Enzymological Review of Serum Leucine Aminopeptidase Isozymes

Takeshi WAJIMA
Department of the Clinical Pathology, Yamaguchi Medical School, Ube
(Received January 24, 1966)

L-leucyl-β-naphthylamide (LNA) is widely used in clinical chemistry and histochemistry as the coloring substrate for leucine aminopeptidase (LAP). However, LNA is also hydrolyzed by other enzymes besides LAP so that it is not necessarily specific for LAP[1,2]. Therefore, when only LNA hydrolysis is used as a guide, there is a possibility for enzymes other than LAP to be measured as LNP. Previously, under the assumption that the enzyme which hydrolyzes LNA is LAP, a study[3] was done by agar gel electrophoresis of human sera, urine and tissue homogenates, and the zymograms on agar plates were examined by histochemical techniques using LNA substrate. It was found[4] that sera of hepatobiliary disease patients demonstrate bands of LAP activity (LAP isozymes) in positions which differ from that in normal serum.

Moreover, CMC column chromatography also has shown 3 or 4 LAP active fractions. However, LAP isozymes which are found in pathological sera may possibly be some other enzymes which increases in hepatobiliary diseases besides LAP. That is, there is a question whether or not they are truly LAP isozymes. Therefore, in order to examine the nature of the enzymes (hereinafter to be called LAP) which hydrolyze LAN in the sera of healthy persons and patients with hepatobiliary disease, a study has been conducted on the LAP active fractions separated by CMC column chromatography with regard to the attitudes toward a variety of substrates, optimum pH, activators, and inhibitors. A similar study was made on the LAP active bands on the zymograms.

MATERIAL AND METHOD

The sera of 3 cases of hepatobiliary disease (one case of biliary cirrhosis, one case of cholelithiasis and one case of bile duct carcinoma) and 2 sera of apparently healthy persons were used.

CMC-column chromatography: After CMC (medium size, Sigma) is washed in water, it is regenerated by 1N-HCL and concentrated aqueous NH_3, and then suspended in 0.01 M-phosphate buffer, pH 5.0 until equilibration. A 60×1 cm glass tube is packed with this up to a height of 50 cm. 2 ml serum which had been dialyzed overnight against 0.01 M-phosphate buffer, pH 5.0 was added and

eluted with 0.01 M-phosphate buffer at a rate of 6–10 ml/h. at pH 5.0–8.0 using a linear gradiant system. The effluent was collected in 5 g fractions. The amount of protein was determined by absorbency at 280 mμ and the LAP activity was measured using LNA as the substrate by the Takenaka-Takahashi method[5].

For each LAP fraction, a study was made on 1) The degree of DL-alanyl-β-naphthylamide and L-cystine-β-naphthylamide hydrolysis (at pH 7.2, same concentration of substrate, all according to the Takenaka-Takahashi method); 2) The degree of LNA hydrolysis at pH 6.0 to 9.5; 3) The activation or inhibitory effect of addition of $MnCl_2$, $MgCl_2$, EDTA (all 10^{-3} M); and 4) Apparent Km.

Moreover, after LAP isozymes had been separated by the method previously reported, effects of $MnCl_2$, $MgCl_2$ and EDTA on LNA were determined from the degree of staining (the intensification or fading of the bands of activity) after immersion for 1 hour at 37°C in substrate mixture to which $MnCl_2$, $MgCl_2$ and EDTA had been added to LNA in proportions of 10^{-3} M. Controls for this study consisted of material to which neither activators nor inhibitors had been added.

RESULTS AND DISCUSSION

The LAP fractions obtained by CMC column chromatography (Figure 1) were studied for any difference in their enzymological nature. Observation on the degree of hydrolysis of LNA, ANA, and CNA revealed that the degree of hydrolysis of LNA and ANA was the same in each fraction. CNA hydolysis was only slight, but there were no differences in the action upon the substrates between each fraction. The activative effects of Mn^{++} and Mg^{++} as well as the

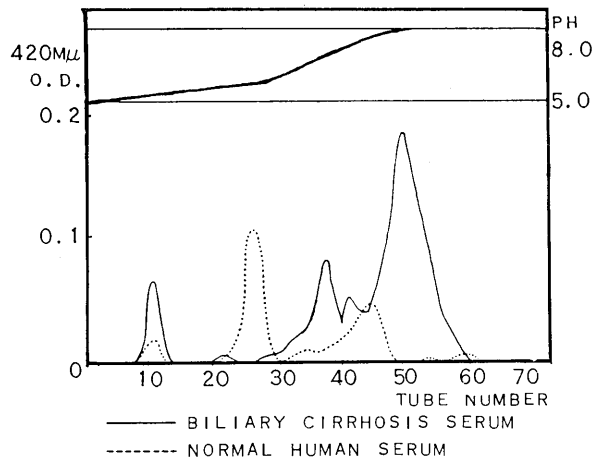

Fig. 1. Example of Fractionation of Serum LAP by CMC-Column Chromntography

Table 1. Enzymatic Characteristics of LAP Fractions by CMC-Column Chromatography.

Diseases \ Characteristics / Fractions		Hydrolysis			Activation effect		Inhibition effect	Km*
		LNA	ANA	CNA	$Mncl_2$	$Mgcl_2$	EDTA	10^{-3}M
Biliary cirrhosis	1	40%+	38%	0%	76%	49%	65%	2.3
	2	68	60	0	93	71	64	1.6
	3	100	100	100++	100	100	100	1.4
Cholelithiasis	1	63	58	0	80	71	77	3.7
	2	72	62	0	62	59	60	2.4
	3	100	100	100++	100	100	100	1.7
Biliary carcinoma	1	79	90	0	99	92	100	1.8
	2	100	100	100++	100	100	63	1.2
Healthy	1	100	100	0	100	100	95	1.2
	2	90	82	0	98	90	100	1.6

* Apparent Km; 0.1 M-phosphate buffer, pH 7.2, L-leucyl-β-naphthylamide, 37° C 30 min. incubation, 420 mμ absorbancy.

\+ Maximum activity is considered to be 100 % and the rest calculated accordingly.

++ Hydrolysis is minimum.

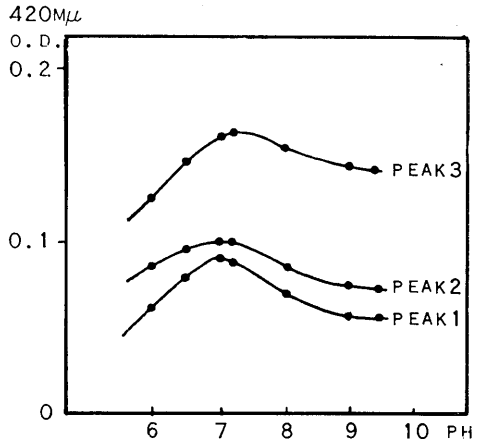

Fig. 2. Degree of L-leucyl-β-Naphthylamide Hydrolysis of Each Fraction at Different PH Levels

inhibitory effects of EDTA showed some differences among the various fractions. The apparent Km showed almost similar values (Table 1). Moreover, the optimum pH was also close to 7.2 (Figure 2). Although it is impossible to consider these fractions as being identical, it can be said that they possess extremely similar properties.

On the zymogram, Mn^{++} and Mg^{++} slightly intensified the color of each activity band, but no differences were found in the color among the isozymes. EDTA caused a similar degree of weakening of the color of all isozyme bands.

The sera of patients with hepatobiliary diseases have been reported to show 2 or 3 LAP bands when LNA is used as the substrate even with different separation methods such as starch gel,[6,7] filter paper[8] and cellulose acetate membrane[9] electrophoresis. Kowlesar et al.[6,7] have presumed this to be the presence of

LAP isozyme and we also feel this is LAP isozyme. Akedo et al.[10] also have found 2 or 3 LAP isozyme bands in pathological sera, but they denied the presence of LAP isozyme because other kinds of enzyme besides LAP can hydrolyze the substrate since LNA has low specificity.

Patterson et al.[11] have reported that when LAP which hydrolyze leucylamide (LA) and the enzymes which hydrolyze LNA are separated by DEAE column chromatography of the serum, the LAP which hydrolyses LA specifically hydrolyzes LNA, too, and is strongly activated by Mn^{++} and Mg^{++} as well as having the optimum pH of 9.0. On the other hand, the enzymes which hydrolyze LNA hardly hydrolyze LA at all and are not activated by Mn^{++} and Mg^{++} as well as having an optimum pH of close to 7.2. Therefore, it was said that the two are different. We have not used LA as the substrate so that we could not ascertain whether what has been considered to be so-called LAP should be classified as an LNA hydrolyzing enzyme rather than LAP. However, since attitudes of the various LAP fractions toward substrate hydrolysis, optimum pH as well as against activators and inhibitors are nearly alike, it is difficult to consider that LNA hydrolysis had been by some enzyme other than LAP.

CONCLUSION AND SUMMARY

The serum of normal persons and patients of hepatobiliary disease showed hardly any difference in enzymological properties (degree of hydrolysis of substrate, attitude toward optinum pH, $MnCl_2$, $MgCl_2$ and EDTA) between fractions having leucine aminopeptidase (LAP) activity causing hydrolysis of L-leucyl-β-naphthylamide. Therefore, the group of enzymes causing hydrolysis of L-leucyl-β-naphthylamide may be considered to be LAP isozymes.

Acknowledgement: The author wishes to thank Professor S. Shibata and Associate Professor H. Takahashi for constant interest and guidance in this study.

REFERENCES

1. Behal, F.J., Hamilton, R.D. Kanavage, C.B., & Kelly, E.C.: Studies on aminopeptidases in human blood. *Arch. Biochen.*, **100**: 308-312, 1963
2. O'Connel, W., & Winzler, R.J.: Studies on an enzymes in human serum which hydrolyzes L-leucy-β-naphthylamide. *Cancer Res.*, **23**: 78-83, 1963
3. Wajima, T., & Takahashi, H.: An agar-gel electrophoretic method for the detection of leucine aminopeptidase isozymes. *Medicine & Biology*, **68**: 101-103, 1964
4. Wajima, T.: Serum leucine aminopeptidase isozymes in diseases and pregnancy, with special reference to hepatobiliary diseases. *Medicine & Biology*, **70**: 15-18, 1965
5. Takenaka, M., & Takahashi, H.: A new method for the determination of serum leucine aminopeptidase. *Medicine & Biology*, **65**: 74-77, 1962
6. Kowlessar, O.D., Haeffner, L.J., & Sleisenger, M.H.: Localization of leucine aminopep-

tidase in serum and body fluids by starch-gel electrophoresis. *J. Clin. Invest.*, **39**: 671-675, 1960

7. Schobel, B., & Weiwalka, F.: Die heterogenität der Leucinoaminopeptidase. Untersuchungen von Organen und Serum bei Leber Gallen und Pankreaserkrankungen. *Klin. Wschr.*, **40**: 1048-1056, 1962
8. Smith, E.E., Pinada, E.P., & Rutenbeurg, A.H.: Localization of serum leucine aminopeptidase activity by paper electrophoresis. *Proc. Soc. Exp. Biol. Med.*, **110**: 683-687, 1962
9. Meade, B.W., & Rosalki, S.B.: Localization of leucine aminopeptidase isoenzymes. *J. Clin. Path.*, **17**: 61-63, 1964
10. Akedo, H., Inui, Y., & Hagihara, H.: Leucine aminopeptidase and its isozyme. *Nippon Rinshyo*, **108**: 829-834, 1964
11. Patterson, E.K., Hsias, S.H. & Keppel, A.: Studies on dipeptidases and aminopeptidases. I. Distinction between leucine aminopeptidase and enzymes that hydrolyze L-leucyl-β-naphtylamide. *J. Biol. Chem.*, **238**: 3611-3620, 1963