MULTIPLE FORMS OF GLYCYLPROLYL DIPEPTIDYL-AMINOPEPTIDASE (DIPEPTIDYL PEPTIDASE IV) IN HUMAN SERA FROM PATIENTS WITH HEPATITIS

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ABSTRACT

Multiple forms of glycyprolyl dipeptidyl-aminopeptidase (GP-DAP) in human serum were separated by electrophoresis on cellulose acetate and the activities were directly measured fluorometrically on the strip using 7-glycyproline-4-methylcoumarinamide as substrate. Human sera from patients with hepatitis had four peaks at the α_1 (peak I), fast α_2 (peak II), α_2 (peak III), and β (peak IV) regions. Normal sera showed mainly peak III. The sera from patients with hepatitis showed increased total enzyme activity and elevation in the two new peaks, peak I and peak II. When sera from patients with hepatitis were heated at 60°C for 30 min, the peaks I, II and III were decreased by about 25%, and peak IV completely disappeared, indicating a different heat stability for each enzyme form. When human sera from patients with hepatitis were treated with neuraminidase, all the peaks shifted to the cathode except for peak IV, and the ratio between the three peaks changed greatly. These results suggest that the multiple forms of human serum GP-DAP may be due to different contents of sialic acid.

Glycyprolyl dipeptidyl-aminopeptidase (GP-DAP, EC 3.4.14.5 dipeptidyl peptidase IV) is a dipeptidyl-aminopeptidase that cleaves N-terminal X-proline and is highly specific for the second-from-terminal amino acid proline. This enzyme was discovered by Hopsu-Have and Glenner in rat liver and kidney using a newly synthesized substrate glycyproline β -naphthylamide (8). Peptides with N-terminal glycyproline-, arginylproline-, or lysylproline-sequence are the preferred substrates (12). The enzyme was present in the sera of various mammals including the human (14). The serum enzyme activity using either glycyproline β -naphthylamide or glycyproline *p*-nitroanilide was shown to be increased in patients with hepatobiliary diseases and to be decreased in patients with rheumatoid arthritis, gastric cancer, acute lymphocytic leukemia, lymphosarcoma and

Hodgkin's disease (1, 2, 5, 7, 16, 17). Using 7-glycyproline-4-methylcoumarinamide the enzyme was found in human saliva (11) and urine (10).

In human sera, especially from patients with hepatitis or liver cirrhosis, multiple forms of the enzyme activity were observed by cellulose acetate electrophoresis (7) or by Sephadex G-200 column chromatography (5).

In the present study, we have established a simple and sensitive method, which may be useful in clinical chemistry, for the detection of multiple forms of GP-DAP in human serum by cellulose acetate electrophoresis with 7-glycyproline-4-methylcoumarinamide as substrate, and have examined the properties of the multiple forms. A preliminary study of this work had been reported (9).

MATERIALS AND METHODS

Glycylproline *p*-nitroanilide tosylate and 7-glycylproline-4-methylcoumarinamide tosylate were synthesized at Peptide Institute, Protein Research Foundation (Minoh, Osaka, Japan), as reported previously (11, 12). Diagnosis of chronic active hepatitis was based on a liver biopsy.

Human blood samples were collected by venipuncture and, after clotting, the serum was separated by centrifuging at 3,000 rpm for 15 min.

Total GP-DAP activity in human serum using glycylproline *p*-nitroanilide tosylate as substrate was assayed by a modification of a continuous direct photometry (12) with use of the LKB 8600 Reaction Rate Analyzer, as described previously (13, 15). After adding 50 μ l of human serum to the reaction mixture (total volume 1.15 ml, in final concentrations) containing 150 mM Tris-HCl buffer (pH 7.9), 60 mM glycylglycine and 1.63 mM glycylproline *p*-nitroanilide tosylate, the reaction was analyzed with the Analyzer at 37°C, and the absorbance was read at 410 nm. The normal total serum activity with glycylproline *p*-nitroanilide tosylate as substrate was 68.9 ± 22.0 (mean $\pm$ 2SD) mU/ml ($n=2,615$).

Separation of the multiple forms of serum GP-DAP was made by electrophoresis on a cel-

lulose acetate film (Separax-S). Five microliters of the serum were placed along a center line of a 5.5×6 cm strip of cellulose acetate, and the samples were electrophoresed for 30 min in 40 mM sodium barbital buffer, pH 9.8 (ionic strength=0.04) at a constant current of 0.5 mA/cm width of a strip. After electrophoresis this film was put upon another film which had been impregnated with a reaction mixture of 150 mM Tris-HCl buffer containing 60 mM glycylglycine, pH 7.9 and 1 mM 7-glycylproline-4-methylcoumarinamide tosylate, and the incubation was carried out for 10 min at 37°C. The fluorescence intensity of the spots of 7-amino-4-methylcoumarin formed enzymatically on the cellulose acetate strip was directly measured at 450 nm with excitation at 380 nm by a Shimadzu CS-910 fluorescence scanner. One film was stained for the identification of serum proteins.

RESULTS

Separation of Multiple Forms of Serum GP-DAP

Separation of multiple forms of GP-DAP in a serum sample from a patient with chronic active hepatitis by cellulose acetate electrophoresis is shown in Fig. 1. Four peaks of GP-DAP activity were observed; peak I in the α_1 -globulin region, peak II in the fast α_2 -globulin region,

Fig. 1 Separation of multiple forms of serum GP-DAP by cellulose acetate electrophoresis. A typical example of serum is shown from a patient with chronic active hepatitis. Electrophoresis was carried out as described in Materials and Methods.

peak III in the α_2 -globulin region and peak IV in the β -globulin region.

Fig. 2 shows the patterns of electrophoresis of serum GP-DAP from normal subjects and patients with hepatic diseases. Total enzyme activity (IU) is shown by each electrophoretic pattern. Normal control sera showed mainly one peak at III. In contrast, 4 samples of sera from patients with chronic active hepatitis had increased total enzyme activity as well as abnormal electrophoretic patterns with high peaks at II and I as compared with peak III. Four samples of sera from patients with obstructive jaundice also showed abnormal peaks (mainly II and less at I) by electrophoresis.

Effect of Heat Treatment on the Pattern of Multiple Forms

The effect of heat treatment on the pattern of multiple forms of human serum GP-DAP was examined with a serum sample from a patient with chronic active hepatitis (Fig 3). The pattern of multiple forms of serum GP-DAP on cellulose acetate electrophoresis was completely stable when the serum was stored at 4°C. When a serum sample from a patient with hepatitis, which showed the four GP-DAP peaks clearly, was heated at 60°C for 30 min, peak I, peak II and peak III were decreased to similar extents by about 25% and peak IV disappeared completely. The result indicates that peaks I, II, and III are fairly heat-stable with similar stability but that peak IV is heat-labile.

Fig. 2 Electrophoretic patterns on cellulose acetate of human sera from 4 normal subjects, 4 patients with obstructive jaundice and 4 patients with chronic active hepatitis. The total activity (IU) is shown by each electrophoretic pattern.

Shifts of each Peak with Neuraminidase Treatment

Since GP-DAP in the pig kidney was reported as a glycoprotein containing 18.3% carbohydrates with about 0.7% sialic acid (3), the difference in the mobility of each multiple form in electrophoresis could be due to different contents in sialic acid. In order to examine this possibility, a human serum sample from a patient with chronic active hepatitis, which clearly showed the four peaks, was incubated with neuraminidase (0.86 IU) at 37°C for 2 h. As shown in Fig. 4, peak I and peak II shifted to the cathode, and two new peaks (peak I' and II') appeared between peak I and peak II and between peak III and peak IV. Peak III shifted to the position of peak IV, but peak IV did not shift to the cathode. Therefore, the apparent activity of peak IV increased markedly.

The serum shown in Fig. 4 was treated with neuraminidase (0.86 IU) at 37°C for various times, and the distance of shift to the cathode of each peak was plotted against time (Fig. 5). Assuming conversions of peak I to peak I', peak II to peak II', and peak III to peak IV, the shifts of each peak were completed within 60 min. The different extent of shifts during the neur-

Fig. 3 Effect of heat treatment on the pattern of multiple forms of human serum GP-DAP. A serum sample from a patient with chronic active hepatitis, which showed all the four enzyme peaks, was heated at 60°C for 30 min, and the electrophoretic patterns of GP-DAP activity were compared before and after the heat treatment. The arrow indicates the peak IV enzyme before heating which disappeared completely after heating.

Fig. 4 Effect of neuraminidase treatment on the pattern of multiple forms of human serum GP-DAP. A serum sample from a patient with chronic active hepatitis, which showed all the four enzyme peaks, was incubated with neuraminidase at 37°C for 2 h, and the electrophoretic patterns of GP-DAP activity were compared before and after the neuraminidase treatment.

Fig. 5 Distance of shift to the cathode of the peaks of GP-DAP activity in the serum for a patient with hepatitis shown in Fig. 4 after incubation with neuraminidase (0.36 IU) at 37°C for various times. Conversions of peak I to peak I', peak II to peak II', and peak III to peak IV are assumed.

aminidase incubation may reflect the gradual loss of and the resultant different contents of sialic acids in GP-DAP. Neuraminidase treatment did not affect the total enzyme activity at all.

DISCUSSION

We have shown four peaks of GP-DAP activity in human serum from patients with hepatitis by electrophoresis. Changes in the electrophoretic pattern during and after neuraminidase treatment strongly suggest different neuraminic acid contents in the enzymes corresponding to each peak. The different heat stability of each peak also suggests a stabilizing effect by neuraminic acid. However, this hypothesis must be proved chemically after complete purification of GP-DAP corresponding to the four peaks.

The present study suggests that the electrophoretic pattern of serum GP-DAP can be a diagnostic acid for liver diseases. The increase in peak I and peak II may be indicative of an active liver damage process. We previously reported that following chronic carbon tetrachloride intoxication in rats, the activity of GP-DAP increased in serum and decreased in the liver, suggesting that the enzyme is released from the liver in hepatic injury (6). It can be speculated that the released GP-DAP from the liver during the process of hepatic injury may have different sialic acid contents resulting in different electrophoretic mobility. In accordance with this hypothesis, Fukasawa *et al.* (4) reported that GP-DAP extracted from microsomal fractions of the pig kidney and liver showed an immunochemical identity but different pI-values, perhaps due to a higher content in sialic acid in the liver enzyme as a result of post-translational modification.

The usefulness of electrophoretic patterns of serum GP-DAP as a diagnostic aid, especially for liver diseases, remains to be further investigated.

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