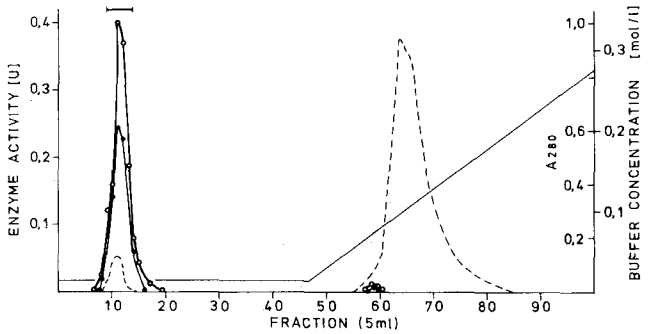

Figure 2. Separation of the active fractions obtained from the DEAE-Sephacel column on a CM-Biogel column. The gradient starts at fraction 47. For symbols see fig. 1.

of activity which did not bind to the column was not further investigated (fig. 1). On the cation exchanger (CM-Biogel), the linamarase activity did not bind to the column, while a large amount of protein did. This resulted in a 16-fold increase in specific activity (fig. 2). As with the anion exchanger the small peak of activity eluted during the gradient with an 80 mM concentration of McIlvaine buffer (pH 5) was not further investigated. Only a minor increase in specific activity occurred following Sephacryl S-200 gel filtration. Overall, a 52-fold purification with a 27 % yield was obtained with an almost constant ratio of activities against the two substrates 4NPG and linamarin.

Electrophoretic analysis and molecular weights. The final preparation of linamarase gave one major protein band and two minor bands on alkaline PAGE under non-denaturating conditions using silver staining (fig. 3 b). Figure 3a shows the enzyme activity against linamarin of the corresponding eluted gel slices. The highest enzyme activity coincided with the main protein peak. The minor bands did not show any linamarase activity. Thus the main peak of the purified linamarase fraction is responsible for the hydrolysis of linamarin. When subjected to SDS-PAGE with silver staining, the final enzyme preparation gave one major band at a position corresponding to about 66 kDa (fig. 4). With gel filtration on Sephacryl S-200 a molecular weight of approximately 130 kDa was determined for the native protein (fig. 5). Thus the linamarase is a dimer consisting of two subunits with the same molecular weight.

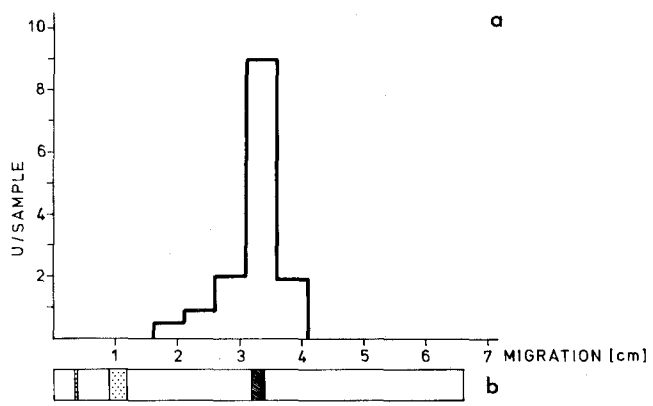

Figure 3. Nondenaturating alkaline PAGE of the purified linamarase. *a* Linamarin hydrolysis in the sliced and extracted gel; *b* schematic diagram of silver-stained protein bands after alkaline PAGE.

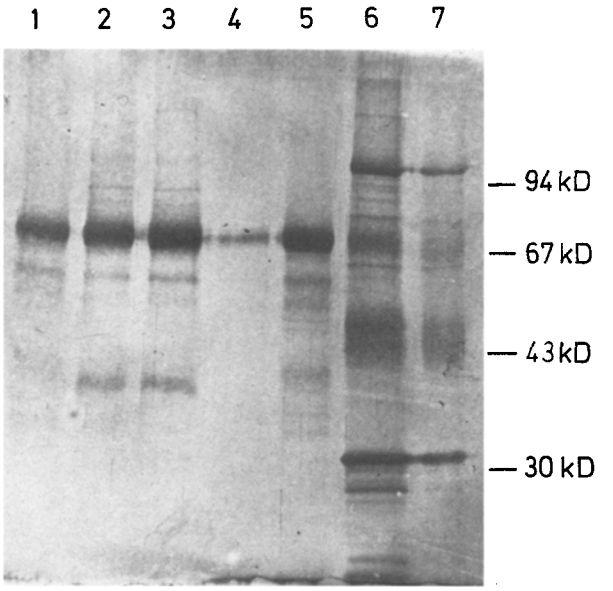

Figure 4. SDS-PAGE and silver staining of the purified linamarase (lane 1: 0.4 µg, lanes 2 and 3: 1–15 µg, lane 4: 0.005 µg, lane 5: 0.5–1 µg protein) and the reference proteins phosphorylase (97 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa); lane 6: 1–13 µg, lane 7: 0.1–0.3 µg of each protein.

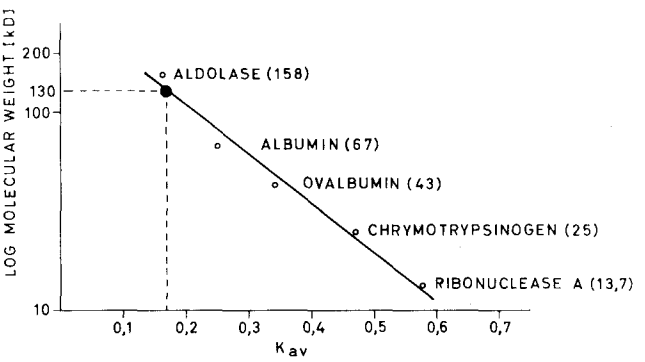

Figure 5. Determination of the molecular weight of the *Zygaena* linamarase on Sephacryl S-200 (compare 'Materials and methods'). The corresponding position of the linamarase is marked by a black dot.

Properties of the linamarase. The effect of pH on the enzyme activity was determined with 4NPG and linamarin as substrates in McIlvaine buffer. As shown in figure 6, there is a broad range of maximal activity between pH 3.5 and 6.5 for 4NPG, whereas the pH optimum for linamarin is more narrow, lying between pH 4.5 and 5. At the pH of the haemolymph, pH 6.2, the activity against linamarin is only about 45% of the maximal

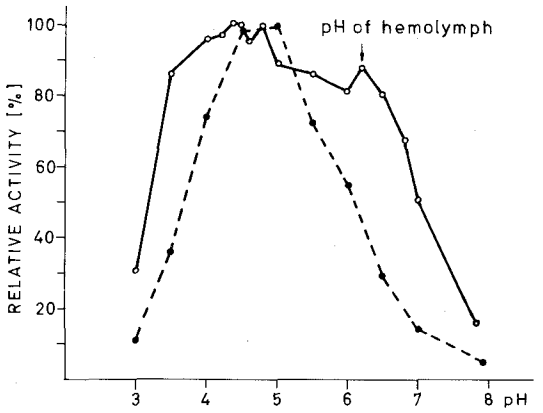

Figure 6. pH-dependency of the purified linamarase fraction. The enzyme activity was measured in McIlvaine buffer against linamarin ●---● and 4NPG o---o with the standard enzyme assay. The enzyme activity is expressed in percent of maximal activity.

Table 3. Substrate spectrum of the purified linamarase. K_m and V_{max} values were determined by using the Lineweaver-Burk equation.

Substrate	K_m mmol/l	V_{max} U/mg	Relative activity* U/mg	%
Linamarin	7.8	71.8	8.5	50.3
Lotaustralin	2.5	65	17.8	105.3
Linustatin	0	0	0	0
Prunasin	0.02	1.2	1.2	7.1
Amygdalin	ND	ND	$\ll 1$	$\ll 1$
Acalyphin	ND	ND	$\ll 1$	$\ll 1$
4-NP-β-glucoside	0.27	20.9	16.9	100
4-NP-α-glucoside	ND	ND	$\ll 1$	$\ll 1$
4-NP-β-galactoside	0	0	0	0
Salicin	ND	ND	$\ll 1$	$\ll 1$
Trehalose	0	0	0	0
Cellobiose	0	0	0	0

* Relative activity was measured at 1 mM substrate concentration and was related to the hydrolysis of 4NPG, which was set at 100%. ND = not determined.

activity. When incubated with 4NPG the linamarase exhibited a temperature optimum at 40 °C.

The purified linamarase is rather stable when stored at pH 5 and at higher pH (tested up to pH 6.2). Thus, only 20% of the enzyme activity is lost when kept at pH 5 for 1 h at 30 °C. In contrast, the enzyme is unstable at more acidic pH values; after 10 min of incubation at pH 4.5 (30 °C), 50% of the activity is lost. Storage at low temperatures does not lead to a major decrease of enzyme activity. When the enzyme is kept at pH 6 for 100 days at 4 °C, 40% of the activity is lost. Storage at − 20 °C and repeated freezing and thawing of the enzyme solution led to the same loss of activity. Chromatography of the haemolymph on an anion exchanger, without ethylene glycol, resulted in nearly complete loss of β-glucosidase activity. Similarily, fresh haemolymph retained its activity for 72 h when incubated at pH 6 with 20% ethylene glycol, whereas about 75% of the activity was lost in the same assay without ethylene glycol. The purified enzyme fraction showed maximal activity when the incubation mixture contained 2–10% ethylene glycol. Lower (0.08%) and higher (20%) concentrations decreased the activity to about 50%. During our experiments ethylene glycol was the compound found to be most efficient in stabilising the enzyme. Other compounds such as glycerol, carbohydrates or methyl cellosolve, more or less empirically used for protein stabilisation [34, 35], showed only a slight effect, or none. Thus, the standard enzyme assays were run with 2% ethylene glycol.

Substrate specificities of the purified linamarase in terms of relative activity, the apparent Michaelis constants and maximum velocities are displayed in table 3: The purified linamarase catalyses the hydrolysis of the artificial substrate 4NPG and the endogenous substrates linamarin and lotaustralin with high efficiency. A clear preference for the glucoside moiety and for the β-configuration of the glycosidic linkage is observed, since 4-NP-α-glucoside and 4-NP-β-galactoside are not hydrolyzed. Disaccharides such as linustatin (glucosylated linamarin) and cellobiose, linked β-1,6 and β-1,4, respectively, are not cleaved; the same is true for trehalose (α-D-glucopyranosyl-α-glucopyranoside), the most abundant sugar in the haemolymph of the insects. Variation of the aglycone of β-monoglycosides reduces the catalytic activity drastically, as is indicated by the low hydrolysis of the cyanogenic glucoside prunasin (2-β-D-glucopyranosyloxy-2R-phenylacetonitrile) and by the fact that there is almost no cleavage of salicin (1-β-D-glucopyranosyloxy-2-hydroxymethyl-benzene).

The effects of the β-glucosidase inhibitors nojirimycin [22], δ-gluconolactone [23] and glucosylpiperidine [24] on the linamarase were tested using 4NPG as substrate. Only glucosylpiperidine proved to be a potent competitive inhibitor, with a Ki value of 0.11 μM. In contrast, the enzyme activity was only slightly inhibited by nojirimycin (Ki 89 μM) and δ-gluconolactone (Ki 790 μM); these values correspond to 94, 3 and 6% inhibition at the inhibitor concentration tested.

In order to determine whether the hydrolysis of linamarin and 4NPG is due to a single enzyme protein, mixed substrate studies were performed [25]. The rate of hydrolysis of mixed substrates was less than the sum of the rats with individual substrates, therefore it was concluded that the two substrates are hydrolyzed by a single enzyme.

Discussion

Several β-glucosidases responsible for hydrolysis of cyanogenic glycosides have been purified from plants (for recent review see Poulton [23]). Most β-glucosidases isolated from insects have been obtained from the digestive tract, and were investigated mainly for their ability to hydrolyse cellulose [26, 27]. Only a few insect β-glucosidases with obviously different functions for the insect have been described, e.g. the tyrosine glucoside hydrolase activity in *Manduca sexta* [28]. This paper reports for the first

time on the purification and characterization from an insect of a β-glucosidase which exhibits a pronounced activity towards endogenous secondary compounds which are thought to be used for defense. The β-glucosidase of *Z. trifolii*, purified 52-fold by methods commonly used for purification of plant glucosidases, exhibits a strong activity towards the endogenous substrates linamarin and lotaustralin and can therefore be regarded as a linamarase. Lotaustralin is a better substrate than linamarin (table 3), as shown by the higher relative activity towards lotaustralin. Unfortunately most linamarases from plants were tested with linamarin only, except the *Hevea*-enzyme[17], which shows a higher relative activity towards linamarin than towards lotaustralin. The poor hydrolysis of the artificial substrates 4-NP-α-glucoside and the 4-NP-β-galactoside, in comparison to 4NPG, indicates a high specificity for glucose as the sugar component and the β-configuration of the anomeric centre. Several natural glycosides are poor substrates, including cellobiose, trehalose, salicin, the cyanogenic monoglucosides prunasin and acalyphin, and the cyanogenic diglucosides linustatin and amygdalin. This indicates a pronounced aglycone specificity as well as the ability to cleave specifically aglycone-β-glucoside linkages but not glucose-β-glucoside bonds. Thus, the β-glucosidase exhibits a clear specificity for linamarin and lotaustralin and, therefore, may be regarded as being specially adapted for the production of hydrogen cyanide from both cyanogenic glucosides.

In contrast, plant linamarases obtained from *Trifolium repens*[29], *Phaseolus lunatus*[30], *Hevea brasiliensis*[17] and *Linum usitatissimum*[31] exhibit a broad specificity in that they usually accept prunasin, salicin, cellobiose and 4-PN-β-galactoside as substrates at rates of relative activity between 1% and more than 25%.

Although the *Zygaena* linamarase differs in its substrate specificity from plant linamarases, it has in common with them the acidic pH optimum and the molecular weight of 66 kDa for its monomer; in plants the value is usually between 55 and 65 kDa[23]. However, its instability above 40 °C is a difference from the plant enzymes, which usually exhibit temperature optima above 50 °C; in fact, this property is one in which it resembles other β-glucosidases of insects, e.g. the β-glucosidase from *Bombyx mori*[32] or from *Trinervitermes trinervoides*[33], which are inactivated at temperatures above 40 °C. Another difference from plant linamarases is the relatively low stability of the *Zygaena* enzyme during the purification process and its quick inactivation below pH 5. As noted above, the former could be partly overcome by adding ethylene glycol to the solvents. The *Zygaena* enzyme is markedly inhibited by glucosylpiperidine, which is known to be an inhibitor for plant glucosidases[24].

Several findings obtained from plants show that the cyanogenic substrates and their β-glucosidases are compartmentalized, the former being localized in special tissues like the epidermal cells of the leaf, the latter in the mesophyll or in the apoplasmatic space[23]. Cyanogenesis occurs only after disruption of the tissue. We have isolated the haemocytes from the haemolymph of *Z. trifolii* by centrifugation and have found that the linamarase activity was present exclusively in the haemoplasma. Since approximately 30% of the glucoside content of one larva is stored in the haemoplasma[11] it is by no means clear yet how linamarase activity in the larvae is controlled. In this context it is of interest that at the pH (6.2) of the haemolymph the apparent activity of the purified linamarase is low. Furthermore, it may be noted that in all experiments the larvae had to be injured in order to obtain haemolymph; an activation process may occur during injury which then leads to the production of HCN. This process may also involve a hydroxynitrile lyase which is currently under investigation.

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Extraadrenal adrenaline formation by two separate enzymes

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Summary. Adrenaline (A) is synthesized in the adrenal medullae by the enzyme phenylethanolamine-N-methyltransferase (PNMT). After surgical removal of the adrenal medullae tissue A levels ranged from 22% of control in the heart to 125% of control in the liver. Use of a novel assay to measure tissue A formation revealed that many tissues can synthesize A using PNMT and another enzyme that N-methylates both noradrenaline and dopamine. These enzymes are non-neuronal, inducible and synthesize a major fraction of tissue and urine A.
Key words. Adrenaline; epinephrine; phenylethanolamine-N-methyltransferase; PNMT; epinine.

Adrenaline (A) (also called epinephrine) is synthesized in the adrenal glands by the enzyme phenylethanolamine-N-methyltransferase (PNMT). The enzyme PNMT is also found in very small amounts in the brain, in even smaller amounts in sympathetic ganglia and the heart. Almost all PNMT is found in the adrenals [1] but when rats have their adrenal medullae surgically removed, A is still present in their urine [2] and increases during stress [3] and with time [4]. Bilateral adrenalectomy did not change urinary A in man or the A response to stress [5]. Adrenalectomized rats still synthesized renal and urinary A [6].
Noradrenaline (NA) is the physiologic substrate for PNMT; it is N-methylated to form A. Assays for PNMT use phenylethanolamine, or a related lipophilic amine, as substrate for PNMT so that an easily isolated product is formed. We have devised an assay for A forming activity (AFA) that uses NA as substrate. This permits assay of AFA by enzymes other than PNMT and investigation into the sources of extraadrenal A synthesis.

Methods

To assay tissue A forming activity (AFA), 50 µl of supernatant from centrifuged tissue homogenate was incubated for 90 min at 25 °C in the presence of 2 M Tris with 5% EDTA, pH 8.6, 2.2 µCi ^{3}H-S-adenosylmethionine, and 1 mg NA or dopamine. Each sample was then shaken for 5 min with 100 mg Al_2O_3 (alumina). The alumina was washed 4 times with 2 ml cold 1-mM dithiothreitol solution. Catechols were then eluted from the alumina with 0.6 ml of 0.1 M perchloric acid and the acid supernatant was transferred to polystyrene tubes. Remaining ^{3}H-S-adenosylmethionine was precipitated with a solution of A, S-adenosylmethionine and phosphotungstic acid. The tubes were centrifuged at 8500 g and the supernatant was transferred to a scintillation vial. One mM dithiotheitol in 1 ml pH 7.5 phosphate buffer was then added followed by 200 µl of scintillant and 4 ml of 1% diethylhexyl phosphoric acid in toluene. Finally, vials were capped, shaken, and counting was done by liquid scintillation spectrometry.
The assay for AFA has a sensitivity (twice blank) of 14 fmol of A formed. The assay was linear from 0.014 pmol/h to 6 pmol/h ($r = 0.998$) using dilutions of rat adrenal medulla in 1 mg bovine serum albumin/ml. There was a within assay coefficient of variance of 2% and between assay coefficient of variance of 18% using 0.045 pmol/h of AFA. The addition of partially purified rat liver catechol-O-methyltransferase had no effect on the assay. AFA is expressed as pmol of A formed per gram of wet tissue per hour of incubation time at 25 °C. Catecholamines were measured by the catechol-O-methyltransferase based radioenzymatic technique of Ziegler et al. [7].

Results

In experiment number 1, male Sprague-Dawley rats weighing 200–250 g underwent denervation by superior cervical ganglionectomy or renal nerve ablation. Denervation depleted tissue NA by 93–99%, yet had little effect on AFA of most tissues (fig. 1). In experiment number 2, seven rats underwent bilateral adrenal demedullation and 6 rats had sham surgery. Twelve days later AFA of the cardiac ventricle was 31 ± 3 pmol A/gm/h in the adrenal medullectomized group and