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γ-GLUTAMYL TRANSPEPTIDASE OF THE ACKEE PLANT*

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Abstract—The enzyme γ-glutamyl transpeptidase was purified from seeds of immature ackee fruit (*Blighia sapida*; Sapindaceae) by salt fractionation and gel filtration on Biogel P-10 and P-200. The procedure, which differs from an earlier one applied to kidney bean fruit, achieves 9.8% yield and 577-fold purification. The enzyme is also present in other parts of the fruit and in leaves. A MW of 12 500 was found by SDS-polyacrylamide gel electrophoresis, a value much lower that that reported for the enzyme from kidney bean fruit. Neutral or amino sugar accounts for 10% of the dry weight. *In vitro*, the enzyme catalysed synthesis of an unusual γ-glutamyl dipeptide which occurs in ackee seeds, using glutathione as glutamyl group donor. The enzyme mechanism was of the double displacement (ping-pong) type.

INTRODUCTION

The enzyme γ-glutamyl transpeptidase is believed to catalyse the synthesis of γ-glutamyl dipeptides which occur in certain plants [1, 2]. However, evidence for significant enzyme activity coexisting with these peptides is limited to a single plant family, Leguminosae [3]. Surprisingly, onion tissues, which contain an extensive range of γ-glutamyl peptides, appear devoid of transpeptidase activity. Moreover, glutathione (GSH), the reputed natural donor of the glutamyl group, has rarely been directly implicated in the biosynthesis of a naturally-occurring plant peptide. We report here that the ackee plant (*Blighia sapida*, Konig; Sapindaceae), which contains γ-glutamyl-β-(methylenecyclopropyl)-alanine (trivial name, hypoglycin B) [4–6], contains significant γ-glutamyl transpeptidase activity as well. Purification from ackee seeds revealed some important characteristics of the enzyme, including an ability to utilize GSH for synthesis of hypoglycin B.

RESULTS AND DISCUSSION

Occurrence of enzyme

Assays done on crude extracts from various fruit parts and from leaves revealed the presence of the enzyme (Table 1). Activity was found in fibrous pericarp, arillus and seed of fruit harvested at various stages of maturity, as well as in the leaf. 'Young' fruit were unopened specimens containing pale-green seeds and arilli; beyond an intermediate stage ('unripe' fruit), the pericarp splits open to reveal 2–4 purple-black seeds and yellow arilli ('ripe' fruit). The data shown are estimates of V_{max} resulting from plots of v^{-1} vs s^{-1}; the standard assay (Experimental) was modified to produce 8–10 graded concentrations of the substrate γ-glutamyl-*p*-nitroanilide (γ-Glu-PNA) for each plot. Virtually identical values of K_m resulted from all plots regardless of the enzyme source, implying an unvarying enzyme species. The same K_m was found for the purified enzyme (see below). Comparison of the occurrence of the enzyme with that of hypoglycin B in the ackee plant would be relevant. Unfortunately, only preliminary data are available for the latter at present. It is believed that this γ-glutamyl dipeptide occurs mainly in the seed, although trace amounts are found elsewhere, including the arillus [6]. The unconjugated amino acid β-(methylenecyclopropyl)-alanine (hypoglycin A) is found in the leaf, seed and arillus (*ca* 0.4% of the fr. wt in arilli from unripe fruit [6, 7]). The concentration of hypoglycin A in the arillus declines considerably as the fruit matures and this may be explained [6] by translocation to the seed and deposition there as hypoglycin B. Consistent with this is the higher concentration of the dipeptide in seeds from ripe fruit compared to that in seeds of young fruit (3.3 mg vs 0.4 mg/g fr. wt (Lewis, C., personal communication)). However, enzyme activity was highest in seeds of young fruit (Table 1) and enzyme purification was based on this source.

Enzyme purification

Except for extraction and preliminary salt fractionation, the steps followed in purifying the enzyme (Table 2 and Experimental) differed from those applied to kidney bean fruit by other workers [8]. In particular, we found that dialysis of the $(NH_4)_2SO_4$ precipitate—a step employed by earlier workers—invariably caused considerable loss of activity. A possible explanation may be the much smaller molecular dimensions of our enzyme as compared to the kidney bean product (see below). The present procedure produced a better yield

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Table 1. Transpeptidase activity in the fruit and leaf of ackee

Part	Stage	Enzyme activity* (units/g fr. wt) $\times 10^3$	(units/mg protein†) $\times 10^3$
Pericarp	Young	2.49	1.04
	Unripe	1.84	0.80
	Ripe	2.40	1.07
Arillus	Young	1.09	0.61
	Unripe	0.96	0.58
	Ripe	1.39	0.55
Seed	Young	13.3	6.29
	Unripe	7.78	3.62
	Ripe	1.50	0.90
Leaf	Undefined	4.34	1.87

*Calculated from V_{max}.
†Protein in crude extract.

Table 2. Purification of transpeptidase from ackee seeds

Step	Total protein	Total enzyme	Specific activity* (units/mg protein) $\times 10^2$	Purification (fold)	Yield (%)
1. Crude extract	5748	25.3	0.44	1	100
2. Extract from ppt., 40–70% $(NH_4)_2SO_4$ saturation	146	19.4	13.2	30	77
3. Al_2O_3 gel supernatant	99	18.9	19.1	43	75
4. Biogel P-10 eluate	3.29	5.53	168	382	22
5. Biogel P-200 eluate	0.98	2.47	254	577	9.8

*From standard assay, giving *ca* 70% V_{max}.

and degree of purification than were reported for the original one (2.3% yield, 360-fold purification). Direct comparison of the final specific activity achieved by the two different procedures is not valid since the assay methods differ, most notably in the substrate used. The purified enzyme gave a single protein band when subjected to polyacrylamide gel electrophoresis in 6 M urea. SDS-polyacrylamide gel electrophoresis was performed on enzyme which had been pre-treated at 100° for 5 min in 8 M urea–1% SDS–1% 2-mercaptoethanol mixture. This treatment would be expected to abolish peptidase activity and also to dissociate oligomeric forms into monomers, with release of constituent polypeptide chains. Again, only a single protein species with MW 12 500 was evident. This value is only *ca* one-tenth of that reported for the enzyme from kidney bean fruit, as determined by gel filtration and sedimentation velocity [8]. It is not clear whether the larger value applies to an aggregated form of the enzyme. A possible limitation on our data is the finding that the enzyme contains carbohydrate; some glycoproteins give anomalous MW results from SDS-polyacrylamide gel electrophoresis [9], but this factor alone is unlikely to be responsible for the large difference between the two sets of data. A wide range of MW appears to exist for the enzyme from animal sources [10].

Carbohydrate content

The enzyme from sheep kidney is a glycoprotein, containing 17.8% neutral sugar, 8% amino sugar and 9.7% sialic acid [10, 11]. Evidence was obtained that the enzyme from ackee also contains carbohydrate. A solution (20 μg/ml) of our apparently homogeneous product gave a much stronger Molisch colour reaction than did an equivalent concentration of ovalbumin (4% carbohydrate). We applied two standard procedures for protein assay [12, 13] to determine the percentage of protein in dried enzyme samples which had been exhaustively dialysed, and found by both procedures that protein accounted for 87% of dry wt. The carbohydrate content, determined by a procedure [14] which measures both neutral and amino sugars, was 10.3%, leaving *ca* 3% of the enzyme dry wt unaccounted for.

Transfer and hydrolytic activites

Previous work [3] established that the plant transpeptidase catalyses both transfer and hydrolysis. Our standard assay for enzyme activity did not distinguish between these two functions, being based on release of PNA from γ-Glu-PNA, present as the sole substrate (other than H_2O) in reaction media. This substrate can itself act as acceptor so that equations (1) and (2) respectively represent separate and possibly competing transfer and hydrolysis reactions:

$$2\ \gamma\text{-Glu-PNA} \rightarrow \gamma\text{-Glu-}\gamma\text{-Glu-PNA} + \text{PNA} \quad (1)$$

$$\gamma\text{-Glu-PNA} + H_2O \rightarrow \text{Glu} + \text{PNA}. \quad (2)$$

When the standard assay was conducted in media of various pH values ranging between 3 and 11, distinct peaks of activity were found at pH 6.5 and 9.5. Lineweaver–Burk plots showed that V_{max} (μmol PNA formed/min/mg protein) and K_m (mM) at pH 6.5 were respectively 0.75 and 0.33; at pH 9.5, these constants were 2.94 and 1.67. While consistent with two different functions of the enzyme, these findings were not considered adequate proof. To obtain more direct evidence, assay media were modified by the inclusion of 10 mM Leu, and reaction time at both pH 6.5 and 9.5 extended to 90 min. The reaction was stopped by acidification to pH 1, and aliquots from the reaction mixtures were examined by TLC. Simultaneous chromatography of authentic standards showed the predominant ninhydrin-positive components at pH 6.5 to be Glu, Leu and unreacted γ-Glu-PNA. In contrast, at pH 9.5 the only ninhydrin-positive spot was γ-Glu-Leu, as expected from equation (3).

$$\gamma\text{-Glu-PNA} + \text{Leu} \rightarrow \gamma\text{-Glu-Leu} + \text{PNA} \quad (3)$$

Similar work and results with the animal enzyme have been reported [15].

GSH as glutamyl donor

After 5 hr incubation at pH 9.5, the enzyme produced γ-Glu-Leu from GSH and Leu; similarly, GSH and hypoglycin A yielded hypoglycin B. TLC of aliquots taken from appropriate reaction mixtures allowed the products to be identified by co-chromatography of authentic standards in 8 different solvent systems. In addition, identification was confirmed by elution from the plates of areas containing the dipeptides, hydrolysis (0.4 N HCO_2H, 2 hr) and rechromatography. A low R_f product, presumably Cys-Gly, gave a positive test for sulphydryl; however, no authentic sample was available for comparison of R_f.

Other workers have reported similar findings that animal kidney transpeptidase utilizes GSH as glutamyl group donor [15, 16], and the kidney bean enzyme was shown to catalyse formation of γ-glutamyl-*S*-methylcysteine, a plant dipeptide [1, 2], from GSH and *S*-methylcysteine [3]. A similar function of GSH in the formation of hypoglycin B is now supported by direct evidence.

It is notable that the conditions for the predominance of transfer over hydrolytic function of the enzyme apparently include a high, possibly unphysiological pH; it has been suggested [17] that relatively high concentrations of amino acid *in vivo* favour the transfer function, thus achieving dipeptide synthesis.

Enzyme mechanism

The mammalian enzyme appears from kinetic analysis to operate by a two-step displacement (ping-pong) mechanism, with formation of an activated γ-Glu-enzyme intermediate [18, 19]. Indirect evidence also suggests a similar mechanism for the plant enzyme [3]. On this basis, transpeptidation between GSH and Leu, for example, would consist of two partial reactions, namely:

$$\text{GSH} + \text{Enz} \rightleftharpoons \text{Glu-Enz} + \text{Cys-Gly} \quad (4A)$$

$$\text{Glu-Enz} + \text{Leu} \rightleftharpoons \text{Glu-Leu} + \text{Enz}. \quad (4B)$$

Reaction (4B) should proceed reversibly in the absence of GSH and this implies that incubation of enzyme with γ-Glu-Leu in the presence of ^{14}C-labelled Leu would result in exchange of the label with γ-Glu-Leu to a stage of isotopic equilibrium [20, 21]. No exchange of ^{14}C should be evident if Glu-[^{14}C], rather than Leu-[^{14}C] were added. Experiments were performed in which either L-leucine-[1-^{14}C] or L-glutamic acid-[U-^{14}C] was added to Glu-Leu plus enzyme and the predicted results were obtained (Fig. 1). This confirms that the double-displacement mechanism applies to this enzyme.

Physiological role of transpeptidase

Now revealed to be less limited than was thought, the distribution of this enzyme in unrelated plant families may suggest a function in plant economy. However, participation in the first step of GSH breakdown [8] hardly qualifies as a vital function, while any advantage linked to γ-glutamyl dipeptide synthesis remains obscure. Further, there is as yet no evidence indicating a role for the enzyme in transmural amino acid transport in plants, as has been postulated [10] and disputed [22] for the enzyme in animals.

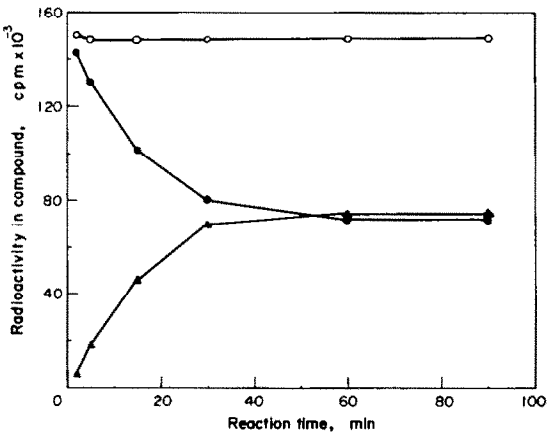

Fig. 1. Demonstration of ping-pong mechanism for transpeptidase. Enzyme-catalysed ^{14}C exchange between L-leucine-[1-^{14}C] (●——●) and γ-Glu-Leu (▲——▲); no exchange between L-glutamic acid-[U-^{14}C] (○——○) and γ-Glu-Leu (not shown). Each reaction mixture contained γ-Glu-Leu, ^{14}C-labelled amino acid and enzyme. After quenching at times shown, aliquots were subjected to TLC and the radioactivity measured in the separated compounds.

EXPERIMENTAL

All chemicals were obtained commercially with the exception of hypoglycins A and B. These compounds were isolated from ackee fruit [23]; there is an error in this ref. which makes it appear that hypoglycin B is eluted by 0.5 M HOAc from a Dowex 1 (Ac) column before instead of after glutamic acid.

Enzyme preparation. Fruit and leaves were collected from ackee trees standing on the University grounds. Seeds and other fruit parts were separated within 2–3 hr after harvesting, rinsed in H_2O and processed either immediately or following overnight storage at −20°. Subsequent procedures were performed at 4–8°. Enzyme was extracted by blending with 0.05 M $NaHCO_3$ (1 ml/g fr. wt), filtration through gauze and centrifugation to remove debris. EDTA and 2-mercaptoethanol (each 5 mM final concn) were added to the crude extract. Purification, monitored by enzyme and protein assay, was as follows (quantities are for 1 kg fr. wt). The ppt. formed between 50 and 70% satn with $(NH_4)_2SO_4$ was extracted ×5 with 20 ml 10 mM Pi (pH 7.0)–5mM EDTA–5 mM 2-mercaptoethanol (buffer mixture) (coaxial homogenizer, residues spun down at 10 000 g, 5 min). Pooled extracts were lyophilized and thereafter retained enzyme activity for at least 8 weeks. The dried powder was redissolved in the buffer mixture and alumina C_γ gel, 0.2 mg (dry wt)/mg dry powder, was added. After 4 hr stirring, the gel, with considerable yellow-brown pigment absorbed, was removed by centrifuging. 10 ml of the supernatant were applied to a column of Biogel P-10 (2.5 × 30 cm), equilibrated and washed with buffer mixture (0.5 ml/min, 5 ml fractions). The fractions containing enzyme activity (usually fractions 8–18) were pooled and lyophilized. The product was dissolved in buffer mixture and applied to a column of Biogel P-200, (2.0 × 30 cm), equilibrated and washed with the same buffer mixture (0.26 ml/min, 5 ml fractions). The enzyme, usually in fractions 18–32, was lyophilized and portions redissolved in buffer mixture for use as required.

Enzyme assay. The mixture for the standard assay contained 4 mM γ-Glu-PNA, 0.2 M Tris–HCl (pH 9.5) in a total vol. of 1.5 ml. Increase in A_{410} was continuously recorded after adding enzyme soln to the mixture. A unit of enzyme activity = 1 μmol PNA released/min; ε for PNA = 8800 [24]. The standard assay gave rates which were *ca* 70% of V_{max}.

Protein assays. Protein elution from columns was followed by measuring A_{280}. In other situations, the Lowry [12] and biuret [13] procedures were used.

Carbohydrate assay. To 1.5 ml of soln containing various concns of enzyme, and to the same vol. of standards containing 10–250 μg glucose (as D-glucosamine–HCl), the procedure of ref. [25] was applied in order to deaminate hexosamines to 2,5-anhydrohexose. The PhOH–H_2SO_4 procedure [14] was then followed. Three sample readings which fell within the calibration range (0–1.4 for A_{490}) were used to calculate the carbohydrate content, and the mean taken.

Gel electrophoresis. Purity of the enzyme was assessed using a BioRad apparatus with a continuous Tris–EDTA-borate buffer system (pH 8.35), and 7.5% polyacrylamide gel. After electrophoresis (3 mA/gel, 1.25 hr), protein was visualized by Amido Black stain. MW determination was done on the enzyme after treatment by method 1 of ref. [26], utilizing the system of ref. [27]. Marker proteins used to determine relative mobilities [26] were chymotrypsin, cytochrome *c*, ovalbumin and ribonuclease. Staining with Coomassie Blue revealed the enzyme's mobility to be between those of ribonuclease and cytochrome *c*.

TLC. This procedure was performed either on microscope slides or glass plates (20 × 20 cm) coated with microcrystalline cellulose. Solvent systems used at various times in this work were: 1, *n*-BuOH–Py–H_2O (1:1:1); 2, 3 and 4, *n*-BuOH–HOAc–H_2O (2:1:1), (3:1:1) and (9:1:2.5); 5, *n*-BuOH–HCO_2H–H_2O (7:2:1); 6 and 7, PrOH–H_2O (7:3) and (4:1); 8, PrOH–HOAc–H_2O (8:1:1). Identification of amino acids and peptides was by spraying with ninhydrin soln (0.4% in Me_2CO). Cys-Gly was detected with azide–I_2 spray reagent [28].

^{14}C exchange. Each of 6 tubes contained 15 μmol L-leucine-[1-^{14}C] (3 μCi), 27 μmol γ-Glu-Leu, 120 μmol Tris–HCl (pH 9.5) and 0.65 μg purified enzyme, in 3 ml total vol. Reaction was stopped by adding 50 μl HOAc to successive tubes at 2,5,15,30,60, and 90 min. Aliquots (0.1 ml) taken from each tube for TLC were applied to plates on which γ-Glu-Leu and Leu were also separately spotted. After development (solvent 4) and drying, areas—indicated as the probable location of γ-Glu-Leu and Leu, respectively, by ninhydrin-sprayed guide spots—were separately scraped off and eluted ×3 with 10 ml H_2O. The pooled extracts were lyophilized, dissolved in scintillation fluor (toluene–Triton X-100–PPO) and radioassayed. Counting efficiency (76%) did not vary between samples or between different experiments, so recorded count rates were corrected only for background (15–18 cpm). Expts with L-glutamic acid-[U-^{14}C] were performed similarly, with Glu substituted for Leu as guide spot.

Buffers. Reaction mixtures in the range pH 3–6 contained 0.2 M KPi; in the range pH 6.8–10.8, 0.2 M Tris–HCl.

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