Detection of Leucine Aminopeptipase Isozyme by Agar-Gel Electrophoresis; with Reference to Various Diseases and Pregnancy

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Leucine aminopeptidase (LAP) is a proteolytic enzyme which hydrolyzes L-leucyl peptides. LAP is mostly contained in the liver, duodenum, pancreas, and kidneys but also detected from the serum, bile, duodenal juice, and urine.[1] A number of reports on the isozyme of this enzyme have been published. Lawrence et al.[2] have detected 1–2 fractions (fast α_2, slow α_2) in starch gel electrophoresis of normal human serum LAP and Schobel et al.[3] also have found 1–2 fractions (postalbumin, β-globulin) using the same method but Kowlessar et al.[4] have found only 1 fraction (postalbumin or fast $\alpha_2$postalbumin). According to Smith et al.[5] paper electrophoresis revealed LAP activity in only 1 fraction (α_1-globulin). It is reported that LAP is separated into 4 fractions (α_1,-β_1,-β_2-and β_3-globulins) on DEAE-cellulose column chromatography.[6] Thus, the number and location of serum LAP isozymes are different accrding to investigators. We previously reported that the use of agar gel electrophoresis is practical for the isolation of isozymes in serum enzymes[7] and we now have applied this technique to isolation of serum LAP isozyme in combination with the method used for demonstrating LAP in tissues by which serum LAP may be easily demonstrated on agar plate. The greatest characteristic of this method is the ease of operation and many samples can be processed in a short time. If isozyme detection is to be done as a clinical test, the method must be simple.

MATERIAL AND METHOD

The material for study consisted of sera from apparently normal persons including 20 adult males, 20 adult females, and 10 infants (6 males, 4 females), and bile fluid (of 2 cases) as well as homogenates (water) of the liver, duodenum, pancreas, and kidneys from 2 autopsy cases obtained within 6 to 8 hours after death.

A total of 248 cases were studied including 200 sick patients (76 cases of hepatitis, 8 cases of liver cirrhosis, 6 cases of hepatic carcinoma, 6 cases of metastatic hepatic carcinoma, 17 cases of cholelithiasis and cholecystitis, 9 cases of malignant obstruction of bile duct, 2 cases of congenital obstruction of bile duct, 13 cases of carcinoma of digestive organ, 8 cases of genital carcinoma, 8 cases of

tumor of urinary organ, 14 cases of diabetes, 8 cases of pulmonary tuberculosis, 5 cases of chronic nephritis, and 21 other cases) and 48 pregnant women.

Electrophoresis of sera (or supernatant fluids of visceral homogenates) from 4 or 5 persons was done by Shibata-Iuchi's[8] agar electrophoresis method (150 volts, 50–60 mA, for 30 minutes) after which the agar plates were placed in the substrate mixture for 1 hour at 37°C. After the reaction, the plates were taken out and washed in running water 2 or 3 times.

The agar plates had been prepared from Difco Noble agar which was heat dissolved in veronal buffer solution (pH 8.9, μ 0.05) in proportions of 0.75%, and spread on glass slides.

To prepare the substrate mixture, 2 ml of L-leucyl β-naphthy-amide hydrochloride (40 mg/dl) and 20 mg of fast garent GBC were mixed with 10 ml of 0.1 M phosphoric acid buffer solution and filtered.

After electrophoresis, protein stain (amidoblack 10B) was performed on the agar plates to determine the position of the isolated LAP isozymes.

RESULTS AND DISCUSSION

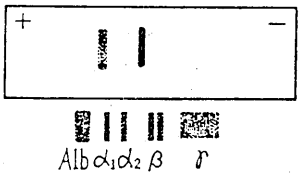

Fig. 1. Leucine Aminope ptidase (LAP) Isozyme in Normal Human Serum.

The upper row is isozyme pattern and the lower is protein fractions by amido black 10 B stain.

I. LAP isozyme of normal human serum

The site of LAP activity appears as an orange-colored band against a pale yellow background. In normal human serum, only a single orange-red band appears corresponding to the α_1-globulin fraction of serum protein (in 22 out of 50 cases) or at the intermediate site between albumin and α_1-globulin (in 28 out of 50 cases) (Figure 1).

No difference was noted in LAP isozyme pattern by age or sex.

Liver hemogenate demonstrated LAP activity bands at locations corresponding to albumin and postalbumin of serum protein and the pancreas at the location of α_2-and β-globulin fractions, while both the duodenum and kidney demonstrated only one LAP band at the position of α_1-globulin fraction. Bile showed LAP activity bands in the α_1-and α_2-glbulin fractions. In homogenates of all these organs, slight LAP activity is present at the site of placement of material (Figure 2).

II. Serum leucine aminpeptidase isozyme in various diseases and pregnancy

In normal human serum, only a single band of activity is noted at a position between albumin and α_1-globulin (Aα_1) or in the position of α_1 globulin (α_1). In morbid serum. however, new bands of LAP activity are detected in positions

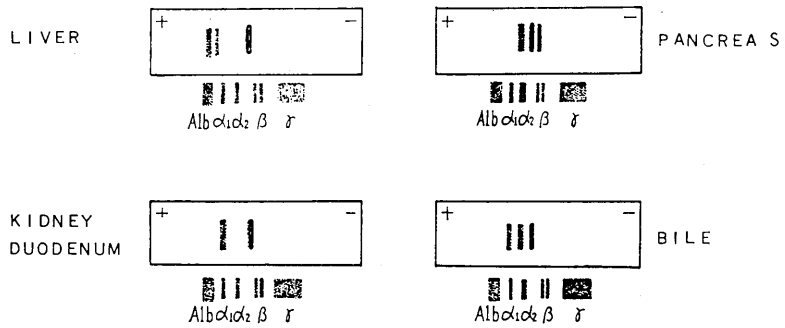

Fig. 2. LAP Isozyme Pattern of Human Tissues and Bile
The upper row is isozyme pattern and the lower is protein fractions by amidoblack 10 B stain.

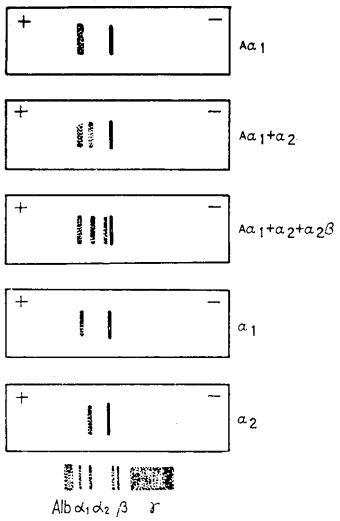

Fig. 3. Serum LAP Isozyme Pattern in Various Diseases and Pregnancy

The lowest row indicates of serum protein fractions by amidoblack 10 B stain.

which cannot be seen in normal human serum. In other words, bands of LAP activity are detected at the position of α_2 globulin (α_2) and at between α_2 globulin and β globulin ($\alpha_2\beta$) so that there are 2 or 3 bands. In other words, morbid serum LAP zymogram shows 3 types; $A\alpha_1$, $A\alpha_2+\alpha_2$, and $A\alpha_1+\alpha_2+\alpha_2\beta$. (Figure 3) As shown in Table 1, the cases selected were classified into 3 groups, namely, diseases of the liver and bile duct, carcinomas (other than those of the liver and bile duct), and non-carcinomatous diseases. The $A\alpha_1+\alpha_2$ type and $A\alpha_1+\alpha_2+\alpha_2\beta$ type detected were almost all limited to diseases of the liver and bile duct. In other diseases, only the $A\alpha_1+\alpha_2$ type was found in 4 cases of diabetes and 2 cases of chronic nephritis (Table 1). Among the diseases of the liver and bile duct $A\alpha_1+\alpha_2$ type was found in 27 (35 %) out of 76 cases of hepatitis, some of the cases of primary hepatic carcinoma, and most of the cases of metastatic hepatic carcinoma, cholelithiasis, and malignant obstruction of bile duct. The $A\alpha_1+\alpha_2+\alpha_3$ type was limited to cholelithiasis and malignant obstruction of bile duct among our cases. Cases of diabetes and chronic nephritis of $A\alpha_1+\alpha_2$ type were reviewed and it was found that some had complications with hepatitis, etc. as shown in Table 2, but as far as the examination of the remaining cases is concerned, no evidence of disturbances of the

Table 1. Serum LAP Isozyme Pattern in Various Diseases.

Diseases		Cases	$A\alpha_1$ type	$A\alpha_1+\alpha_2$ type	$A\alpha_1+\alpha_2++\alpha_2\beta$ type
Hepatobiliary diseases	Hepatitis	76	49	27	
	Liver cirrhosis	8	7	1	
	Hepatic carcinoma	6	4	2	
	Metastatic hepatic carcinoma	6		6	
	Cholelithiasis	16	1	6	9
	Cholecystitis	1	1		
	Malignant biliary obstruction	9		3	6
	Congenital biliary obstruction	2		2	
Carcinomas*	Digestive tract	13	13		
	Genital tract	8	8		
	Urinary tract	8	8		
Non-carcinomatous diseases	Chronic nephritis	5	3	2	
	Diabetes mellitus	14	10	4	
	Pulmonary tuberculosis	8	8		
	Others	21	21		

* Other than those of the liver and bile duct.

Table 2. Liver function tests of the cases where $A\alpha_1+\alpha_2$ type was detected. (Excluding hepatobiliary diseases)

	LAP*	Icteric index (concentration of bilirubin)	GPT+	A/G	Remarks
Diabetes mellitus	29.0	10 (1.5)	19.0	0.86	with chronic hepatitis
	30.0	8 (1.0)	4.5	1.12	with chronic hepatitis
	50.7	10 (2.0)	10.7	0.97	
	52	4	15.0	1.14	
Chronic nephritis	36	7	2.3	0.29	
	40	5	1.0	0.38	

* Normal range <20 units + Normal range <10 units

liver and bile duct could be found.

On the basis of the above findings, the concurrent appearance of LAP isozyme α_2 or LAP isozyme $\alpha_2\beta$ with LAP isozyme $A\alpha_1$ can be considered to be almost characteristic to diseases of the liver and bile duct. Further detailed studies of liver and biliary diseases reveal that among the cases mainly having impairment of the liver parenchyma such as hepatitis, liver cirrhosis, and hepatic carcinoma the majority are the $A\alpha_1$ type as in the case of normal human serum. On the

contrary, most of the cases of cholelithiasis and malignant obstruction of bile duct show a zymogram of the $A\alpha_1+\alpha_2$ type (2 bands of activity) or $A\alpha_1+\alpha_2+\alpha_3$ type (3 bands of activity). In other words, it appears that in the cases showing hepatobiliary obstruction bands of LAP activity which are not seen in normal human serum appear so that there is an increase in number of bands. The $A\alpha_1+\alpha_2$ type was seen in 1 out of 8 cases of liver cirrhosis, while it was noted in 27 out of 76 cases of hepatitis. This suggests that even among diseases in which there is mainly disturbance of the liver parenchyma the activity bands increase in number when there is impairment of the bile flow. In all cases of hepatitis in which isozyme α_2 had appeared, elevation of serum alkaline phosphatase was noted.

Schobel et al.[3] and Kowlessar et al.[9] report that by starch gel electrophoresis, 2 or 3 LAP components which are not seen in normal human serum appear in sera of patients with diseases of the liver and bile duct. Because of the difference in method, it is difficult to compare the results in detail, but their findings agree with our results.

In pregnant woman, the serum LAP zymogram in the first half of preghacy (up to the end of the 5th month of pregnancy) shows a band of activity in the position between albumin and α_1-globulin ($A\alpha_1$) or at the position of α_1-globulin (α_1) as in normal human serum. In the latter half of pregnancy (after the 5th month), however, although there is a single band of activity, its position is shifted to the cathode side and appears at the position of α_2-globulin, showing a unique pattern (α_2 type). This conversion to the α_2 type is consistent with the time when serum LAP activity begins to rise in pregnancy.

In view of the fact that in placental extract and cord blood, the LAP activity band is detected corresponding to the position of α_2-globulin, it seems that LAP isozyme α_2 which is found in pregnant woman's serum is related to placental function. In pregnancy, the activity band does not increase in number but only changes in position and becoms α_2 type. However, the reason why isozyme $A\alpha_2$ is not detected only in the latter half of pregnancy and the difference in the enzymological nature of this isozyme α_2 and isozyme α_2 that appear in diseases of the liver and bile ducts are unknown at present.

CONCLUSION AND SUMMARY

A method for detecting leucine aminopeptidase (LAP) isozyme in human serum using agar gel electrophoresis was developed. This method excels other methods heretofore in use in simplicity and efficiency.

By this method LAP isozyme can be detected in normal human serum in the α_1-globulin fraction or in the position intermediate between albumin and α_1-globulin of serum protein. LAP activity bands can be detected in positions corre-

sponding to albumin and postalbumin of serum protein with liver homogenate, in positions corresponding to α_2-globulin and β-globulin fractions with pancreatic homogenate, in a position corresponding to α_1 globulin fraction with duodenal and renal homogenates, and in positions corresponding to α_1-globulin and α_2-globulin fractions with bile homogenate.

The serum leucine aminopepidase zymograms of 200 cases of various diseases, including 123 cases of hepatobiliary diseases, and 48 pregnant women were observed. LAP zymograms of normal human serum are of two types, namely $A\alpha_1$ type and α_1 type, but in hepatobiliary diseases 1 or 2 other LAP activity bands appear so that zymograms are different, i.e., $A\alpha_1 + \alpha_1$ type or $A\alpha_1 + \alpha_2 + \alpha_2\beta$ type. LAP zymograms were mainly $A\alpha_1$ type in such conditions as hepatitis and liver cancer which are for the most part distubances of the liver parenchyma, but almost all hepatobiliary obstruction diseases due to cholelithiasis or cancer presented the $A\alpha_1 + \alpha_2$ and $A\alpha_1 + \alpha_2 + \alpha_2\beta$ types. The $A\alpha_1 + \alpha_2 + \alpha_2\beta$ type is peculiar to bile duct obstruction. The serum of women in the latter half of pregnancy (after the sixth month) showed just one activity band (α_2 type) in a position corresponding to the position of α_2 globulin in the serum.

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