Comparison of Electrophoretic and Immunological Properties of Gamma-Glutamyl Transpeptidase from Human Adult Liver, Fetal Liver and Primary Hepatoma

P. Selvaraj, K.A. Balasubramanian
Welcombe Research Unit, Christian Medical College and Hospital, Vellore, India

Key Words. γ-Glutamyl transpeptidase • Adult and fetal liver • Hepatoma • Neuraminidase treatment • Electrophoretic and immunological properties

Abstract. γ-Glutamyl transpeptidase (γ-GT) from human fetal liver, normal adult liver and primary hepatoma was separated into a hydrophobic form (detergent-solubilized, native enzyme) and a hydrophilic form (papain-digested enzyme) using papain digestion and phenyl Sepharose CL-4B hydrophobic chromatography. On polyacrylamide slab gel electrophoresis the hydrophobic form of the enzyme from the above three sources did not enter the gel, whereas the hydrophilic form entered the gel and moved as a single band with identical mobility. Neuraminidase treatment decreased the mobilities of hydrophilic form of γ-GT from all the three sources to a similar extent suggesting that the enzymes were sialylated equally. The enzyme activities from all the three sources were inhibited and precipitated equally by antibody to fetal liver γ-GT. In Ouchterlony double diffusion test the precipitin lines formed between enzymes from the three sources and anti-fetal liver γ-GT showed a reaction of complete identity. The activity of the hydrophilic form of the enzyme was more inhibited and required less amount of antibody for precipitation compared to their hydrophobic forms of the enzyme from all the three sources.

γ-Glutamyl transpeptidase (γ-GT; (5-glutamyl)-peptide: amino acid 5-glutamyl transpeptidase; EC 2.3.2.2) was found to be elevated in fetal livers and hepatomas of rat, mouse and human compared to normal adult liver [1–3]. Histochemical and biochemical studies on experimental hepatocarcinogenesis in the rat showed that this enzyme could be used as an oncofetal marker for neoplastic transformations of livers [4]. It has also been used to show the accrualment of embryonic properties by rat liver in certain experimental conditions like portacaval shunt [5]. In all the above circumstances only the quantitative change and the appearance of γ-GT activity in the hepatocytes was used to describe the carcinoembryonic characteristics of the liver. However, it has not yet been conclusively proved either in the rat or in humans that the increase of γ-GT during the oncofetal development is due to the appearance of an isoenzyme as has been shown for many enzymes.
[6] or otherwise. Such studies may prove whether the oncofetal development of the liver results in any change in properties of γ-GT and also provide some understanding about the expression of the gene responsible for γ-GT in such development. If there is any variation, it can also be used to differentiate the heptoma from other hepatobiliary diseases, since γ-GT has been reported to be elevated in most of the hepatobiliary diseases [7]. In the present communication, we have analysed the electrophoretic behaviour and immunological properties of partially purified γ-GT from human adult liver, fetal liver and primary heptoma in order to show the extent of sialylation and immunological reactivity of the enzyme.

Materials and Methods

Chemicals for polyacrylamide gels were purchased from Eastman Kodak Co., New York. Papain was from Sigma Chemical Company, USA. Phenyl Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents and chemicals used were the same as described before [2].

Enzyme Assay and Unit

During purification procedures, the γ-GT activity was assayed as described [2] at 30 °C, and for immunonablibation and immunoprecipitation studies the enzyme was assayed by the modified method as described [8]. 1 U is the amount of enzyme that converts 1 µmol of the substrate/min at 30 °C. Specific activity is expressed as units per milligram of protein.

Partial Purification of Detergent and Papain

Solubilized Forms of γ-GT from Human Adult Liver, Fetal Liver and Hepatoma

All purification steps were carried out at 4 °C unless otherwise stated. The purification of membrane-bound γ-GT was carried out as follows from frozen (at −20 °C) normal adult liver, pooled fetal liver (from human fetuses of 14–20 weeks old) and primary hepatoma. Homogenization, deoxycholate extraction, acetone precipitation and ammonium sulphate fractionation were carried out as described earlier [2] except that the 40,000 g pellet was used for deoxycholate extraction. The enzyme obtained after (NH₄)₂SO₄ fractionation was loaded on a phenyl-Sepharose CL-4B column equilibrated with 50 mMol/l Tris-HCl buffer, pH 7.5 (buffer A). Unbound proteins were washed out with 5 bed volumes of the same buffer followed by 15 bed volumes of 20% ethylene glycol in 20 mMol/l Tris-HCl buffer pH 7.5. After washing the ethylene glycol by buffer A, the bound enzyme was eluted with 10 bed volumes of a linear gradient of 0–1% Triton X-100 in buffer A. The eluted enzyme was divided into two parts. One part was precipitated with 2 vol of cold acetone (−20 °C) to remove Triton X-100. The precipitate was dissolved in buffer A and dialysed against the same buffer. This preparation was taken as the hydrophilic form or the detergent-solubilised form of γ-GT. The second part was incubated with 0.04 mg of papain per mg of protein at 37 °C for 4 h in presence of 0.8 mg/ml of cysteine, precipitated with (NH₄)₂SO₄ and rechromatographed on a phenyl-Sepharose CL-4B column as described above. The unbound enzyme was collected and dialysed against buffer A. This was taken as hydrophilic (papain-digested) form of γ-GT [9]. This obtained hydrophilic and hydrophobic forms of γ-GT were used for all studies.

Neuraminidase Treatment

The partially purified γ-GT was incubated with 0.2 U of neuraminidase (Vibrio cholerae, Boehringer, FRG) per mg of enzyme protein at 30 °C for 6 h in buffer A containing 1 mMol/l CaCl₂. Two control experiments were performed. In the first control they-GT was treated in the same way without neuraminidase. In the second control the enzyme was treated with neuraminidase in buffer A containing 2 mMol/l EDTA.

Polyacrylamide Slab Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out as described by Davis [10] using 7% vertical slab gels. 50 mMol/l Tris-glycine buffer, pH 8.3, was used in the electrode chambers. The slab gels were stained for γ-GT activity as described [11].

Antiserum Production

Antiserum was raised against membrane-bound γ-GT purified from human fetal liver. The papain-digested γ-GT obtained after the second phenyl-Se-
pharocre CL-4B chromatography was further purified by preparative polyacrylamide gel electrophoresis. The purified enzyme showed a single band for protein on 7% polyacrylamide gel electrophoresis which could be stained for γ-GT activity. The enzyme obtained in the final step showed a specific activity of 693 U/mg of protein with 30% recovery. 300 μg of purified γ-GT in 1.5 ml of buffer A was emulsified with equal volumes of Freund's complete adjuvant (Difco Laboratories, USA) and injected subcutaneously to a 6-month-old rabbit. On the 22nd day after the first injection, 150 μg of enzyme was injected subcutaneously as a booster. The rabbit was bled on the 7th, 8th and 9th days after the booster. IgG was separated from the antisemur as described [12]. As a control, IgG was also purified from nonimmune rabbit serum. The IgG preparations thus obtained did not show γ-GT activity and was used for all immunological studies described here.

Immunoinhibition and Immunoprecipitation Studies on γ-GT

Immunoinhibition and immunoprecipitation of γ-GT were performed using antibody to fetal liver γ-GT. A constant amount of γ-GT in 100 μl of buffer A was mixed with an equal volume of varying concentrations of antibody (IgG) to fetal liver γ-GT in the same buffer and incubated for 16 h at 4°C. After the incubation, 50-μl aliquots were drawn for γ-GT assay to measure the inhibition of enzyme activity by antibody. To the rest of the incubation mixture, 20 μl of 10% stabilised formaldehyde-fixed [13] bacterial suspension of Staphylococcus aureus (Cowman I) was added and further incubated for 1 h at 4°C. The mixture was centrifuged at 16,000 rpm for 5 min in a Beckman/Spinco model-152A microfuge and the enzyme activity was assayed in the supernatant. The treatment with bacterial suspension removed all the enzyme-antibody complexes and the free antibody from the incubation mixture [14]. Further addition of bacterial suspension to the supernatants obtained from the incubation mixture of highest antibody concentration used did not result in a decrease of the enzyme activity in the supernatant. Therefore, the γ-GT activity assayed after the centrifugation represents the activity of the free enzyme present in the supernatant and not of the enzyme-antibody complex. Three different types of control experiments were performed in the same way as described above. In the first and second control, the antibody was replaced, respectively, by equal amounts of rabbit nonimmune IgG and buffer A. In the third control, the enzyme was treated with buffer A instead of the antibody and bacterial suspension. Equal amounts of γ-GT from all the three sources were used for immunoprecipitation and inhibition studies.

Immunodiffusion

The double immunodiffusion was performed in 1% agarose gels according to the method of Ouchterlony [15]. After 24 h incubation at 4°C, the slides were washed and the precipitate was stained for γ-GT activity as described [11].

Results

The specific activity of the enzyme in the homogenates of the adult and pooled fetal liver was 0.015 and 0.096, respectively. Three different primary hepatoma tissues used in this study had specific activities of 0.055, 0.052 and 0.183. There was no difference observed in the behaviour of the enzyme from all the three sources (adult liver, fetal liver and hepatoma) during purification. Papain digestion did not have any influence on the enzyme activity.

Electrophoretic Mobilities of γ-GT

Figure 1 shows the electrophoretic mobilities of partially purified γ-GT obtained from adult liver, fetal liver and hepatoma. The hydrophobic form of enzyme from all the three sources did not enter the gel and remained in the origin, whereas the hydrophilic form of γ-GT entered the gel and moved as a single band with identical mobility. The mobility of the hydrophilic form of γ-GT from the three sources was decreased to the same extent by neuraminidase treatment. The hydrophilic form of enzyme did not enter the gel even after neuraminidase treatment. However, the activity of both the forms of enzyme was not affected by neuraminidase
treatment. No difference in the mobility was observed between the two controls. Thus, the use of EDTA-inhibited neuraminidase as a control showed that the change in the electrophoretic mobility observed by neuraminidase treatment is due to desialylation of the enzyme and not because of proteolytic cleavage by the contaminating proteases [16].

Immunoinhibition and Immunoprecipitation of γ-GT

Figure 2 shows immunoinhibition and immunoprecipitation patterns of γ-GT activity by antibody to fetal liver γ-GT. The immunoinhibition pattern shows that the activities of hydrophobic and hydrophilic forms of the enzyme from all the three sources were inhibited to a similar degree by anti-fetal liver γ-GT. Table I shows the percentage inhibition of γ-GT activity by antibody when the enzyme was assayed from an incubation mixture in which the antibody concentration was enough for complete precipitation of the enzyme. It can be seen from table I and figure 2 that the hydrophilic form of γ-GT (fig. 2B) was more inhibited by antibody than the hydrophobic form (fig. 2A).

<table>
<thead>
<tr>
<th>Source of γ-GT used</th>
<th>% inhibition when added antibody was enough for complete precipitation of enzyme</th>
<th>Amount of antibody required to precipitate 4 mU of enzyme, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% hydrophobic γ-GT</td>
<td>% hydrophilic γ-GT</td>
</tr>
<tr>
<td>Adult liver</td>
<td>53</td>
<td>86</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>49</td>
<td>87</td>
</tr>
<tr>
<td>Primary hepatoma</td>
<td>53</td>
<td>86</td>
</tr>
</tbody>
</table>

Control activity was taken as 100%. Experiments are described in Methods.
The immunoprecipitation curves (fig. 2) obtained for the enzyme from all the three sources followed a parallel pattern and can be superimposed. Figure 2 also shows that anti-fetal liver $\gamma$-GT can completely precipitate $\gamma$-GT from adult liver and hepatoma. The amount of antibody required to precipitate equal units of $\gamma$-GT from adult liver, fetal liver and hepatoma is shown in table I. Almost equal amounts of anti-fetal liver $\gamma$-GT precipitated 4 mU of $\gamma$-GT from all the three sources. It can also be seen from figure 2 and table I that the hydrophobic form (fig. 2A) of $\gamma$-GT required a higher concentration of anti-
body compared to that of the hydrophilic form (Fig. 2B) for complete precipitation. No difference was observed between the three controls, which rules out the possibility of any nonspecific interactions of γ-GT with rabbit IgG or S. aureus (Cowan I) bacterial suspension. The γ-GT from the three hepatomas behaved in an identical manner during all electrophoretic and immunological studies.

Immunodiffusion

Figure 3 shows the enzyme staining of precipitin lines formed by hydrophilic forms of γ-GT from fetal liver, adult liver and hepatoma with anti-fetal liver γ-GT in Ouchterlony double diffusion test. The lines showed a reaction of complete identity. The hydrophobic forms also behaved in the same way.

Discussion

In our studies, we have used both the detergent-solubilised and papain-digested forms of γ-GT for two reasons. Firstly, the electrophoretic studies with the detergent solubilised form of γ-GT is difficult because of its tendency to form aggregates [17]. Secondly, the immunological studies with the two forms of γ-GT may reveal the effect of papain digestion on the immunological behaviour of the enzyme from all the three sources. It has been shown that the γ-GT from various tissues of bovine [18] and human [19] differ in their electrophoretic mobilities, due to a difference in the sialic acid content, and identical mobilities were obtained after neuraminidase treatment. Based on the affinity of γ-GT towards Con A-Sepharose 4B, it has been reported that γ-GT present in rat fetal liver [20] and mammary tumours of mouse and human [21] were more sialylated compared to their normal adult forms. Yamamoto et al. [8] observed a slightly different electrophoretic mobility between adult liver and hepatoma γ-GT before and after neuraminidase treatment. The electrophoretic behaviour of γ-GT reported here, before and after neuraminidase treatment, shows that the enzymes from human adult liver, fetal liver and hepatoma were sialylated in a similar manner. This is supported by our earlier observations [2] that during purification, the γ-GT from all the three sources behaved in an identical manner towards Con A-Sepharose 4B. Recently, it has also been reported that the rat fetal liver γ-GT could not be differentiated from adult liver γ-GT using Con A-Sepharose 4B column chromatography [22].

The immunological studies reported in this paper show complete identity of γ-GT
from adult liver and hepatoma with the fetal liver enzyme. We have also observed that the fraction of γ-GT obtained from the 40,000 g supernatants of the homogenates of all the three sources can be precipitated by the anti-fetal liver γ-GT (data not shown). These findings contradict that of Fujisawa et al. [23] that antibody to a soluble form of γ-GT from hepatoma did not cross react with the membrane bound hepatoma γ-GT but cross reacts with soluble form of fetal liver γ-GT; however, the specificity of the cross reaction with respect to the adult liver soluble enzyme was not indicated. It has been shown that the γ-GT present in various human organs were immunologically identical [17, 24, 25]. Therefore, it appears that the γ-GT present in human fetal liver, adult liver and hepatoma are immunologically indistinguishable from that of other human organs and possess common antigenic determinants. The difference observed between the hydrophobic and the hydrophilic forms of γ-GT in their immunoinhibition and immunoprecipitation properties, which is also to the same extent in all the three sources, may be due to changes produced in molecular properties of the enzyme by papain digestion and needs a detailed study.

In conclusion, it appears from our results that unlike in the case of development of rat liver [20], no difference was observed in the sialylation of γ-GT during oncofetal development of human liver. The present study, supported by earlier reports [2, 22], also suggests that the elevated level of γ-GT found during oncofetal development of human liver represents the derepression or more efficient expression of the gene responsible for γ-GT production which is normally repressed, although not completely, in the adult liver. This observation is similar to that described for α-fetoprotein [26] but different from that reported for aldolase [27] where a new isoenzyme has been described.

Acknowledgements

The authors thank Prof. V.J. Mathan, Head of this Unit, for his continued interest in this work and Mr. S. Moses Narendran for his secretarial assistance. Staphylococcus aureus (Cowen 1) was kindly supplied by Mr. M.S. Rajagopalan. The Wellcome Research Unit is supported by the Wellcome Trust, London.

References


Received: March 10, 1982
Accepted: September 1, 1982
K.A. Balasubramanian,
Wellcome Research Unit,
Christian Medical College and Hospital,
Vellore 632 004 (India)