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MOLECULAR AND KINETIC PROPERTIES OF PURIFIED γ-GLUTAMYL TRANSPEPTIDASE FROM YEAST (*SACCHAROMYCES CEREVISIAE*)

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Key Word Index—*Saccharomyces cerevisiae*; yeast; glutathione; γ-glutamyl transpeptidase; kinetic study; subunit structure.

Abstract—The enzyme γ-glutamyl transpeptidase was purified from the yeast *Saccharomyces cerevisiae* by a procedure involving protamine sulfate treatment, chromatography on DEAE-Sephadex A 50, salt fractionation, successive chromatography on Sephadex G 150 and lentil lectin sepharose 6B. The procedure achieves 25% yield and 4200-fold purification. The final preparation is a glycoprotein (M_r 90 000) containing 31.4% carbohydrates and composed of two non-identical subunits (M_r 64 000 and 23 000). The specificity patterns of the yeast enzyme are rather similar to those of mammalian and higher plant transpeptidases. The enzyme mechanism might be of the double displacement (ping-pong) type.

INTRODUCTION

Mammalian renal γ-glutamyl transpeptidase (γ-GT) (EC 2.3.2.2) has been postulated as a carrier in transmural amino acid transport [1] and extensive studies on highly purified enzyme preparations have been published [1–3]. In contrast, few papers concerning higher plant and microbial γ-GT have appeared [4–7]. In fact, the physiological rôle of this enzyme is still obscure. γ-GT and glutathione (GSH) were initially thought to be involved in the bulk absorption of amino acid by the yeast [8, 9]. However these claims have been recently contradicted [10–13]. In the yeast *Saccharomyces cerevisiae* γ-GT is confined to the central vacuole [14] and to the plasmalemma [12]. Furthermore, the biosynthesis of yeast γ-GT is controlled by the nature of the nitrogen source supplied to the cell [15] and the enzyme seems to catalyse *in vivo* hydrolysis and transpeptidation [13] of GSH. In this paper we report molecular and kinetic properties of highly purified yeast γ-GT.

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RESULTS AND DISCUSSION

Purification of the enzyme

The details of the isolation procedure (Table 1) are described in the Experimental section. This procedure leads to a preparation that is about 4300-fold purified and exhibits a single band when subjected to polyacrylamide gel electrophoresis.

The specific activity of pure *Saccharomyces cerevisiae* enzyme is very much lower than that of mammalian γ-glutamyltranspeptidase but is rather similar to that of a preparation of ackee plant transpeptidase [4]. The yeast enzyme is not activated by ions as was observed for the sheep kidney enzyme [16]. Treatment of *S. cerevisiae* enzyme with a stem bromelain [17] or trypsin [16] at various stages of the purification did not increase the

Table 1. Purification of yeast γ-GT*

Step	Volume (ml)	Total protein (mg)	Transpeptidase activity			Purification index
			Total units	Units/mg	Yield	
1. Crude extract	450	7020	197	0.028	100	1
2. Protamine sulphate	435	5130	180	0.035	91	1.25
3. Chromatography on DEAE Sephadex A 50	520	1060	167	0.158	85	5.6
4. $(NH_4)_2SO_4$ precipitation	14.5	652	146	0.224	74.2	8
5. Chromatography on Sephadex G 150	41	160	96	0.6	49	21.4
6. Chromatography on lentil lectin	25	0.48	58.1	121	29.5	4330

*See Experimental.

specific activity of the enzyme. On the contrary, the yeast transpeptidase seems to be rather sensitive to proteolysis. For example, care was taken to harvest the yeast cells in the exponential phase of growth. Otherwise, a continuous decline in the enzyme activity was observed during the subsequent purification steps. For similar reasons, it is also important to fractionate the crude extract of yeast on DEAE Sephadex at the beginning of the purification. In fact, many workers have experienced considerable difficulties in purifying yeast enzyme owing to endogeneous proteases appearing during the exponential or the stationary phase of growth [18].

Rat renal and ackee plant γ-GT contain respectively 25.8% and 10.3% carbohydrate [2, 4] as shown by the $PhOH–H_2SO_4$ procedure [19]. By the same procedure applied to the exhaustively dialysed purified yeast enzyme, we have detected a carbohydrate content of 31.4%.

M_r *and subunit composition*

The M_r of the purified enzyme, as determined by gel filtration, was estimated to be 90000 ± 9000. When the enzyme was treated with SDS and 2-mercaptoethanol, as described in the Experimental, it migrated as two components on polyacrylamide gel electrophoresis (PAGE) in the presence of SDS. The apparent M_r of the large subunit in 64000 ± 6400 and that of the small subunit is 23000 ± 2300. The yeast γ-GT is very similar to the human and other mammal kidney enzymes in M_r and the subunit structure [1, 20]. However, yeast enzyme contrasts with the ackee enzyme for which an M_r of 12500 was reported [4]. Respective M_r values of *ca* 125000 and 50000 were reported for the kidney bean [21] and the fungus *Tricholoma shimeji* [22]. To our knowledge, no other data concerning the quaternary structure of γ-GT of higher plants and fungi have been reported.

Table 2. Hydrolytic and transfer activities of yeast γ-GT toward γ-glutamyl compounds*

Substrate	Hydrolytic activity: Apparent K_m value (mM)	Hydrolytic activity: Apparent V_{max} value (units/mg)	Transpeptidase activity (units/mg)
GSH	1.5	49.8	120.5
GSSG	2.7	39.6	119.0
Ophtalmic acid	1.10	38.6	74.2
L-γ-Glu-L-Met	1.80	49.8	66.5
L-γ-Glu-Ala	1.32	45.8	79.5
L-γ-Glu-Leu	1.50	39.2	98.6
L-γ-Glu-Tyr	2.3	38.6	101.0
L-Glu-NH_2	2.5	18.5	26.4
L-γ-Glu-PNA	1.5	152.5	47.8

*The hydrolytic activity at pH 7 was determined as described in the Experimental. The concentration of each γ-glutamyl compound was varied between 1 and 10 mM, and the values given in the table were obtained from linear double reciprocal plots of the data. Transpeptidation at pH 9 between 10 mM γ-glutamyl donor and 10 mM L-[methyl-^{3}H]-Met acceptor was determined as described in the Experimental.

Catalytic properties of purified yeast γ-glutamyl transpeptidase

Mammalian and plant γ-GT catalyse both transfer and hydrolysis of γ-glutamyl group of numerous donors [3, 4]. The apparent rate of GSH hydrolysis by the yeast enzyme was relatively independent of pH between 7.0 and 8.5 and decreased only slightly above pH 8.5. In contrast, the apparent rate of transpeptidation between GSH and L-Met at pH 7 was only 4% of the rate at pH 8.5.

The products of hydrolysis and transpeptidation of GSH were examined by TLC. At neutral pH, Glu was detected as a major reaction product, but also GSSG, cys*bis*gly and γ-Glucys*bis*gly. The oxidized products resulted probably from a pseudo-oxidase reaction as was shown for renal γ-GT [23]. A faint spot of γ-Glumet was apparent at pH 7, but γ-Glu-GSH, a product of autotranspeptidation [5] was not detectable. At pH 9, γ-Glumet and Glu appeared as major reaction products. Again, oxidized products were revealed but no autotranspeptidation products. This result strongly suggests that autotranspeptidation is not a catalytic function of yeast γ-GT. This kind of reaction, probably unphysiological, was demonstrated in the case of mammal γ-GT [24] but only suggested for the plant enzyme [4].

Hydrolysis of γ-glutamyl compounds

The apparent K_m and V_{max} for the hydrolysis of GSH and numerous other γ-glutamyl compounds were determined (Table 2). Similar kinetic constants were obtained for most of the compounds, except for L-Glu NH_2 which is hydrolysed at a lower rate and γ-Glu PNA which is hydrolysed at higher rate. Similar activities and results with rat renal transpeptidase have been reported [25].

The transpeptidase reaction

Data on transpeptidation at pH 9 between several γ-glutamyl compounds and methionine are summarized in

Table 3. Apparent K_m and V_{max} values for yeast γ-glutamyltranspeptidase acceptors*

Acceptor†	K_m values (mM)	V_{max} values (units/mg)
L-Met	1.5	10.7
L-Arg	4.6	7.6
L-Lys	4.9	6.4
L-His	3.7	8.8
L-Pro	n.d.	n.d.
L-Val	5.2	5.6
L-Asp-NH_2	2.4	6.0
Gly-Gly	2.7	7.9
L-Met-L-Met	1.1	12.1

*Transpeptidation between GSH (10 mM) and various acceptors was followed as described in Experimental for the case of L-[methyl-^{3}H]Met. The concentration of acceptor was varied between 1 and 10 mM, and the values given in the table were obtained from double reciprocal plots of the data.

†All the acceptors were uniformely-labelled with ^{14}C except L-[methyl-^{3}H]Met.

n.d., No detectable activity.

Table 2. It is of interest that GSSG is almost as active as GSH in the transfer reaction catalysed by the yeast enzyme. In contrast, the disulphide is a less good γ-glutamyl donor in the case of the rat renal enzyme [26]. The apparent K_m and V_{max} values for a number of amino acids and peptide acceptors were determined in studies with GSH as a γ-glutamyl donor (Table 3). Highest values of transpeptidation rate were obtained with L-methionine and L-methionyl-L-methionine. No accurate values could be obtained for L-Glu, L-Ala and L-Glu NH_2 owing to an excess substrate inhibition exerted by those amino acids on the transpeptidation reaction (not shown). This probably reflects their binding at the donor site of γ-GT.

Kinetic study

Steady-state studies. In this investigation we have chosen glutathione as a donor and L-methionine as an acceptor. This amino acid was chosen because it apparently does not bind to the donor site (see above; Table 3) and therefore, is not expected to create complications in unravelling the basic mechanism of the enzyme.

Transpeptidation rate (V_T). Double reciprocal plots of $1/V_T$ vs 1/GSH for various L-Met and $1/V_T$ vs 1/L-Met for various concentrations of GSH converged at the left of the ordinate (Fig. 1). Analogous plots for renal transpeptidase show two sets of parallel lines [26, 27], which diagnose a typical ping-pong mechanism. In fact, parallel lines are not expected for a ping-pong mechanism modified by a hydrolytic shunt [28] except if the constant term in the denominator of the rate equation is negligible. L-Cysgly, a product of the reaction, is a non-competitive inhibitor with respect to GSH when L-Met is present at a non-saturating level (Table 4). The inhibition is overcome at high concentration of the acceptor. The dipeptide is a competitive inhibitor with respect to L-Met; GSH concentration has no effect on the inhibition pattern. L-γ-Glutamyl-*O*-carboxyphenyl hydrazide (L-γ-Glu-OCPH), an inactive analog of the donor [29], is a competitive inhibitor versus GSH and non-competitive inhibitor versus L-Met (Table 4).

Hydrolysis rate (V_H). The acceptor L-Met behaved as a non-competitive inhibitor of the hydrolysis of GSH.

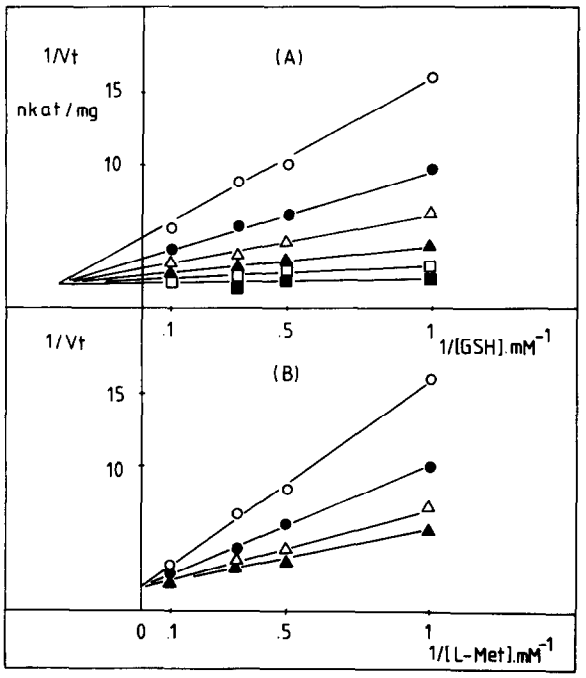

Fig. 1. Double reciprocal plots of the rate of transpeptidation (V_T) of GSH with L-Met at pH 9 and 37°. (A) Versus 1/GSH for various concentrations of L-Met: (○) 1 mM; (●) 2 mM; (△) 3 mM; (▲) 5 mM; (□) 10 mM; (■) 20 mM. (B) Versus 1/L-Met for various concentrations of GSH: (○) 1 mM; (●) 2 mM; (△) 5 mM; (▲) 10 mM.

Other acceptors, for example L-Leu and L-Met-L-Leu behaved similarly. L-Cysgly, the product of the reaction, is a non-competitive inhibitor (Table 4). These results are compatible with one of the mechanisms in which the donor binds first to the enzymes (ping-pong, Theorell–Chance, ordered bi-bi) or a rapid equilibrium random alternative; they exclude a mechanism in which the acceptor and H_2O bind first [28]. Studies of the ratio V_T/V_H and of the rate of the total reaction ($V_S = V_T + V_H$)

Table 4. Effects of inhibitory products on transfer and hydrolysis reactions catalysed by yeast γ-GT*

	TRANSFER			
	GSH varies		L-Met varies	
Inhibitory product	Unsaturated	Saturated with L-Met	Unsaturated	Saturated with GSH
L-Cys-Gly	NC	—	C	C
L-γ-Glu-OCPH	C	C	NC	NC

	HYDROLYSIS
Inhibitory product	GSH varies
L-Cys-Gly	NC
L-Met	NC
L-Leu	NC
L-Met-L-Leu	NC

*Transfer and hydrolysis reactions were measured at pH 9 as described in the Experimental. The concentration of each substrate was varied between 0.5 and 10 mM. Each inhibitory product was tested at three appropriate concentrations. C, Competitive (only slope varies); NC, non-competitive (slope and intercept both vary with inhibitor); —, no inhibition.

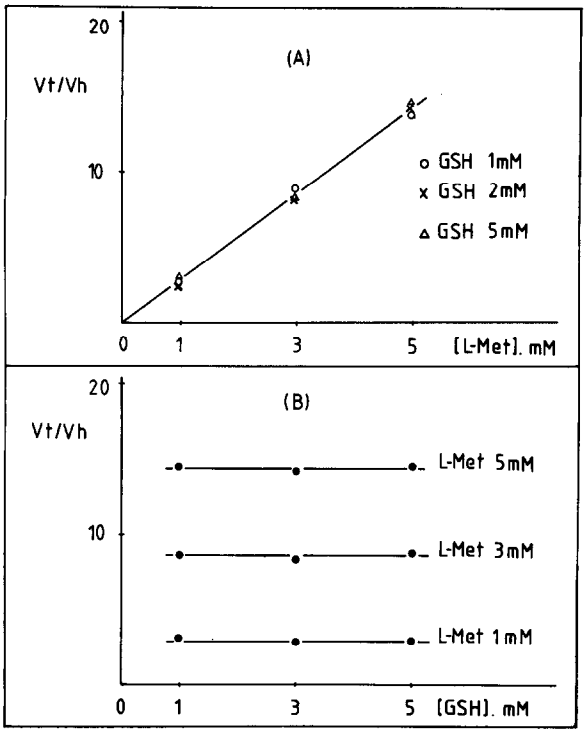

Fig. 2. (A) Plots of V_T/V_H versus L-Met for various concentrations of GSH. (B) The same but versus GSH for various concentrations of L-Met.

provided additional evidence for the above-mentioned hypothesis. V_T/V_H vs L-Met for different GSH concentrations is directly proportional to the concentration of the acceptor (Fig. 2A). The same ratio vs GSH for different concentrations of the acceptor is independent of the donor concentration (Fig. 2B).

Finally, $1/V_S$ vs 1/GSH for different concentrations of the acceptor gave a set of lines not converging at the same point (Fig. 3). Some evidence that yeast γ-GT probably functions according to a ping-pong mechanism was obtained in experiments reported below.

Evidence of covalent catalysis

The hypothesis that the reaction of renal γ-GT involves a γ-glutamyl-enzyme formation was previously inferred from steady-state kinetic studies [26, 27] and observations that certain γ-glutamyl analog binds covalently to the enzyme [30]. However, these results suggest only that a nucleophile site is located at the active centre of the enzyme. Such a nucleophile could potentially be involved in the formation of a γ-glutamyl-enzyme intermediate. Isotopic exchange studies have shown that ackee plant γ-GT might also function according to a ping-pong mechanism [4]. Other lines of evidence for a covalent catalysis by the yeast enzyme were obtained. As shown in Table 2, the maximal rates of hydrolysis of the different γ-glutamyl compounds are very similar, except for L-Glu NH_2 and L-γ-Glu-PNA. This result suggests that the hydrolysis of a common γ-glutamyl-enzyme is the rate determining step for the reaction of all the γ-glutamyl compounds and, consequently, that a common acyl-enzyme exists. The lower V_{max} for hydrolysis of L-Glu NH_2 could be explained by the fact that the amide NH_2 is a poor leaving group. As a consequence, the acylation step may become rate determining [31]. Amino acids and peptides, as leaving groups, may partially control the rate-determining step and PNA would exert a very restricted or negligible effect on this step.

Regardless of which step is rate-determining, a common intermediate which is formed from several donor molecules must give the same ratio of products at a given concentration of two acceptors [32]. We have applied this criterion to the γ-GT catalysed reactions of a series of γ-glutamyl donors with L-Met and H_2O. At a constant concentration of L-Met, different donors give a similar ratio of γ-glutamyl-methionine to glutamic acid as products (Table 5), suggesting that L-Met and H_2O are reacting with a common intermediate γ-glutamyl-enzyme at a constant ratio of rates.

Physiological rôle of γ-GT

In a previous paper we have shown that two peptides, γ-glutamyl-glutamic acid and γ-glutamylglutamine are probably synthesized in the yeast cell by γ-GT during growth on L-Glu as a nitrogen source [13]. At first glance,

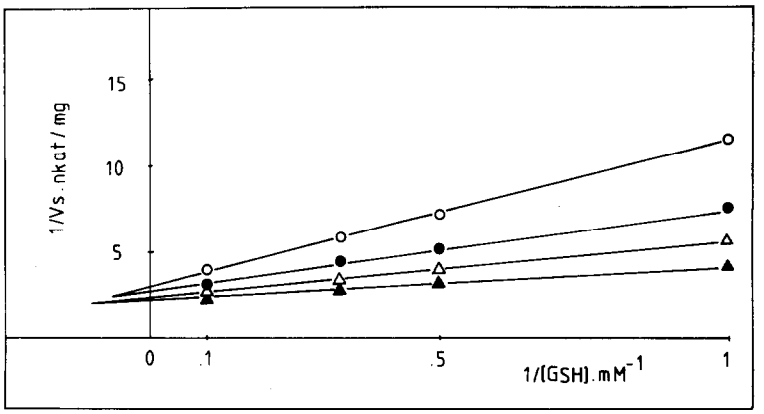

Fig. 3. Double reciprocal plots of the rate of the total reaction (V_S) versus 1/GSH for various concentrations of L-Met: (○) 1 mM; (●) 2 mM; (△) 3 mM; (▲) 5 mM. V_S was calculated as the sum $V_T + V_H$.

Table 5. Ratio of transfer to hydrolysis of different γ-glutamyl compounds catalysed by yeast γ-GT in the presence of L-Met*

γ-Glutamyl compounds	V_T/V_H
GSH	14.3
Ophtalmic acid	13.8
L-γ-Glu-L-Met	14.7
L-γ-Glu-Ala	14.1
L-γ-Glu NH_2	13.6
L-γ-Glu-PNA	14.4

*Rates of transfer (V_T) and hydrolysis (V_H) were measured at pH 9 as described in the Experimental in the presence of 10 mM of γ-glutamyl compound and 5 mM L-Met.

this seems to be inconsistent with the *in vitro* specificity pattern of yeast γ-GT, because transpeptidation is inhibited by excess L-Glu and L-Glu NH_2 (Table 3). However, γ-GT might function *in vivo* as a catalyst for the biosynthesis of the above mentioned peptides if the free acceptor concentration is kept below its K_i value and GSH concentration above its true K_m. Alternatively, it is conceivable that γ-GT is involved in a vectorial metabolism where the acceptor has no access to the donor site. The rôle of γ-GT in higher plants is still undefined [6] but current data favour the idea that transpeptidases act mainly as peptidases in plants [21, 33]. Studies of lower plant γ-GT should provide useful data for understanding this enzyme function.

EXPERIMENTAL

Chemicals. L-[U-^{14}C]His, L-[U-^{14}C]Pro, L-[U-^{14}C]Val, L-[U-^{14}C]Arg, L-[U-^{14}C]Leu, L-[U-^{14}C]Lys, L-[U-^{14}C]Asp NH_2 and L-[methyl-^{3}H]Met were purchased from Amersham-Belgium. [1,4-^{14}C]Gly-Gly was obtained from ICN-Irvine and [U-^{14}C]L-Met-L-Met was synthesized by unblocking t-BOC-L-Met-L-Met with HCl in HOAc [34]. L-γ-Glutamyl-*O*-(carboxy)phenylhydrazide was synthesized according to ref. [23]. L-γ-Glutamy-L-glutathione was obtained as in ref. [5] but with crude porcine kidney γ-GT (Sigma). All other chemicals were obtained commercially.

Analytical procedures. γ-GT activity was determined during the purification steps as described previously [15] but in the presence of 2 mM L-γ-Glu-PNA, 20 mM L-Met and 0.1 M Tris–HCl (pH 9); the activity is expressed as nkat (PNA released at 37°). The assay for hydrolytic activities of the pure enzyme was conducted in 20 mM Tris–HCl buffer containing the γ-glutamyl substrate. After incubation at 37°, the reaction was terminated by heating at 90° for 2 min. Samples which are heat-inactivated immediately following enzyme addition, served as blanks. The glutamate in the cooled sample was determined according to ref. [25]. Transpeptidation rate in 20 mM Tris–HCl buffer was assayed at 37° in the presence of various γ-glutamyl donors and L-[methyl-^{3}H]-Met as an acceptor in 0.2 ml final vol. The reaction was terminated with 0.5 ml of cold 0.05 M HOAc. The γ-Glu-L-[methyl-^{3}H]-Met product was separated on Dowex acetate (AGIX2) columns and estimated by scintillation according to ref. [25]. Protein was determined by the method of ref. [35] with bovine serum albumin as the standard. Sugar content of purified γ-glutamyl transpeptidase was estimated according to ref. [19] by the PhOH–H_2SO_4 procedure. TCL was carried out on silica gel. The sheets were developed in the ascending direction with EtOH–NH_4OH (25%) (77:23). Spots were revealed with a ninhydrin spray. R_f: GSH, 0.49; GSSG, 0.08; γ-Glu-GSH, 0.36; γ-Glucys*bis*gly, 0.21; Cys*bis*gly, 0.41; Glu, 0.32; Met, 0.81; γ-Glu-Met, 0.54.

Determination of M_r *and subunit composition of the enzyme.* Apparent M_r of γ-glutamyl transpeptidase was estimated by gel filtration on a Sephadex G200 column (2.5 × 40 cm) essentially as described in ref. [36]. Enzymes of known M_r and blue dextran 2000 were used to calibrate the column. The markers (urease, catalase, *E. coli* ornithine carbamoyltransferase, alkaline phosphatase and hexokinase) were determined by appropriate assays [37]. The column was equilibrated with 0.1 M Tris–acetate buffer (pH 8).

PAGE was performed according to ref. [38] in 6% gels min in 0.05 M Tris–acetate buffer (pH 8.5). SDS-PAGE was carried out on 10% acrylamide gels as described in ref. [39]. The subunit M_rs were estimated from a simultaneous electrophoresis run using the following protein markers: ovalbumin, glutamate dehydrogenase, fumarase, aldolase, glyceraldehyde-3-Pi dehydrogenase, trypsin and myoglobin.

Purification of yeast γ-GT. Unless otherwise stated, all operations were carried out at 4°. Cells of *Saccharomyces cerevisiae* 1278b having grown on a medium containing L-Glu as sole nitrogen source [15] were harvested at 0.6–0.7 mg/ml dry wt. About 250 g of fresh pellets of *S. cerevisiae* were suspended in 10 mM Tris–HCl buffer pH 8 containing 5 mM GSSG. The suspension was sonicated in a Raytheon 10 kHz magnetostriction apparatus. After removal of cell debris by centrifugation at 20000 *g* for 15 min the supernatant fluid was stored at 4°. Cell debris were suspended in a vol. equal to that of the first supernatant fluid of the above mentioned buffer. The homogenate was gently swirled for 2 hr. After centrifugation, the washing supernatant was added to the first extract. The sum of the two supernatant is referred to as 'crude extract'. The crude extract was treated with protamine sulphate (0.15 g/g prot) which was added dropwise under stirring as a 5% soln. The suspension was stirred for 15 min and then centrifuged at 20000 *g* for 15 min. The supernatant was applied to a column (5 × 60 cm) of DEAE Sephadex A 50 previously equilibrated with NaPi buffer 0.05 M pH 7.5. The column was then washed with 1 l. of the same buffer, and γ-GT was eluted with a linear gradient of 0.05 M NaPi buffer pH 7.5 (1 l.) in the mixing chamber and 0.25 M NaPi buffer pH 7.5 (1 l.) in the reservoir. Fractions of 10 ml were collected at a flow rate of 70 ml/hr. The most active fractions were pooled and brought to 50% satn by solid $(NH_4)_2SO_4$ in the presence of 5 mM GSSG. The mixture was stirred gently for 45 min. The ppt was removed by centrifugation and the pellet discarded. Further precipitation at 70% satn gave a ppt which was dissolved in 10 ml of Tris–HCl buffer pH 7 containing 5 mM GSSG. The cloudy suspension was dialysed against the same buffer until cleared. It was then applied on a column (2.5 × 100 cm) of Sephadex G150 previously equilibrated with the buffer. The column was run at 10 ml/hr and fractions (2 ml) containing the activity were pooled. The soln was adjusted to 1 mM $CaCl_2$ and 1 mM $MnCl_2$ and applied to a column (1.6 × 15 cm) of lentil lectin Sepharose 6 B. The resin was previously equilibrated with Tris–HCl 0.05 M pH 7 containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. The column was washed with 100 ml of the same buffer and γ-GT was eluted with the buffer containing 2 mg/ml of α-methylglucoside. The active fractions obtained after this step were pooled and concd with a PM 30 (amicon) membrane. The soln was stored at −19°. Under these conditions, the enzyme activity remains stable for at least 2 months.

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