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Evaluation of Serum γ-Glutamyltransferase by Electrofocusing, and Variations in Isoform Patterns

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Serum γ-glutamyltransferase (EC 2.3.2.2) showed microheterogeneity on electrofocusing, owing to variations in sialic acid content. We investigated the isoform patterns of papain-treated serum samples on agarose gels containing nonionic detergent and ampholytes in the low pH range. Serum from cases of cholestasis show seven bands with γ-glutamyltransferase activity. These same bands were also found in liver tissue similarly treated, and they had pI values ranging from 3.8 to 4.2. Papain-treated sera that also had been neuraminidase digested showed only a single band, still enzymatically active and with a pI of 5.9. Increased γ-glutamyltransferase in serum as a result of alcohol abuse was combined with a abnormally high degree of sialylation of the enzyme, giving more anodal isoforms. The decline in the concentration of this enzyme during several weeks of abstinence was accompanied by a gradual decrease in γ-glutamyltransferase sialylation and the appearance of more cathodal fractions.

Additional Keyphrases: *alcoholism · sialylation and enzyme microheterogeneity · tissue source of circulating enzyme*

The glycoprotein γ-glutamyltransferase (EC 2.3.2.2; GGT) is an integral membrane enzyme that catalyzes the transfer of γ-glutamyl groups between γ-glutamyl peptides and small peptides or amino acids. The enzyme is proposed to have an important role in the cellular uptake of amino acids via the γ-glutamyl cycle, particularly in organs with high amino acid uptake (*1*).

The enzyme consists of two nonidentical subunits, the catalytic site being located in the light chain (*2, 3*). A hydrophobic domain constitutes the amino-terminal part of the heavy chain anchoring GGT to the membrane (*4*). This hydrophobic domain can be cleaved off by proteolytic enzymes such as papain, thereby converting the detergent binding, "amphiphilic," form of the enzyme into a water-soluble "hydrophilic" form (*5*). Both forms of the enzyme are present in serum (*6*). The liver is considered to be the major source of GGT in serum.

Trypsin-solubilized preparations of GGT from liver, pancreas, kidney, and duodenum have different mobilities on polyacrylamide gel electrophoresis (*7*). GGT-microheterogeneity due to varying sialic acid content has been reported, based on examination of human and rat kidney enzymes (*2, 3*). Cancerous rat mammary tissue contains GGT with an abnormally high sialic acid content as compared with the enzyme from normal rat mammary gland (*8*). The different binding properties of GGT to concanavalin A (Con A) were used to separate fetal rat liver GGT from adult rat liver enzyme. These properties depend on the varying sialic acid content of the enzyme (*9*). In alcoholic hepatitis the fetal form is increased in serum (*10*) as it is in the fatty-liver stage of alcoholic liver disease (*11*).

Here we report a new method for separating GGT isoforms in serum by electrofocusing, and the successive variations of the isoform patterns in alcoholics.

Materials and Methods

Apparatus

Electrofocusing apparatus "Ultrophor 2217," which includes movable electrodes, constant power supply 2197, and adjustable thermostatic cooler 2209, was from LKB, Bromma, Sweden. A U-frame for casting 0.5-mm thick gels, glass plates 125 × 260 × 3 mm, electrode strips, and "Paratex" filter papers for sample application were also from LKB. Polyester films with a hydrophilic surface, "Gelbond," were from FMC Corp., Marine Colloids Division, Rockland, ME 04841.

Reagents

"Isogel" agarose, "SeaPrep" 15/45 agarose, and "Seakem" agarose were purchased from FMC Corp., Marine Colloids Division. "Servalyt" 2–4 and "Servalyt" 3–5 were from Serva Feinbiochemica, Heidelberg, F.R.G. "Pharmalyte" 3–10 and Con A-Sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Papain (EC 3.4.22.2; type IV), L-cysteine, and Triton X-100 were from Sigma Chemical Co., St Louis, MO 63178. L-γ-Glutamyl-*p*-nitroanilide was from Boehringer Mannheim GmbH, F.R.G. *N*-Glycylglycine and *N*-1-naphthylethylenediamine dihydrochloride were purchased from British Drug Houses Ltd Chemicals, Poole, U.K. Neuraminidase (EC 3.2.1.18; from *Vibrio cholerae,* 1 kU/L) was from Behringwerke AG, Marburg, F.R.G.

All other chemicals used were of reagent grade.

Procedures

Study population. Male alcohol addicts admitted to the Department of Alcohol Diseases for inpatient detoxification were investigated. From 50 consecutive admissions we selected six men, 37–64 years old, fulfilling the following criteria.

- They had been drinking each day for about three weeks before admisssion.
- GGT in the serum exceeded 3 μkat/L at the time of admission (reference value <1 μkat/L).
- During abstinence, blood was sampled weekly for liver tests and blood ethanol at the outpatient clinic for at least six weeks after detoxification, and no signs of drinking were observed by trained clinic personnel.

Samples with icteric sera were collected from three patients whose serum had above-normal GGT and alkaline phosphatase (EC 3.1.3.1) activities. Two of these patients had a verified biliary obstruction, the third had 2-bromo-1,1,1-trifluoro-2-chloroethane (Halothane)-induced liver damage. The serum samples were stored at −18 °C and

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analyzed within three months of collection. Normal hepatic, pancreatic, renal, and prostatic tissue was collected at autopsy, within 10 h of death, and stored at −70 °C.

Sample preparation. We treated 80-μL samples of serum with 0.3 mg of papain activated in 20 μL of freshly prepared 5 mmol/L L-cysteine containing 1 mmol of Na_2EDTA per liter. This treatment was at 37 °C for 1 h. The samples were then dialyzed against a Tris HCl buffer (10 mmol/L, pH 8) at room temperature for 1 h. The microdialysis was performed in 1-mL Eppendorf conical microtubes with an 8-mm hole drilled in the cap. The dialysis membrane was pressed between the cap and the tube. The microtube was fitted upside down into a piece of Styrofoam and placed on the surface of the dialyzing Tris buffer. Before electrofocusing, the samples were diluted to a GGT activity of 1.5 μkat/L with the Tris buffer.

Cryostat sections from frozen organ tissues were prepared with a thickness of 3–5 μm and a surface area of about 1 cm^2. Various amounts of cryostat sections (e.g., about 20 pieces from liver tissue) were treated with 0.6 mg of papain in 0.2 mL of phosphate buffer (0.1 mol/L, pH 7.2) containing 1 mmol of freshly prepared L-cysteine and 0.2 mmol of Na_2EDTA per liter. This digestion was at 37 °C for 1 h. The solution was then dialyzed against the Tris buffer at room temperature for 1 h.

Molding the electrofocusing gel. A solution containing, per liter, 80 g of Isogel agarose, 20 g of SeaPrep 15/45 agarose, and 120 g of sorbitol in distilled water was heated to boiling, then cooled to 60 °C. An electrofocusing gel, 0.5 mm thick, was molded of 16 mL of this mixture, 2 mL of 100 mL/L Triton X-100, and 0.7 mL each of Servalyt 2–4, Servalyt 3–5, and Pharmalyte 3–10. The 3–10 gradient gel was molded in the same way except that 2.0 mL of Pharmalyte 3–10 was substituted for the ampholyte combination.

Electrofocusing conditions. Double application papers (Paratex), on which 30 μL of sample solution had been applied, were placed on the cathodal side of the agarose gel. A paper strip soaked in H_2SO_4, 50 mmol/L, was applied to the anode and a strip soaked in NaOH, 0.5 mol/L, to the cathode. An extra cathodal strip (Whatman 1 MM paper), moistened with 0.5 mol/L NaOH, was also used to drain the water produced by electroendosmosis. The power supply was set at maximum voltage and current with a limiting power of 0.25 W/cm (length of the agarose gel). The application papers were removed after 60 min of focusing, and a new cathodal strip was placed just anodal to the application site. The cathodal electrode was adjusted to contact the new cathodal strip. After a total focusing time of 4 h, the power was increased to 0.30 W/cm for another 30 min.

GGT activity staining after electrofocusing. The GGT staining method described by Kok et al. (*12*) was used and further adapted according to the Scandinavian recommendation for determination of GGT activity (*13*). A 10 g/L agarose gel containing, per liter, 4 mmol of L-γ-glutamyl-*p*-nitroanilide, 75 mmol of *N*-glycylglycine, 10 mmol of $MgCl_2$, 15 mmol of *N*-1-naphthylethylenediamine dihydrochloride, and 14 mmol of $NaNO_2$ in Tris HCl buffer (150 mmol/L, pH 8.3) was molded shortly before electrofocusing was ended. The substrate gel was then placed on the electrofocusing gel and the gels were incubated at 37 °C for 20 min, then separated. GGT activity was made visible as purple bands after developing the electrofocusing gel in 125 g/L trichloroacetic acid for 30 to 60 s.

GGT activity determination. GGT activity was determined according to the Scandinavian recommendation (*14*).

Neuraminidase treatment. Papain-treated serum samples of 0.20 mL were dialyzed against 1.0 L of sodium acetate (0.1 mol/L, pH 5.5) containing 9 mmol of $CaCl_2$ per liter, for 2 h at room temperature. To a dialyzed sample we added 0.10 mL of neuraminidase (1 kU/L) and then placed the sample in continuous dialysis, in the same buffer, for 48 h at 37 °C. A small sample, taken after 30 min of neuraminidase digestion, was dialyzed against the Tris HCl buffer for 2 h at room temperature, then frozen at −18 °C. After 24 h of neuraminidase digestion, another 0.10 mL of neuraminidase was added to each sample. After 48 h the digested samples were dialyzed against Tris buffer at room temperature for 2 h and stored at −18 °C.

pI determination. The gel was cut in 5-mm lengthwise slices after electrofocusing. These pieces were placed in 1 mL of distilled water each and kept overnight. pH was measured with a Radiometer pH meter for micro volumes.

Con A-Sepharose chromatography. Affinity chromatography of papain or detergent (5 mL/L Triton X-100) treated samples was performed according to Köttgen et al. (*9*). A 120 × 9 mm column was equilibrated with a Tris HCl buffer (50 mmol/L, pH 7.5) containing 0.5 mol of NaCl per liter. Upon analyzing detergent-treated samples, 5 mL/L Triton X-100 was added to the buffer. We eluted by adding 0.20 mol of α-methyl-D-mannoside per liter to the equilibrating buffer.

Results

Electrofocusing Conditions.

We extensively evaluated the commercially available ampholytes—Servalyt, Pharmalyte, and Ampholine. The combination described in *Methods* was found optimal. Because of physical characteristics, in the presence of detergent, electrofocusing in agarose was found superior to polyacrylamide. For higher resolution in agarose we added SeaPrep 15/45 to the gel and the two-step electrofocusing condition in which the cathodal electrode was moved slightly anodal to the sample application site after the initial focusing period.

The enzyme staining conditions allowed clear visualization of GGT activity of 1.5 μkat/L in serum samples. Those with a GGT activity of less than 1.0 μkat/L could be visualized by applying 45 to 60 μL of sample, instead of the usual 30 μL, to the electrofocusing gels.

Detergent-solubilized samples compared to papain-treated samples. GGT activity remained unchanged after treatment

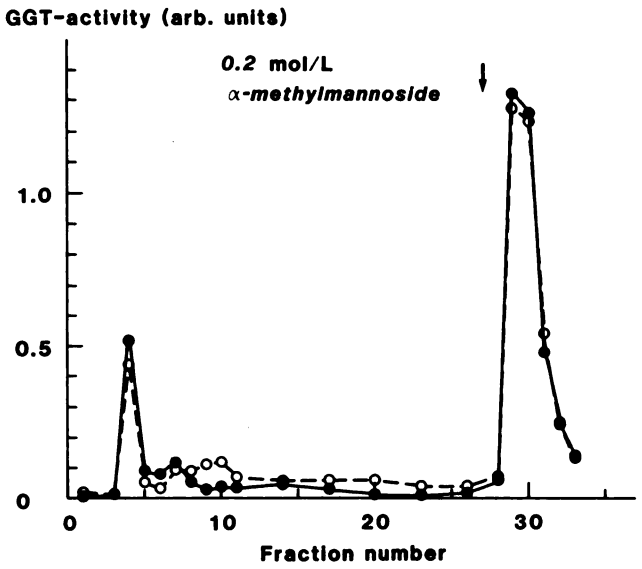

Fig. 1. GGT activities after Con A-Sepharose chromatography of a 1-mL portion of a serum sample that was either papain-treated (●) or detergent-solubilized (○)

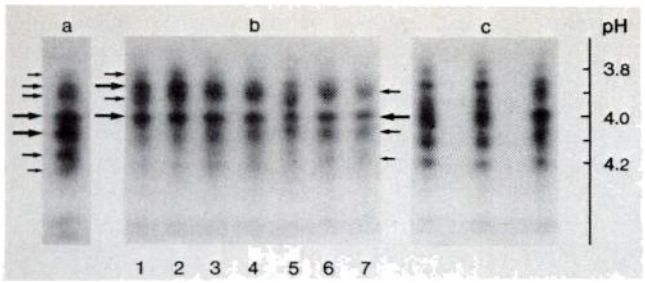

Fig. 2. GGT isoforms

a: in liver tissue treated with papain. *b:* in papain-treated sera from patient described in Table 1 (samples no. 1–7). *c:* in papain-treated icteric sera with increased GGT as a result of extrahepatic biliary obstruction or toxic induced liver damage. Electrofocusing performed as described in *Methods*. Anode at top

of sera with papain or 10 mL/L Triton X-100 as compared with untreated sera. Con A-Sepharose affinity chromatography of papain- or detergent-treated samples revealed no change in the ratio of non-bound to bound GGT forms comparing the two different treatments (Figure 1). Detergent-solubilized samples showed, after electrofocusing, a complex GGT isoform pattern, with much GGT activity retained at the application site. Papain-treated samples showed fewer GGT staining isoforms.

GGT microheterogeneity in tissue preparations. The GGT activity in liver tissue was visualized after electrofocusing of papain-treated liver tissue. Enzyme microheterogeneity in the liver consisted of seven fractions with pI values between 3.8 to 4.2 (Figure 2*a*).

Papain-treated organ tissues from the kidney had GGT isoforms with pI values between 3.8 and 5.3, the pancreas isoforms between 3.8 and 4.6, and the prostate isoforms between 3.8 and 4.1.

GGT microheterogeneity in serum. Analysis of papain-treated icteric sera with increased GGT activity as a result of extrahepatic biliary obstruction or toxic induced liver damage also revealed seven GGT-staining fractions having the same pI values as the fractions from liver tissue (Figure 2*c*).

Papain-digested samples of sera with above-normal GGT of different causes were treated with neuraminidase for 30 min and for 48 h at 37 °C before electrofocusing in the pH 3–10 gradient. After 48-h digestion the GGT activity was retained as a single band with a pI of 5.9 (Figure 3).

Changes in distribution of GGT microheterogeneity due to alcohol abuse. All serum samples drawn from the six male alcohol addicts fulfilling the criteria in *Methods* were analyzed. At the time of admittance to hospital, sera from all six patients with GGT elevation (mean value 7.9 μkat/L; SD 3.9) showed an enzyme pattern, after electrofocusing, with abundance of the more anodal fractions. At normalization of serum GGT activity (mean value 1.8, SD 1.2 μkat/L) during abstinence for several weeks, the more anodal fractions disappeared and more cathodal isoforms of the enzyme appeared. These changes were seen in all six patients and the pattern changes are exemplified in sequential order by one typical patient presented in Table 1 and Figure 2*b*.

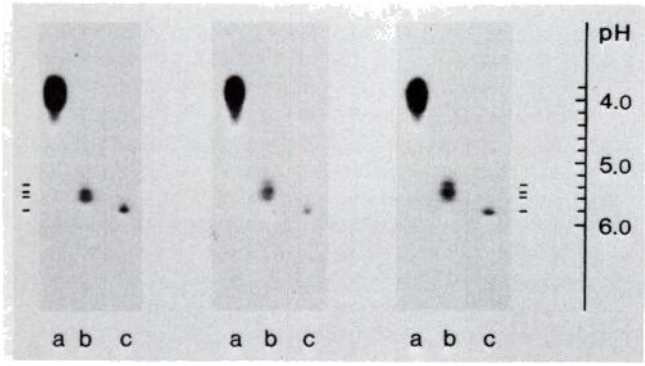

Fig. 3. Papain-treated samples (*a*), serum samples that also were neuraminidase digested for 30 min (*b*), and for 48 h (*c*) as described in *Methods*

Discussion

Numerous reports on the separation of serum GGT isoforms have been published (reviewed in *14*). The number of isoforms demonstrated have depended on the electrophoretic technique used. Most of the "isoenzyme fractions" have represented lipoprotein-bound enzyme activities in the α_1 and β_1 regions. A tendency of the detergent-binding form to bind lipoproteins in the hydrophobic domain (*15*) can explain the more complex GGT microheterogeneity pattern seen after electrofocusing of detergent-solubilized samples as compared with papain-digested samples. These lipoproteins could be involved in the liberation of GGT from the liver cell membrane. Papain treatment did not affect GGT activity; proteolytic digestion reportedly does not substantially change the lectin-binding abilities of the enzyme (*16*). This was confirmed by our results from Con A-Sepharose affinity chromatography of papain-digested as compared with detergent-treated serum samples.

A very narrow pH gradient was found important for separation of the GGT isoforms in serum, and nonionic detergent had to be included in the gels—a requirement that may be explained by the poor solubility of the enzyme in the acidic pH range. Addition of hydroxyethylated agarose (SeaPrep 15/45) improved electrofocusing resolution, probably because of the molecular sieving properties of this agarose (*17*). Because of uneven conductivity in the very acidic pH range and the interaction of some ampholytes with the staining reaction, the choice of commercially available ampholytes was critical.

Demonstration of the same seven isoforms of GGT in human liver tissue and in serum suggests the liver as the major source of the serum enzyme. A contribution from the prostate cannot be excluded from our results. Neuraminidase digestion of serum reduced the number of GGT isoforms to one single asialo form with a marked change in pI. Variations in sialic acid content are therefore considered to be responsible for the microheterogeneity of serum GGT.

We have shown a predominance of GGT isoforms with high sialic acid content in sera from alcoholics. The normalization of serum GGT during abstinence was accompanied

Table 1. GGT Activity in Samples and Clinical Data on Patient in Figure 2b

Sample	1	2	3	4	5	6	7
Days after admission	0	1	22	29	39	47	54
GGT activity, μkat/L	9.1	7.5	3.1	2.2	1.4	0.8	0.7

This patient, a 37-year-old man with a five-year history of habitual alcoholism, had been drinking heavily for four months and had a daily intake of 250 g of alcohol for the last three weeks. At the time of admission his blood alcohol concentration was 43 mmol/L, aspartate aminotransferase (EC 2.6.1.1) 2.4 μkat/L (<0.67 μkat/L), alanine aminotransferase (EC 2.6.1.2) 3.1 μkat/L (<0.67 μkat/L), cholesterol 8.6 mmol/L (3.2–7.2 mmol/L), and triglycerides 7.4 mmol/L (0.4–2.2 mmol/L) (normal reference values in parentheses). At detoxification he had moderate withdrawal symptoms. His blood pressure, 170/105 mmHg, and pulse, 130 beats/min, were within normal limits in less than three days. He was discharged after 15 days of hospital care. At the outpatient clinic he was prescribed disulfiram.

by a reduced sialic acid content of the enzyme. Ethanol intake has been shown to induce GGT synthesis (*11, 18*). We conclude that the increased sialylation of GGT could be an expression of increased synthesis. Similar changes in sialic acid content of α_1-antitrypsin have been reported in conditions with increased synthesis due to inflammation and estrogen treatment (*19*). Orosomucoid has also been found to change its carbohydrate composition upon treatment with estrogen and in inflammation, but in a more complex manner (*20, 21*).

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