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PURIFICATION, PROPERTIES AND FUNCTION OF A UNIQUE γ-GLUTAMYL CYCLOTRANSFERASE FROM THE HOUSEFLY, *MUSCA DOMESTICA* L.*

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SUMMARY

A γ-glutamyl cyclotransferase which catalyzes the conversion of γ-L-glutamyl-L-phenylalanine into free pyrrolidone carboxylic acid and free phenylalanine has been isolated from pupae of the housefly, *Musca domestica* L. The enzyme was purified 4100-fold by $(NH_4)_2SO_4$ fractionation and chromatography on Sephadex G-75, DEAE-cellulose and hydroxylapatite. The purified enzyme has a pH optimum of 7.1–7.3, a K_m of $2.94 \cdot 10^{-3}$ M toward γ-L-glutamyl-L-phenylalanine, and an apparent mol. wt of 30 000 ± 2000.

γ-Glutamyl cyclotransferase from housefly pupae exhibits a very narrow and unique specificity, compared with purified mammalian γ-glutamyl cyclotransferase. Thus γ-L-glutamyl-L-phenylalanine is the most active substrate for the housefly cyclotransferase, whereas human and sheep brain cyclotransferase attack this substrate more slowly, by some three orders of magnitude. Other γ-glutamyl compounds, such as γ-L-glutamyl-L-alanine, γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide and γ-L-glutamyl-γ-L-glutamyl-α-naphthylamide which are excellent substrates for the mammalian cyclotransferase, are attacked very slowly or not at all by the housefly cyclotransferase. γ-L-Glutamyl-γ-L-glutamyl-L-phenylalanine is not a substrate for the housefly enzyme.

Functionally, the housefly γ-glutamyl cyclotransferase participates in the breakdown of a large free pool of γ-L-glutamyl-L-phenylalanine that is present in the newly-formed white pupa. This event is reflected in a several-fold increase in the activity of γ-glutamyl cyclotransferase following the transition of the larva to a white pupa. Free phenylalanine released in the reaction is then available for conversion to tyrosine and thence quinones which sclerotize the cuticle of the white pupa.

* Based on research conducted at the Research Institute, Canada Agriculture, Belleville, Ontario (Canada).

INTRODUCTION

γ-Glutamyl cyclotransferase participates in the overall metabolism of γ-glutamylamino acids by catalyzing their conversion into pyrrolidone carboxylic acid and free amino acid according to the following reaction:

γ-L-Glutamyl-L-amino acid ——→ L-pyrrolidone carboxylic acid + L-amino acid[1]

The enzyme was first described in pig liver extracts by Connel and Hanes in 1956[2]. Since then, γ-glutamyl cyclotransferase has been the subject of several investigations and highly purified preparations have been obtained from mammalian sources[3,4].

In this laboratory γ-L-glutamyl-L-phenylalanine has been isolated and identified as a major constituent of the larva of the housefly[5]. Growing larvae synthesize and accumulate a large free pool of this dipeptide, and after the fully-grown larva transforms into a so-called white pupa, the pool of γ-glutamylphenylalanine is rapidly and totally consumed. Its disappearance coincides with the hardening and darkening (sclerotization) of the cuticle of the white pupa. Apparently γ-glutamylphenylalanine serves as a large reservoir of phenylalanine that can be metabolized to quinones which mediate sclerotization[5].

The results presented here demonstrate that γ-glutamyl cyclotransferase catalyzes the conversion of γ-glutamylphenylalanine into free pyrrolidone carboxylic acid and free phenylalanine, a reaction which is known to occur *in vivo* in the housefly pupa[6]. The purification and properties of γ-glutamyl cyclotransferase from the housefly pupa are described and its properties are compared with those of the mammalian enzyme.

MATERIALS AND METHODS

Biological

Larvae of the housefly, *Musca domestica* L. were reared on CSMA medium at 25 °C as described previously[5]. Pupae 8–24-h old were stored at −20 °C until a sufficient weight (usually 1 kg) for isolation of the enzyme had been accumulated.

Chemicals

γ-L-Glutamyl-L-phenylalanine and γ-L-[G-^{3}H]glutamyl-L-phenylalanine (41.7 Ci/mole) were prepared as described previously[6,7]. Radioactivity in the latter was: [G-^{3}H]glutamate, 19.6%; [G-^{3}H]phenylalanine, 80.4%. γ-L-Glutamyl-γ-L-glutamyl-L-phenylalanine was supplied by Fox Chemical (Los Angeles).

γ-L-Glutamyl-α-naphythylamide and γ-L-glutamyl-γ-L-glutamyl-α-naphthylamide were synthesized by the method of Orlowski and Szewczuk[8]. γ-L-Glutamyl-*p*-nitroanilide was synthesized according to Orlowski and Meister[9]. γ-L-Glutamyl-*p*-nitroanilide, γ-D-glutamyl-γ-L-glutamyl-*p*-nitroanilide, γ-L-glutamyl-L-glutamine and γ-L-glutamyl-α-L-aminobutyric acid were a generous gift from Dr M. Orlowski. Other γ-glutamyl compounds were from Cyclo Chemical (Los Angeles).

γ-Glutamyl cyclotransferase assay using γ-[G-^{3}H]glutamylphenylalanine

Enzyme activity was determined by measuring the amount of [G-^{3}H]pyrroli-

done carboxylic acid formed from γ-[G-^{3}H]glutamylphenylalanine*. The incubation mixture contained: 2.5 μmoles of γ-glutamylphenylalanine; 360 000 dpm (approx. 4 nmoles) of γ-[G-^{3}H]glutamylphenylalanine, of which 70 500 dpm existed as γ-[G-^{3}H]glutamyl-; 50 μmoles of Tris–HCl buffer, pH 7.2; and enzyme in a final volume of 0.5 ml. After incubation at 37 °C for 10 min, the reaction mixture was held at 100 °C for 3 min, cooled to 25 °C and passed through a column of Dowex 50 resin (X4, 200–400 mesh, H^+ form, 1 cm × 2 cm). The column was washed with 5 ml of water and the effluent was collected in two scintillation vials. 10 ml of scintillator (5 g of 2,5-diphenyloxazole and 100 g of naphthalene per l of dioxane) were added and ^{3}H radioactivity counted and corrected for quenching in a Beckman LS-233 scintillation counter. Blanks were prepared in the same way, but omitting the enzyme. A unit of enzyme is defined as that amount which catalyzes the formation of 1 μmole of pyrrolidone carboxylic acid/min, under the conditions given above. Specific activity is expressed in terms of units per mg of protein, determined according to Lowry *et al.*[10].

To determine γ-glutamyl cyclotransferase activity at various stages in the insect's life cycle, the insects (larvae or pupae of known age) were homogenized in 10 times their weight of 0.01 M Tris–HCl buffer, pH 7.2, containing 0.75% NaCl (w/v) and 0.02% 1-phenyl-2-thiourea (w/v). The extract was centrifuged at 27 000 × *g* for 1 h at 0–4 °C. Aliquots of the supernatant liquid were assayed as above; activity was expressed as units per insect. The supernatant liquid was also assayed for γ-glutamyl transpeptidase activity using γ-L-glutamyl-*p*-nitroanilide as substrate as described previously[11], except that the assays were done at pH 7.2. For reasons that will become apparent below, the presence of trace amounts of γ-glutamyl transpeptidase activity remaining in the centrifuged extracts does not interfere with the formation of pyrrolidone carboxylic acid from glutamylphenylalanine catalyzed by γ-glutamyl cyclotransferase (see Results and Discussion).

Substrate-specificity studies

The ability of purified γ-glutamyl cyclotransferase to form pyrrolidone carboxylic acid from several types of γ-glutamyl compound was assayed according to Orlowski *et al.*[3]. The incubation mixtures contained: γ-glutamyl compound, 2.5 μmoles; Tris–HCl buffer, pH 7.2, 50 μmoles; and purified enzyme in a final volume of 0.5 ml. After 0–60 min of incubation at 37 °C, the reaction mixture was held at 100 °C for 3 min and passed through a short column of Dowex 50, as described above, until a total volume of 3.0 ml of effluent was collected. The absorbance at 205 nm (due to the internal peptide bond of pyrrolidone carboxylic acid) was read against a blank prepared in the same way, except that the enzyme was added immediately prior to heating at 100 °C. The amount of pyrrolidone carboxylic acid was determined from a standard curve of authentic pyrrolidone carboxylic acid processed in the same manner.

* It is worthwhile noting that cyclization of the γ-[G-^{3}H]glutamyl residue of γ-[G-^{3}H]glutamylphenylalanine to [G-^{3}H]pyrrolidone carboxylic acid does not result in loss of radioactivity, although in fact a hydrogen is lost during cyclization. This is readily understood when it is remembered that ^{3}H in labile positions such as the free amino and carboxyl groups is exchanged (removed) during purification of the dipeptide[6].

RESULTS

Changes in the activity of γ-glutamyl cyclotransferase during the transition of the larvae of M. domestica to a pupa

γ-Glutamyl cyclotransferase activity, expressed as units/insect in Fig. 1, was relatively low and constant during the 5-day period of larval growth. Upon formation of the so-called white pupa on the 5th–6th day, activity began to increase dramatically and reached a maximum some 12–14 h later. Thereafter activity declined quickly at first, and then more slowly. After 3–4 days a base-line level comparable to that found in the larval stages was reached. Accordingly, pupae 8–24-h old were used in the purification of cyclotransferase as described below.

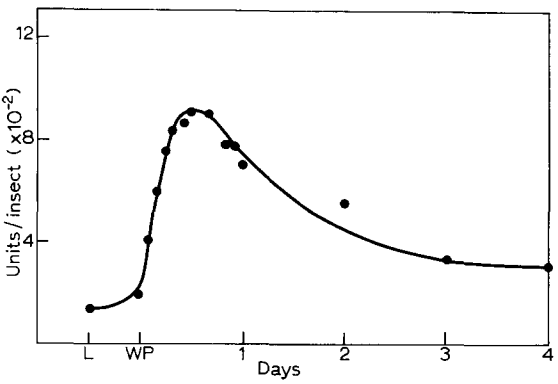

Fig. 1. Changes in the activity of γ-glutamyl cyclotransferase during the transition of the fully-grown, 5-day-old larva of *M. domestica* to the immobile, non-feeding, quiescent white pupa. Almost immediately after the white pupa forms, hardening and darkening of its cuticle (called a puparium) begins. Hardening and darkening are virtually complete within 12 h. Enzyme activity in centrifuged insect extracts was determined by measuring the amount of [G-^{3}H]pyrrolidone carboxylic acid formed from γ-[G-^{3}H]glutamylphenylalanine as described in the text. L, larva; WP, white pupa, on graph.

Purification of γ-glutamyl cyclotransferase

All steps were done at 0–4 °C unless otherwise stated. A summary of the purification of γ-glutamyl cyclotransferase from pupae of *M. domestica* is given in Table I.

TABLE I

PURIFICATION OF γ-GLUTAMYL CYCLOTRANSFERASE FROM PUPAE OF *M. domestica*

Step	*Volume (ml)*	*Protein (mg)*	*Total activity (units)*	*Spec. act. (units/mg)*	*Yield (%)*	*Purification factor*
1. Homogenate*	3600	110 000	1883	0.0172	100	1
2. $(NH_4)_2SO_4$ (40–60%)	760	33 060	1410	0.0426	75	3
3. First Sephadex G-75	600	1 199	1471	1.23	78	71
4. DEAE-cellulose	206	80.7	847	10.5	45	610
5. Second Sephadex G-75	37	25.6	440	17.2	23	1000
6. Hydroxylapatite	15	5.8	410	70.7	17	4100

* Pupae of *M. domestica* 8–24-h old, 1 kg.

Step 1. Homogenization. 1 kg of pupae 8–24-h old stored at −20 °C were thawed and homogenized in 4 l of 0.01 M Tris–HCl buffer, pH 8.0, containing 0.75% of NaCl (w/v) and 0.02% of 1-phenyl-2-thiourea (w/v) in a Waring-type blender. The homogenate was centrifuged at 12 000 × *g* for 60 min.

Step 2. $(NH_4)_2SO_4$ fractionation. The supernatant from Step 1 was brought to 40% $(NH_4)_2SO_4$ saturation at 0 °C. After standing for 1 h, the resulting precipitate was removed by centrifuging at 12 000 × *g* for 30 min, and discarded. The supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation at 0 °C and the precipitate that formed after 1 h was collected by centrifuging, dissolved in a minimum volume of 0.01 M Tris–HCl buffer, pH 8.0, and dialyzed overnight against distilled water.

Step 3. 1st Sephadex G-75 chromatography. The enzyme solution from Step 2 was concentrated by ultrafiltration (Diaflo UM 10 membrane, Amicon Corp.) and divided into five equal portions. Each portion was chromatographed separately on a column of Sephadex G-75 (5 cm × 90 cm) equilibrated with 0.01 M Tris–HCl buffer, pH 8.0. Elution was carried out using the same buffer at a flow rate of 50 ml/h and fractions of 20 ml each were collected. As shown in Fig. 2, cyclotransferase activity emerged as a single sharply-defined peak after most of the high-molecular-weight proteins had emerged in the void volume (approx. 600 ml).

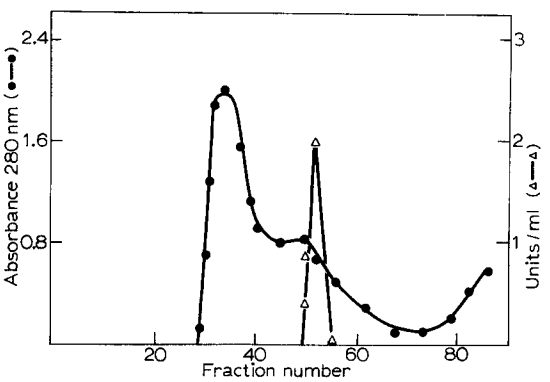

Fig. 2. Chromatography of γ-glutamyl cyclotransferase from $(NH_4)_2SO_4$ fractionation (Step 2) on Sephadex G-75. Approx. 6 g of protein contained in 100 ml of 0.01 M Tris–HCl buffer, pH 8.0, was chromatographed on a Sephadex G-75 column (5.0 cm × 90 cm) eluted with 0.01 M Tris–HCl buffer, pH 8.0, at a flow rate of 50 ml/h. Fractions of 20 ml were collected. Protein (●——●) was monitored at 280 nm and enzyme activity (△——△) was assayed by measuring the amount of [G-^{3}H]pyrrolidone carboxylic acid formed from γ-[G-^{3}H]glutamylphenylalanine as described in the text.

Step 4. DEAE-cellulose chromatography. Active fractions from the previous step were pooled and applied to a column of DEAE-cellulose (2.5 cm × 45 cm) equilibrated with 0.01 M Tris–HCl buffer, pH 8.0. Elution was carried out with a linear gradient of 0.15 M NaCl (250 ml) and 0.30 M NaCl (250 ml) in 0.01 M Tris–HCl buffer, pH 8.0, followed by a further 200 ml of 0.30 M NaCl in the same buffer. Most of the inactive protein remained bound to the column while the enzyme emerged as a single peak at the end of the gradients (Fig. 3). As the enzyme was found to be relatively unstable in strong salt solutions, it was subjected to ultrafiltration immediately after

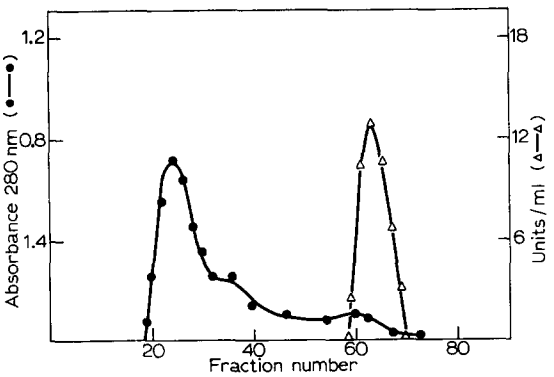

Fig. 3. Chromatography of γ-glutamyl cyclotransferase from the first Sephadex G-75 fractionation (Step 3) on DEAE-cellulose. Approx. 1.2 g of protein contained in 600 ml of 0.01 M Tris–HCl buffer, pH 8.0, was flown onto a DEAE-cellulose column (2.5 cm × 40 cm). A linear gradient of 0.15 M NaCl (250 ml) and 0.30 M NaCl (250 ml) both in 0.01 M Tris–HCl buffer, pH 8.0, was applied to the column, followed by a further 200 ml of 0.30 M NaCl in the same buffer. Fractions of 8.6 ml were collected and assayed for enzyme activity (△——△) as described in the text. Protein (●——●) was monitored at 280 nm.

it had emerged from the column and dialyzed overnight against 0.01 M Tris–HCl buffer, pH 8.0.

Step 5. 2nd Sephadex G-75 chromatography. The concentrated enzyme solution from Step 4 (about 80 mg) was applied to the top of a column of Sephadex G-75 (2.5 cm × 45 cm) equilibrated with 0.01 M Tris–HCl buffer, pH 8.0, and chromatographed as described in Step 3. Active fractions were pooled and concentrated by ultrafiltration.

Step 6. Hydroxylapatite chromatography. The enzyme solution (about 5 ml) from Step 5 was applied to a column of hydroxylapatite (1 cm × 6 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 8.0. Elution was carried out with a

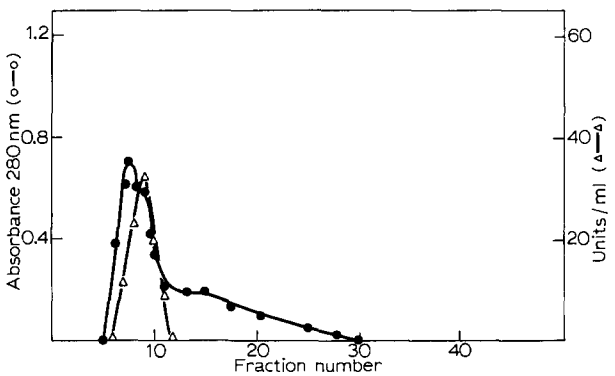

Fig. 4. Chromatography of γ-glutamyl cyclotransferase from the second Sephadex G-75 fractionation (Step 5) on hydroxylapatite. Approx. 25 mg of protein contained in 5 ml of 0.01 M potassium phosphate buffer, pH 8.0, were chromatographed on an hydroxylapatite column (1 cm × 6 cm) eluted with a linear gradient of 0.01 M potassium phosphate buffer, pH 8.0, (100 ml) and 0.10 M potassium phosphate buffer, pH 8.0, (100 ml). The flow rate was about 4 ml/h and 2-ml fractions were collected. Protein (●—●) was monitored at 280 nm and enzyme activity (△—△) was assayed as described in the text.

linear gradient of 0.01 M potassium phosphate buffer, pH 8.0, (100 ml) and 0.1 M potassium phosphate buffer, pH 8.0, (100 ml). The flow rate was 4 ml/h and 2-ml fractions were collected until the enzyme emerged from the column as a single peak (Fig. 4). Most of the inactive protein remained bound to the column.

An overall purification (summarized in Table I) of 4100-fold was achieved. The purified enzyme could be stored at −20 °C in 0.01 M Tris–HCl buffer, pH 8.0, for at least one month without significant loss of activity. At 0–4 °C, however, more than 80% of the activity was lost within 10 days.

Properties of purified γ-glutamyl cyclotransferase

The reaction of purified γ-glutamyl cyclotransferase with γ-L-glutamyl-L-phenylalanine yielded free pyrrolidone carboxylic acid and free phenylalanine, as determined by separating the reaction products by high voltage paper electrophoresis at pH 1.9 and 6.4. Pyrrolidone carboxylic acid was detected with the starch–KI reagent[12], and its identity was confirmed by co-migration with authentic standards of pyrrolidone carboxylic acid and also by its absorption at 205 nm. No free glutamic acid was produced in the reaction, demonstrating that the purified enzyme preparation was devoid of any dipeptidase activity capable of hydrolyzing γ-glutamylphenylalanine. The preparation also contained no γ-glutamyl transpeptidase activity, as determined by incubating it with γ-L-glutamyl-*p*-nitroanilide for prolonged periods (4 h) under reaction conditions described previously[9,11]. γ-L-Glutamyl-*p*-nitroanilide is known to be an excellent substrate for housefly γ-glutamyl transpeptidase[11].

The pH–activity relationship was examined in 0.1 M Tris–HCl buffer and 0.1 M potassium phosphate buffer. The enzyme was most active at pH 7.1–7.3 (Fig. 5).

The K_m value toward γ-L-glutamyl-L-phenylalanine obtained from a Lineweaver–Burk[13] plot (least squares method) was $2.94 \cdot 10^{-3}$ M (Fig. 6). All of the components in the incubation mixture were the same as described in the assay procedure in Materials and Methods except for variation in the concentration of γ-glutamylphenylalanine and γ-[G-^{3}H]glutamylphenylalanine; the ratio of these was kept constant.

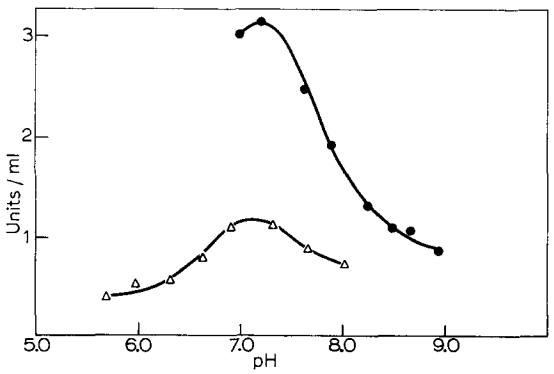

Fig. 5. pH–activity curve of purified γ-glutamyl cyclotransferase from pupae of *M. domestica* in 0.10 M Tris–HCl buffer (circles) and 0.10 M potassium phosphate buffer. The ionic strength of the buffers was not constant.

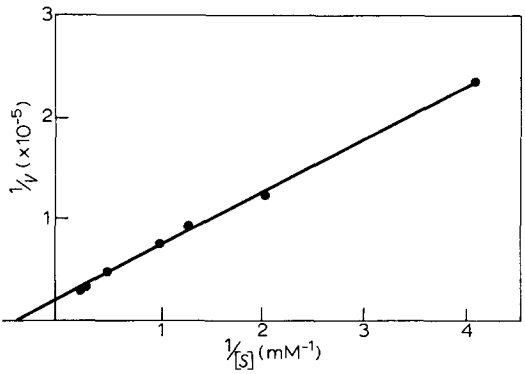

Fig. 6. Lineweaver–Burk plot of γ-L-glutamyl-L-phenylalanine concentration against the activity of purified γ-glutamyl cyclotransferase from pupae of *M. domestica*.

The apparent molecular weight of γ-glutamyl cyclotransferase was estimated by gel filtration on Sephadex G-75 in 0.01 M Tris–HCl buffer, pH 7.2. The elution volumes of several well characterized proteins were plotted as a function of the logarithms of their molecular weights, and as shown in Fig. 7, the elution volume of γ-glutamyl cyclotransferase corresponds to a mol. wt of 30 000 ± 2000.

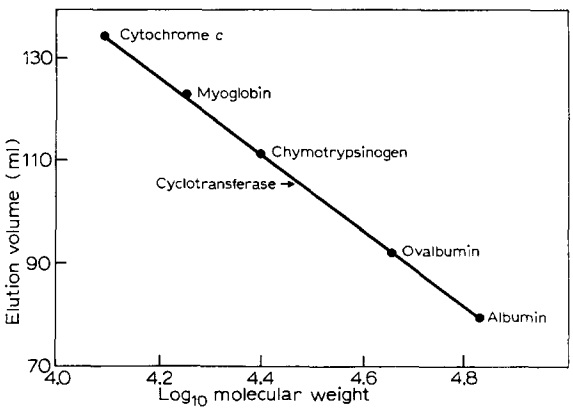

Fig. 7. Estimation of the molecular weight of purified γ-glutamyl cyclotransferase from pupae of *M. domestica* by Sephadex G-75 gel filtration.

Substrate specificity of the purified enzyme

γ-Glutamyl cyclotransferase from housefly pupae exhibited a very narrow specificity, as evident from Table II. Of all the γ-L-glutamyl-L-amino acids and γ-L-glutamyl compounds that were tested, γ-L-glutamyl-L-phenylalanine was attacked most readily. γ-L-Glutamyl-L-methionine was almost as effective a substrate, while the remaining γ-L-glutamyl compounds were attacked very slowly or not at all. α-L-Glutamyl compounds were not substrates. Of the γ-L-glutamyl-γ-L-glutamyl compounds, only γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide had appreciable activity as a substrate (about 4-fold less active than γ-L-glutamyl-L-phenylalanine). γ-D-Glutamyl-γ-L-glutamyl-*p*-nitroanilide was inactive.

TABLE II

SPECIFICITY OF γ-GLUTAMYL CYCLOTRANSFERASE FROM PUPAE OF *M. domestica*

The reaction mixtures contained: 2.5 μmoles of substrate; 50 μmoles of Tris–HCl buffer, pH 7.2; and 0.02–0.05 units of γ-glutamyl cyclotransferase (spec. act. 19.5 units/mg) in a final volume of 0.5 ml. The mixtures were incubated at 37 °C for 10–60 min, after which the formation of pyrrolidone carboxylic acid was determined as described in the text.

Substrate	*Pyrrolidone carboxylic acid formed (μmoles/min per mg)*
γ-L-Glutamyl-L-phenylalanine	19.5
γ-L-Glutamyl-L-methionine	13.0
γ-L-Glutamyl-L-valine	2.0
γ-L-Glutamyl-L-alanine	0.0
γ-L-Glutamyl-β-naphthylamide	0.0
γ-L-Glutamyl-L-leucine	0.0
γ-L-Glutamyl-L-glutamic acid	0.0
γ-L-Glutamyl-L-glutamine	0.0
γ-L-Glutamyl-α-L-aminobutyric acid	0.0
γ-L-Glutamylglycine	0.0
γ-L-Glutamyl-*p*-nitroanilide	0.0
γ-L-Glutamyl-4-methoxy-2-naphthylamide	0.0
γ-L-Glutamyl-L-lysine	0.0
L-Glutamine	0.0
α-L-Glutamylglycine	0.0
α-L-Glutamyl-L-alanine	0.0
α-L-Glutamyl-L-tyrosine	0.0
α-L-Glutamyl-β-naphthylamide	0.0
γ-L-Glutamyl-γ-L-glutamyl-L-phenylalanine	0.0
γ-L-Glutamyl-γ-L-glutamyl-*p*-nitroanilide	5.0
γ-D-Glutamyl-γ-L-glutamyl-*p*-nitroanilide	0.0
γ-L-Glutamyl-γ-L-glutamyl-α-naphthylamide	0.0
γ-L-Glutamyl-L-cysteinylglycine (reduced glutathione)	0.0

In a final series of experiments, γ-glutamylphenylalanine or γ-[G-^{3}H]glutamylphenylalanine was incubated with γ-glutamyl cyclotransferase (0.01 unit) together with varying amounts of γ-glutamyl transpeptidase partly purified[11] from housefly pupae (0.01–0.1 unit) at pH 7.2 and pH 8.0. There was no significant increase in the amount of pyrrolidone carboxylic acid formed, compared with reaction mixtures containing γ-glutamyl cyclotransferase alone.

DISCUSSION

The occurrence of highly active γ-glutamyl cyclotransferase in the early pupal stage of the housefly is not surprising. As mentioned in the Introduction, growing housefly larvae synthesize and accumulate a large pool of γ-glutamylphenylalanine, amounting to about 12 μmoles/g of wet weight[5]. After the fully grown larva transforms into a white pupa, the dipeptide is totally consumed within a few hours[5]. Previous radioisotope experiments[6] involving γ-[G-^{3}H]glutamylphenylalanine have established that free pyrrolidone carboxylic acid and free phenylalanine are formed *in vivo* from this dipeptide by the housefly white pupa. The data in the present study have demonstrated that γ-glutamyl cyclotransferase participates in the breakdown of γ-glutamylphenylalanine, and this event is reflected in a several-fold increase in

the activity of cyclotransferase following the transition of the larva to a white pupa. Available evidence[5,6,14,15] suggests that free phenylalanine released from the dipeptide would be converted to tyrosine and thence quinones. The latter cause sclerotization of the cuticle of the white pupa.

A comparison of the properties of purified housefly γ-glutamyl cyclotransferase with those of purified γ-glutamyl cyclotransferase from human and sheep brain[3] or pig liver[4] reveals that the enzymes have similar molecular weights, slightly different pH optima, and strikingly different specificities. Thus, the molecular weights of the separate forms of γ-glutamyl cyclotransferase isolated from pig liver by Adamson *et al.*[4] are 22 200 (1500 S.E.) and 21 300 (500 S.E.) compared to 30 000 ± 2000 for the housefly cyclotransferase. The pH optimum of the pig liver enzyme is 8.0, and 7.8–8.2 for human and sheep brain cyclotransferase isolated by Orlowski *et al.*[3]. The housefly cyclotransferase has a pH optimum nearer neutrality of 7.1–7.3

γ-Glutamyl cyclotransferase from housefly pupae exhibited a very narrow and unique specificity compared with the mammalian cyclotransferases. Thus γ-glutamylphenylalanine was the most active substrate for housefly cyclotransferase, whereas γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide was attacked some four times more slowly. On the other hand, human brain cyclotransferase attacks γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide about 200 times more readily than γ-glutamylphenylalanine, and sheep brain enzyme at least 1000 times more readily[3]. Evidently the housefly and brain cyclotransferases differ in their specificities towards γ-glutamylphenylalanine by at least three orders of magnitude. Another compound, γ-L-glutamyl-γ-L-glutamyl-α-naphthylamide, which is an excellent substrate for pig liver cyclotransferase[4], is attacked extremely slowly or not at all by housefly cyclotransferase. Generally speaking, γ-glutamyl-γ-glutamyl compounds appear to be preferred substrates for the purified mammalian cyclotransferases mentioned above, whereas they are relatively poor substrates for housefly cyclotransferase. The converse—that γ-glutamyl compounds are preferred substrates for housefly cyclotransferase—is not entirely true: of all the γ-glutamyl compounds tested in the present study, only γ-glutamylphenylalanine and γ-glutamylmethionine were very active substrates.

On the basis that γ-glutamyl-γ-glutamyl compounds appear to be preferred substrates for mammalian γ-glutamyl cyclotransferase, Orlowski *et al.*[3] have suggested that γ-glutamylamino acids might be metabolized in a two step reaction as follows:

$$\gamma\text{-Glutamylamino acid} \xrightarrow[\text{transpeptidase}]{\gamma\text{-Glutamyl}} \gamma\text{-glutamyl-}\gamma\text{-glutamylamino acid} + \text{amino acid}$$

$$\gamma\text{-Glutamyl-}\gamma\text{-glutamylamino acid} \xrightarrow[\text{cyclotransferase}]{\gamma\text{-Glutamyl}} \text{pyrrolidone carboxylic acid} +$$

$$+ \gamma\text{-glutamylamino acid}$$

There is very little evidence to support such a two-step reaction for the metabolism of γ-glutamylphenylalanine in the housefly pupa. First, γ-glutamyl-γ-glutamylphenylalanine is not a substrate for γ-glutamyl cyclotransferase isolated from housefly pupae (Table II). Nor was there any increase in the amount of pyrrolidone carboxylic acid formed from γ-glutamylphenylalanine when both γ-glutamyl cyclotransferase and γ-glutamyl transpeptidase were present in the reaction mixture, even when the

transpeptidase/cyclotransferase ratio was 10:1. Finally, radioisotope experiments of Bodnaryk[6] strongly indicate that γ-glutamyl-γ-glutamylphenylalanine is not formed *in vivo*. Of course, it can be suggested that γ-glutamyl-γ-glutamylphenylalanine forms *in vivo*, but is turned over too rapidly for detection. In the light of all the data given above, this would seem unlikely. The available evidence to date from *in vivo* and *in vitro* experiments is consistant with a direct conversion of γ-glutamylphenylalanine into pyrrolidone carboxylic acid and phenylalanine without formation of a tripeptide intermediate. However, the possibility remains that other γ-glutamyl compounds in the housefly, such as glutathione, are metabolized to pyrrolidone carboxylic acid *via* the two step reaction proposed by Orlowski *et al.*[3]. In this connection, it is noteworthy that the human brain and pig liver cyclotransferases can be resolved into isoenzymes by isoelectric focusing[3,4]. Although the catalytic properties of these separate forms of cyclotransferase have not been investigated fully, it is conceivable that they may differ widely in substrate specificity. The γ-glutamyl cyclotransferase isolated here from housefly pupae may represent a separate form of cyclotransferase highly specific towards γ-glutamylphenylalanine. Further experimentation will be required to test the possibility that housefly pupae (or other animal tissues) possess separate forms of γ-glutamyl cyclotransferase showing specificity towards γ-glutamyl and γ-glutamyl-γ-glutamyl compounds.

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REFERENCES

1 Orlowski, M. and Meister, A. (1971) in *The Enzymes* (Boyer, P. D., ed.), Vol. 4, pp. 123–151, Academic Press, New York
2 Connell, G. E. and Hanes, C. S. (1956) *Nature* 177, 377–378
3 Orlowski, M., Richman, P. G. and Meister, A. (1969) *Biochemistry* 8, 1048–1055
4 Adamson, E. D., Szewczuk, A. and Connell, G. E. (1971) *Can .J. Biochem.* 49, 218–226
5 Bodnaryk, R. P. (1970) *J. Insect Physiol.* 16, 919–929
6 Bodnaryk, R. P. (1973) *Insect Biochem.*, submitted for publication
7 Bodnaryk, R. P. (1972) *Insect Biochem.* 2, 49–52
8 Orlowski, M. and Szewczuk, A. (1961) *Acta Biochim. Pol.* 8, 189–193
9 Orlowski, M. and Meister, A. (1965) *J. Biol. Chem.* 240, 338–347
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
11 Bodnaryk, R. P. and Skillings, J. R. (1971) *Insect Biochem.* 1, 467–479
12 Rydon, H. N. and Smith, P. W. G. (1962) *Nature* 169, 922–923
13 Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658–666
14 Hackman, R. H. (1964) in *The Physiology of Insects* (Rockstein, M., ed.), Vol. 3, pp. 471–506, Academic Press, New York
15 Brunet, P. C. J. (1965) in *Aspects of Insect Biochemistry* (Goodwin, T. W., ed.), pp. 49–77, Academic Press, New York