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γ-GLUTAMYLTRANSFERASE FROM AZO DYE INDUCED HEPATOMA AND FETAL RAT LIVER

SIMILARITIES IN THEIR KINETIC AND IMMUNOLOGICAL PROPERTIES

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Summary

Some properties of γ-glutamyltransferase ((γ-glutamyl)-peptide: amino-acid γ-glutamyltransferase EC 2.3.2.2) from azo dye induced hepatoma and fetal rat liver were studied using kinetic and immunological criteria. There was no significant difference between the hepatoma enzyme and fetal rat liver enzyme in some of their catalytic properties.

Antisera against the purified hepatoma enzyme also reacted to the fetal rat liver enzyme in the inhibition test and the precipitin reaction. A structural similarity between the hepatoma enzyme and fetal rat liver enzyme was observed and the acquirement of fetal characteristics in hepatoma was discussed.

Introduction

γ-Glutamyltransferase (EC 2.3.2.2) is widely distributed in mammalian tissues [1]. The activity was the highest in kidney and pancreas [2] and also in azo dye induced hepatoma or ascites hepatoma, a high activity was found [3,4]. Fiala and Reuber reported that a non protein sulfhydryl compound was detected in the extracts from primary hepatoma developed during azo dye hepatocarcinogenesis in rats [3]. The compound was identified as L-cysteine, which was formed from glutathione by the enzyme γ-glutamyltransferase in hepatoma tissues. Fiala et al. [5] and Taniguchi et al. [6] reported that liver γ-glutamyltransferase was activated in the latter stage of azo dye hepatocarcinogenesis. Taniguchi [7] purified the enzyme from azo dye induced hepatoma and reported its physicochemical properties. On the other hand our previous report indicated hepatoma acquired the fetal properties of glutathione metabolism [8]. γ-Glutamyltransferase activity was hardly found in adult rat liver but in fetal rat liver a high activity was observed. It is a matter of interest

to determine whether or not any differences exist among the hepatoma and fetal liver γ-glutamyltransferase in their catalytic and immunological properties. This paper describes the comparison between hepatoma and fetal rat liver enzymes from the kinetic and immunological points of view.

Materials and Methods

Chemicals

L-γ-Glutamyl *p*-nitroanilide was obtained from C.F. Boehringer, Mannheim, Yamanouchi, Japan. All other reagents used were of analytical grade.

Fetal rat liver and hepatoma

Pregnant Donryu Strain rats from the Nihon Rat Co. Ltd, Tokyo were fed ad libitum with a basal diet produced by the Oriental Yeast Co. Ltd, Tokyo. Fetal rat livers were taken from pregnant rats one to three days before delivery. Azo dye induced hepatomas were collected by the method described previously [7].

Assay of enzyme activity

γ-Glutamyltransferase was assayed at 37°C according to the method described by Orlowski and Meister [9] using L-γ-glutamyl *p*-nitroanilide as the substrate. Glycylglycine was not used as acceptor except the crude enzyme extracts were assayed. The enzyme activities in the crude enzyme extracts of fetal liver, adult liver and hepatoma were determined by the method described previously [6]. The amount of *p*-nitroaniline formed from the substrate by the enzyme was determined colorimetrically. The unit of enzyme activity was expressed in terms of mμmoles of *p*-nitroaniline formed per min at 37°C. The specific activity was expressed as units per mg protein. Protein concentration was determined by the method of Lowry et al. [10] using crystalline bovine serum albumin as the standard.

Purification of γ-glutamyltransferase and antigen used for the immunization

Hepatoma γ-glutamyltransferase was purified by the method described previously [7]. Fetal rat liver was purified partially by the method used for the hepatoma enzyme. Because of the difficulties in obtaining the enzyme sources, only partial purification was carried out. 25 g of fetal rat liver were centrifuged in the presence of 0.08 M $MgCl_2$ and then solubilized with 1.5% deoxycholate. The subsequent steps for the extraction of the purification was almost the same as that used in the hepatoma enzyme, namely acetone fractionation, heat treatment, streptomycin treatment, *n*-butanol treatment, Sephadex G-200 chromatography and DEAE Sephadex chromatography. The secondary step of the purification was not done because only a small amount of enzyme extract was obtained at this step. The final specific activity obtained was 80 units/mg, and the protein was 4.0 mg. This enzyme extract from fetal liver was used for the following experiments.

Preparation

Antisera was prepared against hepatoma γ-glutamyltransferase by injecting

two rabbits with the purified hepatoma γ-glutamyltransferase. Each rabbit was injected subcutaneously with 1 ml of a solution containing 1 mg of hepatoma γ-glutamyltransferase dissolved in 145 mM NaCl and emulsified with an equal volume of Freund's complete adjuvant. One week after immunization a 1 mg booster injection of hepatoma γ-glutamyltransferase was administered in a similar manner. On the 7th day after the boosting injection, the rabbits were bled by heart puncture after overnight fasting. The clear serum collected after centrifugation was stored at −20°C in sealed tubes. Non-immune sera were collected in a similar manner to eliminate non specific effects of the enzyme activity.

Absorption of raw antisera

Preliminary experiments revealed that a small amount of impurity in the "purified" hepatoma γ-glutamyltransferase was observed as a fine extra precipitin line. This was due to the rabbit's biological amplification of trace antigenic contaminants. This antibody against impurity was removed from the antiserum by absorption with the crude hepatoma enzyme extract. Hepatoma (1 g) was homogenized with 10 ml of 0.01 M $MgCl_2$ and centrifuged at 12 000 × *g* for 30 min. The supernatant was used as the crude enzyme extract. One ml of the antiserum was incubated with 3 ml of crude enzyme extract for 8 h at 37°C. The mixtures were centrifuged at 12 000 × *g* for 30 min, and a clear supernatant was obtained. The absorbed antiserum thus obtained was demonstrated to be specific for hepatoma γ-glutamyltransferase.

Immunodiffusion

Double gel diffusion was performed at room temperature using a 1.2% solution of agarose (Difco) in a barbital/sodium barbital buffer, pH 8.6, 0.06 ionic strength, with 0.01% sodium azide added as a preservative, according to the method described by Ouchterlony [11].

Enzyme inhibition by anti-γ-glutamyltransferase

The antiserum inhibition experiments were carried out by adding the antiserum (diluted with 0.1 M potassium phosphate buffer, pH 7.4, containing 145 mM NaCl) to the standard enzyme assay reaction mixture. In each experiment a control without an addition of antiserum was included as well as a control containing an aliquot of non-immune serum. The samples were incubated for 2 h at 37°C. The remaining enzyme activity was measured at 412 nm in a Hitachi 624 type recording spectrophotometer.

Results

γ-Glutamyltransferase activity in fetal rat liver, adult rat liver and hepatoma

Adult rat liver showed very little activity, but during the course of hepatocarcinogenesis the enzyme was strikingly activated [6]. Twenty adult rat livers were found to contain a mean and standard error of 10 ± 1.0 units/mg protein. Ten fetal rat livers contained 100 ± 25 units/mg protein. Azo dye induced hepatoma of twenty rats had a significant high activity of 410 ± 20 units/mg protein.

TABLE I

SOME KINETIC PROPERTIES OF γ-GLUTAMYL TRANSFERASE

Effect of pH on enzyme activity was studied as follows. The enzyme was assayed in both Tris · HCl and borate/NaOH buffers [7]. The reaction mixture contained 0.5 ml of buffer (1 M), 0.1 ml of 50 mM L-γ-glutamyl *p*-nitroanilide and the enzyme (5 μg of protein) in a total volume of 1.0 ml. The values of *Km* for L-γ-glutamyl *p*-nitroanilide were determined by the double-reciprocal method. Inhibition of enzyme activity by L-serine was observed in the presence of borate [7].

Properties	Hepatoma [7]	Fetal liver
Optimum	8.8 — 9.0	8.8 — 9.0
K_m for γ-glutamyl *p*-nitroanilide	$1.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}$
K_i for L-serine	$3.4 \cdot 10^{-4}$	$3.2 \cdot 10^{-4}$

Some kinetic properties of γ-glutamyltransferase from hepatoma and fetal rat liver

K_m value, K_i value and pH optimum of the enzymes. Some properties of γ-glutamyltransferase were compared between the two enzymes. No significant difference was seen in the pH optimum, K_m value for L-γ-glutamyl *p*-nitroanilide and K_i value for L-serine in the presence of borate between hepatoma and fetal liver enzyme as shown in Table I.

Effects of cations and EDTA

Orlowski and Meister reported slight activation of the hog kidney enzyme by Mg^{2+} [9]. No activation by Mg^{2+} was observed with the beef kidney enzyme [12]. The fetal liver enzyme was slightly activated by divalent cations such as Mg^{2+} and Ca^{2+} as shown in Table II. Hepatoma enzyme was also slightly activated by these cations. Orlowski et al. [13] reported that the monovalent cations such as Na^+ and K^+ were also activators, however, Tate and Meister [14] have not observed this effect when glutathione is substituted for L-γ-glu-

TABLE II

EFFECT OF CATIONS AND EDTA

The reaction mixture contained 0.5 ml of 1 M Tris · HCl, pH 9.0, 0.1 ml of 50 mM L-γ-glutamyl p-nitroanilide, 0.5 μg of the enzyme and indicated concentrations of cations or EDTA in a total volume of 1.0 ml. The activity was expressed in percentage of that of the control experiment.

Addition	Conc (mM)	Relative enzyme activity (%)	
		Hepatoma	Fetal liver
None		100	100
$MgCl_2$	20	130	110
	100	135	120
$CaCl_2$	10	147	155
KCl	150	100	102
NaCl	150	102	105
$ZnCl_2$	1	3	13
EDTA	10	100	107

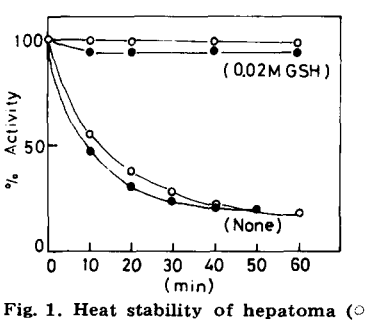
tamyl *p*-nitroanilide in the enzyme assay. Monovalent cations such as Na^+ or K^+ were not activators for both fetal and hepatoma enzymes. Zinc ion was potent inhibitor for these enzymes.

Enzyme stability

Fetal liver enzyme was incubated at 58°C for 60 min in the presence of 0.02 M GSH, but its original activity was retained with no significant loss as shown in Fig. 1. There were no significant differences in the stability of hepatoma enzyme when compared against fetal rat liver enzyme. In each case incubation at 58°C for 40 min inactivated the γ-glutamyltransferase activity almost by 80%.

The effect of anti-hepatoma γ-glutamyltransferase on the fetal liver transpeptidase activity

Absorbed immune sera produced in rabbits against the purified hepatoma γ-glutamyltransferase, inhibited hepatoma enzyme to a maximum extent of 80%, while fetal rat liver enzyme was also inhibited by about 80%. In all cases, non-immune serum stimulated both enzyme activities (by approximately 15%) and the values described here are corrected for this effect. As shown in Fig. 2 almost identical patterns of enzyme inhibition are observed. The highly sensitive reaction between antigen and antibody and the measurement of catalytic function indicate no structural differences between hepatoma and fetal liver enzyme.



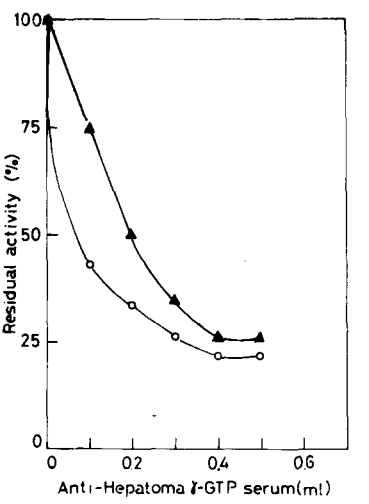


Fig. 1. Heat stability of hepatoma (○———○) and fetal rat liver (●———●) γ-glutamyltransferase was observed in the presence or absence of glutathione (0.02 M). The enzyme was incubated at 58°C in 0.1 M Tris · HCl, pH 9.0, at a concentration of 5 units/ml with or without glutathione. At the time indicated, samples were removed and immediately assayed as described in Materials and Methods.

Fig. 2. The incubation system containing 30 μg of hepatoma enzyme (○———○) and fetal rat liver enzyme (▲———▲) were incubated for 2 h at 37°C in the presence of an appropriate antiserum before assaying. The solvent was 100 mM potassium phosphate containing 145 mM NaCl, pH 7.4. γGTP; γ-Glutamyltransferase.

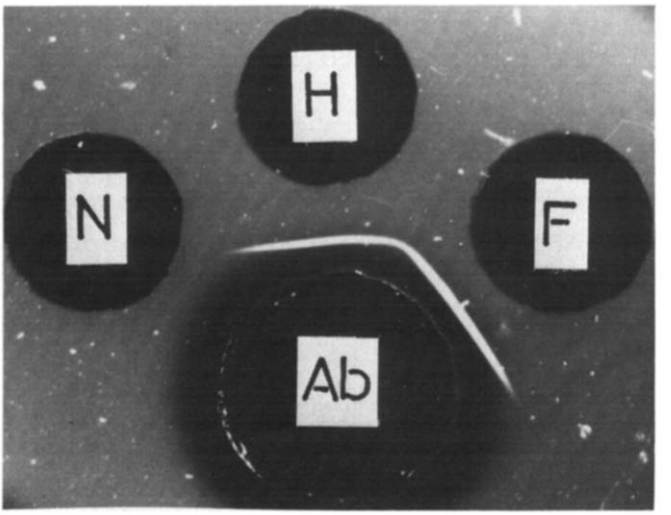


Fig. 3. Typical gel diffusion pattern with antisera with γ-glutamyltransferase from hepatoma and fetal rat liver. Ab, rabbit anti-hepatoma γ-glutamyltransferase, F, partially purified fetal rat liver enzyme; H, purified hepatoma enzyme; N, no addition.

Precipitin reaction between antisera against the hepatoma enzyme and the fetal liver enzyme

Agar gel diffusion was carried out by the method described by Ouchterlony [11]. Absorbed antisera against hepatoma γ-glutamyltransferase showed a single precipitin line against the enzyme, whereas serum obtained prior to immunization did not react with the enzyme. The fetal liver enzyme also reacted to the antisera against the hepatoma enzyme and cross-reactivity was observed between the hepatoma and fetal rat liver enzyme as shown in Fig. 3. The strong cross-reactivity between antigen and antibody may indicate that there are no structural differences between hepatoma and fetal liver enzyme.

Discussion

Previous work described that hepatoma tissue acquires fetal characteristics with respect to glutathione metabolism [8]. It has been shown that γ-glutamyltransferase activity was quite low in adult rat liver whereas in fetal rat liver, and in azo dye induced hepatoma the activity was extremely high. This fact also indicates that the hepatoma tissue acquires the fetal properties with special regard to γ-glutamyltransferase activity.

Recently various carcino-embryonic proteins have been found in hepatoma. α-Fetoprotein [15], Carcinoembryonic antigen [16], aldolase, hexokinase [18] phosphorylase [19] have also been reported. Knox examined numerous enzymes by computer analysis and reported that fetal properties are acquired by many enzymes in hepatoma. The correlation coefficient between the activity of fetal type enzyme and hepatoma enzyme were extremely high [20].

Weinhouse et al. [18] recently described the similarity of some of the isozyme patterns of the poorly differentiated hepatomas to those of fetal liver

and reported that he had found evidence for "switching on" of fetal protein synthesis following or accompanying the "switching off" of gene products of differentiated cells. They concluded that impairment of gene control, rather than alteration of gene structure, may be a crucial factor in neoplastic transformation.

The present study indicated that the enzyme obtained from fetal liver and hepatoma had almost the same properties in their kinetic and immunological properties examined. These facts suggested that two enzymes were almost identical and consisted of the same enzyme protein. The identity of the γ-glutamyltransferase from hepatoma and fetal rat liver demonstrated in the present paper revealed that the ontogenic reversion of the enzyme did not involve alterations in gene expression.

Orlowski and Meister reported that γ-glutamyltransferase plays an important role in amino acid transport of tissues [21]. Numerous evidence supporting this hypothesis are available [22]. They proposed that a γ-glutamyl cycle exists in the kidney, choroid plexus and certain other mammalian cells which plays partial roles in the amino acid transport of tissues. In fetal rat liver and hepatoma whether this cycle also exists or not is an interesting problem. More work is desirable along these lines to clarify this point.

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