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ELASTASE-TYPE ACTIVITY ASSOCIATED WITH HIGH DENSITY LIPOPROTEINS IN HUMAN SERUM

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SUMMARY - Elastase-type activities were found associated with lipoprotein fractions in human serum. A metallo-protease hydrolysing Suc(Ala)$_3$ PNA and soluble elastin peptides was isolated from apoHDL by Sepharose-elastin chromatography. Polyacrylamide gel electrophoresis and immunoelectrophoresis indicated that this elastase-type activity was associated with apoA$_1$.

INTRODUCTION

In recent years, elastases have been isolated and characterized from many cell types (1, 2, 3, 4). The imbalance between such protease levels and inhibitors in tissues or body fluids results in connective tissue destruction (5, 6). In spite of the presence of high levels of inhibitors e.g. α_1-antitrypsin and α_2-macroglobulin in human serum, we previously demonstrated that this physiological fluid does contain elastase-type activities as determined on specific synthetic alanine peptide and/or soluble elastin substrates (7, 8).

We present now evidence showing that part of the elastase-type activity in human serum is associated with purified lipoproteins.

MATERIALS AND METHODS

Elastase (EC 3447), α-N-benzoyl-DL-arginine paranitroanilide HCl (BAPNA), benzoyl tyrosine-paranitroanilide HCl (BTPNA), diisopropylflurophosphate (Dipf), phenylmethane-sulfonyl fluoride (Pmsf), 1-10-phenanthroline and α_1-antitrypsin (A 9024) were purchased from Sigma Chemical (St. Louis, Miss. USA) ; succinoyl-trialanine-paranitroanilide (Suc(Ala)$_3$PNA) came from Precibio (Rueil-Malmaison, France). Other reagents are of highest purity commercially available.

Lipoproteins from human sera were prepared by sequential centrifugation in presence of potassium bromide (9) and delipidation of high density lipoproteins 1.063<d<1.21 was performed according to Scanu and Edelstein (10).

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Determination of enzyme activities. Elastase activities were determined on $Suc(Ala)_3PNA$ according to Bieth et al. (11). However due to low enzyme levels in lipoprotein fractions longer incubation periods were required. Appropriate blanks were included to take into account spontaneous hydrolysis of the substrate ; linear standard curves were obtained up to 24 hours of incubation.

Elastinolytic activities were determined using either ^3H-labeled ligamentum nuchae insoluble elastin (specific activity = $1.85\ 10^5$ cpm/mg) or ^3H-labeled kappa-elastin Sepharose beads (specific activity = $1.4\ 10^6$ cpm/mg) (12).

Polyacrylamide gel electrophoresis were carried out according to Davis et al. (13) and immunoelectrophoresis using anti-HDL or anti-purified apoproteins were performed as previously described (14). Proteins were evaluated according to Lowry et al. using bovine serum albumin as standard (15).

RESULTS

Enzyme activity hydrolysing $Suc(Ala)_3PNA$ could be demonstrated in all lipoprotein fractions in human sera (Table I). HDL ($1.063<d<1.21$) associated elastase-type activity accounted for 4-30 per cent of the total elastase activity of human sera. Such enzyme levels determined in 7 different HDL preparations corresponded to a mean of 22.4 (standard deviation : 8,9) ng of porcine pancreatic elastase equivalents per mg of protein. Following delipidation of HDL with ethanol diethyleter solvents, a detectable elastase activity remained associated with the apoproteins equivalent to 6.7 ng elastase equivalents per mg of apoprotein (standard deviation : 2,5). The presence of neutral detergents (Brij 35) as well as sodium dodecyl sulphate in assay buffer considerably enhanced the activity detected in HDL or apoHDL preparations. Maximum effect was observed with 0.2 per cent SDS in 100 mM Tris-HCl buffer, 5 mM $CaCl_2$, 0.02 per cent NaN_3 pH 8.0 (Fig. 1). Similar SDS concentration completely inactivated the activity of porcine pancreatic elastase.

In order to control if pancreatic elastase can associate with circulating lipoproteins the following experiment was carried out. 100 µg of porcine pancreatic elastase (60 U/mg) were added per ml of human serum incubated 72 hours at 22°C and HDL and LDL fractions were isolated as before ; in three different human sera, the addition of exogenous elastase did not increase to any detectable extent the amount of elastase-type activity determined in the isolated HDL and LDL preparations. In such experiments, we were unable to demonstrate by immunological techniques (using a rabbit antiserum to porcine pancreatic elastase) the presence of pancreatic elastase associated with isolated HDL fractions. It appears therefore unlikely that the association of pancreatic elastase with the circulating lipoproteins could account for the above findings.

TABLE I

Elastase-type activities determined on $Suc(Ala)_3PNA$ of several lipoprotein fractions of human sera.

Specific activity was determined in the following conditions :
Buffer : 100 mM Tris-HCl, $CaCl_2$ 5 mM, Brij 35 0.1 %, NaN_3 0;02 %, pH 8.0.
Temperature : 37°C.
Substrate concentration : 2.5 mM.
Specific activity : nmole substrate hydrolysed per hour per mg of protein
% of total activity refers to the total elastase-type activity determined with the same substrate in the unfractionated sera.
1 µg of porcine pancreatic elastase (75 U/mg) hydrolyzed 245 nmoles of substrate in 1 hour under similar experimental conditions.

The results represented the mean of 3 separate determinations.
nd = not determined.

PREPARATIONS (serum number)	HDL 1.063<d<1.06		LDL 1.006<d<1.063		VLDL d<1.006		Residual serum	
	Spec.Act.	% of total	Spec.Act.	% of total	Spec.Act.	% of total	Spec.Act.	% of total
1	17.2	5.9	9.42	3.5	11.8	3.3	0.625	87.3
2	14.4	23.0	11.8	0.4	73.6	7.6	0.425	69.0
3	3.6	4.0	2.1	0.3	16.8	3.6	0.595	92.1
4	1.0	nd	0.56	nd	2.23	nd	nd	nd

The apoHDL associated enzyme was characterized by its inhibition profile. Five different preparations were analyzed. The elastase-type activity recovered in 4 of them presented the characteristics of a metallo-protease (Table 2). It was inhibitable by metal chelating agents and insensitive to active site directed serine-protease inhibitors. Furthermore, $alpha_1$-antitrypsin at concentrations as high as 0.1 mg/ml did not decrease to any appreciable extent the activity of the enzyme. In one apoHDL preparation however some Pmsf sensitive activity was also detected suggesting the presence in this preparation of two different types of activities hydrolysing $Suc(Ala)_3PNA$.

The isolation of the HDL-associated elastase-activity was undertaken using mixed bed insoluble elastin Sepharose 2B columns. 25 mg of apoHDL were loaded on to the column and eluted with 40 mM Tris-HCl, 5 mM $CaCl_2$, 0.02 % NaN_3, pH 8.0. No elastase activity was recovered in the major unretarded protein fraction (Fig. 2). Elastase-type activity was eluted by increasing the ionic strength of the eluant. In similar experimental conditions, the elastase activity associated with native HDL preparations passed unretarded on this column. The activity hydrolysing $Suc(Ala)_3PNA$ was eluted as two main fractions designated as fractions A and B. Fraction B contained only low amounts of protein and its enzyme activity appeared unstable. For these reasons only the elastase peak eluted with 0.1 molar NaCl and designated as fraction A was further analysed. Its specific activity on $Suc(Ala)_3PNA$ was 115 fold the one

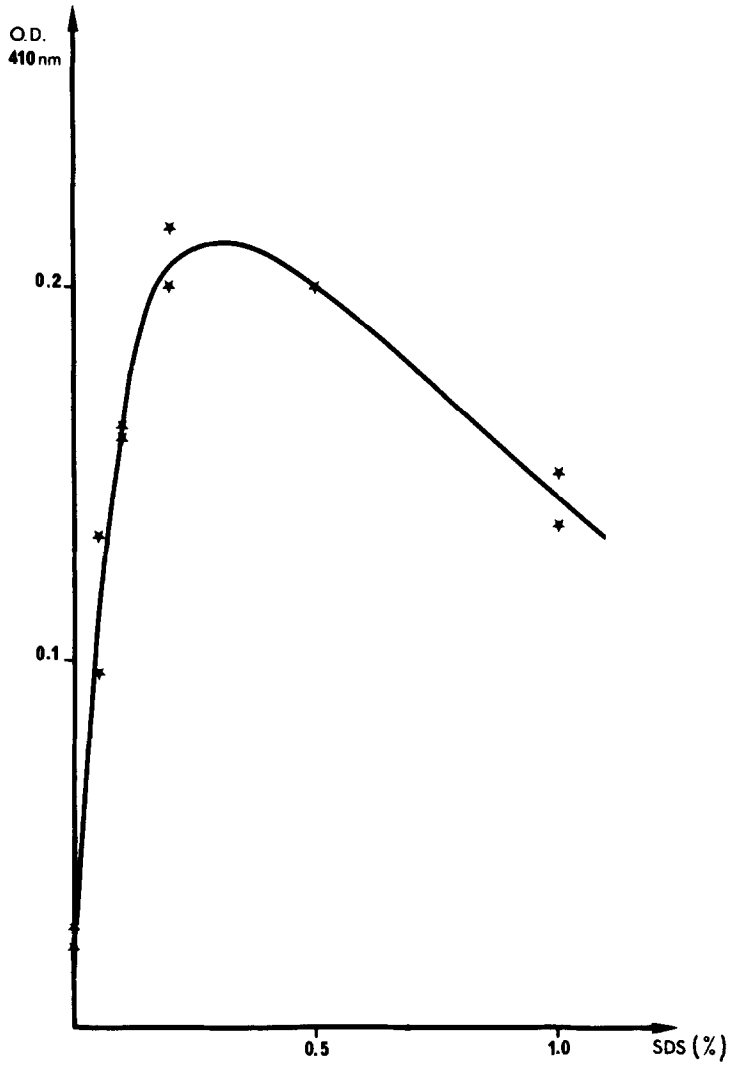

Figure 1 - Stimulation of elastase activity associated with ApoHDL by sodium dodecyl sulphate.
Assay buffer : 40 mM Tris HCl, 5 mM $CaCl_2$, 0.02 % NaN_3, pH 8.0
25 µg of ApoHDL.
Abscissa : final concentration of SDS in assay buffer.
Ordinates : variation in optical density at 410 nm after 18 hrs incubation at 37°C.

of the original apoHDL preparations ; it was able to cleave ^3H kappa elastin covalently linked to activated Sepharose 4B beads (Fig. 3), but had only a very low activity towards ^3H labeled insoluble elastin. We determined that 1 mg of fraction A-protein hydrolysed only 3 µg of insoluble elastin after 3 days of incubation at 37°C. Maximum activity on either $Suc(Ala)_3PNA$ and ^3H

TABLE II

Inhibition profile of the elastase-type enzyme associated with apoHDL.

Inhibitor (concentration)	per cent of residual activity
None	100
DipF (1 mM)	100
Pmsf (1 mM)	100
1-10-phenantroline (1 mM)	5
EDTA + dithiothreitol (1 mM each)	0
$CaCl_2$ (5 mM)	145
EDTA + $CaCl_2$ (1 mM) each)	93.4
α_1-antitrypsin (0.1 mg/ml)	100

ApoHDL (1.063<d<1.21) (350 µg) and inhibitor (at the indicated concentration) are incubated 1 hour at 20°C before determining the residual activity hydrolysing $Suc(Ala)_3PNA$.

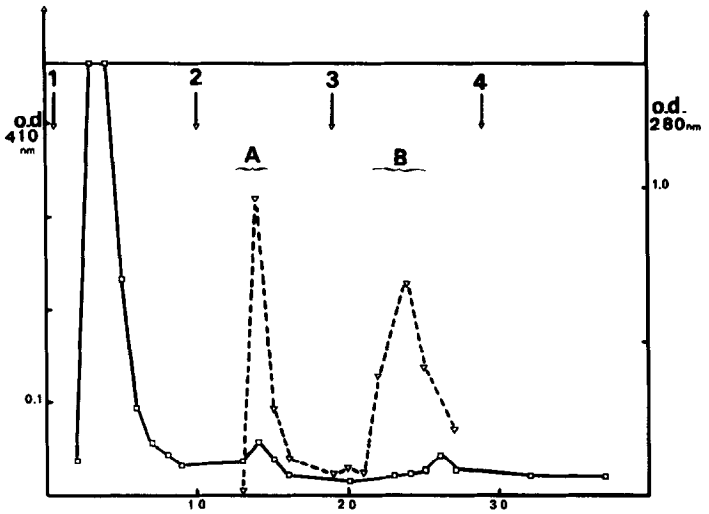

Figure 2 - Isolation of elastase-type protease associated with ApoHDL on a mixed bed ligamentum nuchae insoluble elastin-Sepharose 4B column (2x10 cm).
Insoluble elastin content : 40 mg.
Temperature : 37°C.
25 mg of ApoHDL were loaded on to the column.

Buffer : 1 40 mM Tris HCl, 5 mM $CaCl_2$, 0.02 % NaN_3, pH 8.0
2 " " " " " " " " , 0.1 M NaCl
3 " " " " " " " " , 0.5 M NaCl
4 " " " " " " " " , 2 M NaCl

Abscissa : fraction numbers (5 ml per fraction).
Ordinates : variation in optical density at 280 nm
activity hydrolysing $Suc(Ala)_3PNA$ 410 nm.

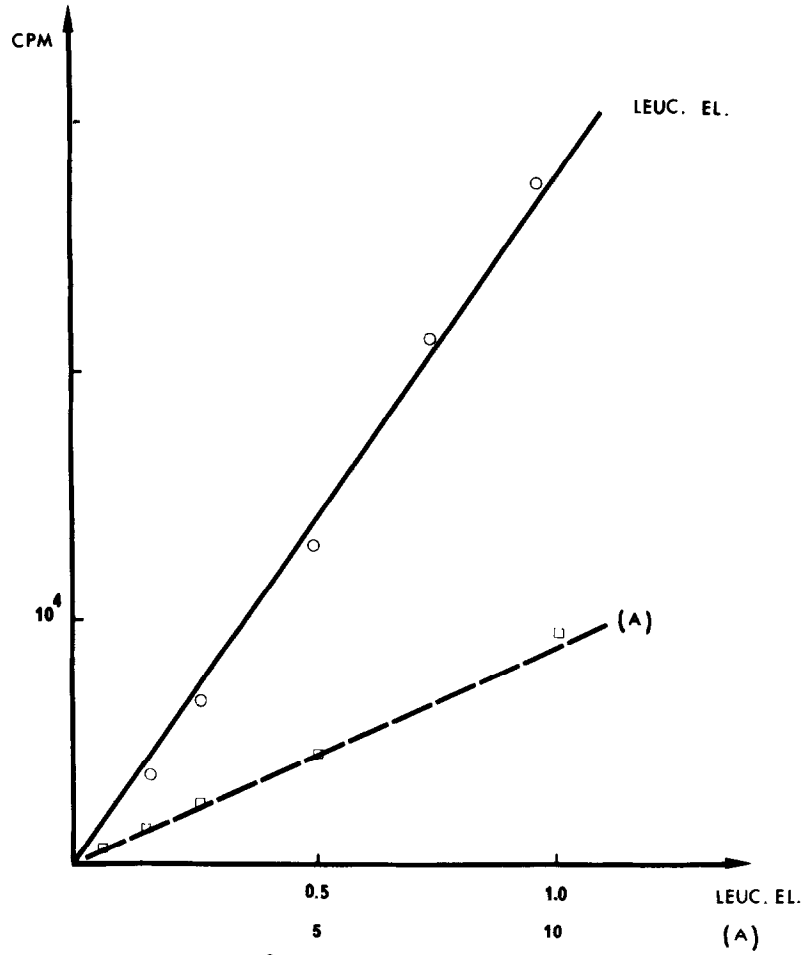

Figure 3 - Hydrolysis of ^3H kappa-elastin-Sepharose beads by leucocyte elastase and fraction A from ApoHDL.

Buffer : 100 mM Tris HCl, 5 mM $CaCl_2$, 0.1 % Brij 35, 0.02 % NaN_3, pH 8.0.
Substrate : 100 µl of kappa elastin-Sepharose beads, total activity 54000 cpm.
Temperature : 37°C.
After 24 hrs incubation at 37°C the tubes are centrifuged in an hematocrite centrifuge and the radioactivity determined in the supernatant in Instagel solution in a Packard scintillation counter.

Abscissa : µg of leucocyte elastase
µg of fraction A.
Ordinates : total radioactivity in the supernatant (cpm).

kappa elastin-Sepharose beads was found at pH 8.0 ; no activities hydrolysing BTPNA and BAPNA could be demonstrated. Together with the original apoHDL preparation, fraction A was analysed by PAGE electrophoresis and immunoelectrophoresis. The major protein present in this fraction appeared to be $apoA_1$ and slicing the PAGE gels for elastase determination using ^3H kappa elastin-

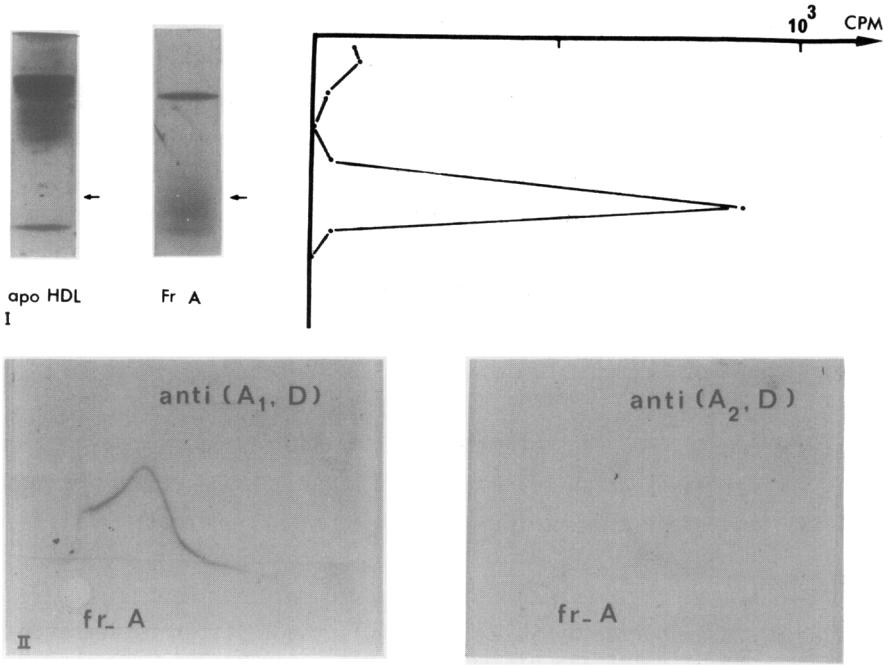

Figure 4 -
(I) Polyacrylamide gel electrophoresis (according to Davis)(13) of ApoHDL and isolated fraction A on mixed bed Sepharose-elastin column. Encard : unstained Coomassie Blue gels were sliced and the activity hydrolysing ^{3}H kappa elastin-Sepharose beads determined on each slice.

The arrows on the gels corresponds to the position of maximum activity hydrolysing ^{3}H kappa elastin-Sepharose beads.

(II) Immunoelectrophoresis of fraction A (20 µl, 0.5 mg/ml).
a. Antisera to $ApoA_1$ and ApoD
b. Antisera to $ApoA_2$ and ApoD.

Sepharose beads as substrate indicated that the elastase activity did not coincide with any of the protein bands and exhibited an anionic migration on polyacrylamide gel electrophoresis (Fig. 4).

DISCUSSION - CONCLUSION

Enzymatic activities towards $Suc(Ala)_3PNA$ and soluble elastin peptide were found associated with high density lipoproteins in human sera and part of it (20-60 per cent) remained associated with apoproteins after delipidation of HDL in organic solvants. The HDL associated enzyme could be inhibited by metal chelating agents and was insensitive to inhibition by α_1-antitrypsin. The partial purification of the HDL associated activity was attempted by the use of a mixed bed insoluble elastin-Sepharose column. $ApoA_1$ was coeluted with part of the elastase-type activity in this chromatographic procedure.

Our results suggest that a metal dependent elastase-type activity is strongly associated with apo high density lipoproteins. When native HDL was passed on a Sepharose-elastin column, enzyme activity was not retained on the column. Only when delipidated apo-HDL was used, could the elastase-activity be retained on the Sepharose-elastin column. The fact that $apoA_1$ was eluted together with the elastase-type activity when apo-HDL was chromatographed on elastin-Sepharose columns indicated that this apoprotein may be involved in the association of the metallo-proteinase with HDL. Finally pancreatic elastase added in vitro to human serum did not associate to any detectable extent to lipoproteins.

Our experiments show that circulating lipoproteins do carry with them elastase-type proteases which may penetrate with the lipoproteins into the arterial wall and exert a proteolytic effect on matrix and cell constituents.

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