DEMONSTRATION OF A PROTEASE-LIKE ACTIVITY IN HUMAN SERUM LOW DENSITY LIPOPROTEIN

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1. Introduction

There has been considerable controversy in the literature concerning the subunit molecular weight of the apoprotein B of human serum low density lipoprotein* [1, 2]. Employing various methods of approach, varying sizes of protein subunits were reported from 8000 to 250000 daltons [3–7]. During our attempts to understand the possible meaning of these discrepancies, we observed a phenomenon that may offer a possible explanation for the controversy. This paper presents the preliminary results of such a study.

2. Materials and methods

Blood, collected from normal healthy donors was obtained from the blood bank. Phenyl methyl sulfonyl fluoride (PMSF) was bought from Serva fine biochemicals, Heidelberg. Fluoroscamine (4-phenyl spiro [furan-2(3H) 1′-phthalan]-3, 3′-dione) was obtained from Hoffmann-La Roche, Basel, Switzerland. All other reagents were of analytical grade.

LDL_2 was prepared from the serum in presence of 0.2% sodium azide and 10^{-3} M EDTA throughout, by differential centrifugation as described earlier [8]. The LDL_2 fraction with a density between 1.025 and 1.050 g/ml was obtained and, after dialysis, further purified by column chromatography on hydroxyapatite [9]. The preparation of LDL so obtained was found to be pure by electrophoretic and immunological methods [10].

Analytical gel electrophoresis was performed as described by Davis [11]. Electrophoresis in presence of SDS was done according to the method of Weber and Osborn [12]. Protein was estimated by the method of Lowry et al. [13].

The measurement of amino groups by the fluorescence method was done, as described by Schwabe [14], using fluorescamine. In this experiment, samples of LDL_2 collected at different periods of storage, were delipidised with chloroform:methanol. The protein precipitates were then incubated with 5% SDS for 2 hr at 37°C in the presence of 0.01 M mercaptoethanol and 0.01 M PO_4 buffer, pH 7.0. The sample was divided into two equal parts in each case, one for the determination of protein content and the other for fluorescence measurement.

3. Results

3.1. *Subunit molecular weight of LDL_2*

When fresh LDL_2 (or frozen at –20°C) was subjected to SDS-gel electrophoresis a single protein band was obtained (fig. 1). Removal of lipid from the native LDL_2 [15] and electrophoresis of the apoprotein B also gives a single band identical in mobility to the native LDL_2. We name this pattern, obtained in SDS-gel electrophoresis immediately after the isolation of LDL_2, as 'primary pattern'.

Using several markers of known molecular weight

* *Abbreviations:* LDL_2 = low density lipoprotein (d = $1.025 < \rho < 1.050$ g/ml); SDS = sodium dodecyl sulphate; EDTA = ethylenediaminetetraacetic acid; PMSF = phenylmethylsulfonylfluoride.

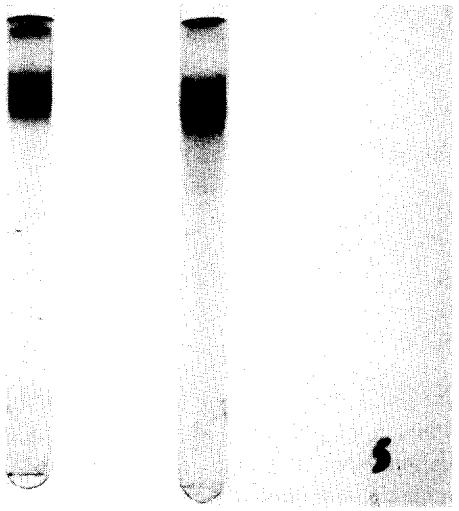

Fig. 1. SDS-gel electrophoresis of fresh LDL_2 and apo-LDL_2. Acrylamide gels (3.75%) were used and 20 μg of protein was applied on to the gels in each case and electrophoresis was performed at a current of 8 mA/gel.

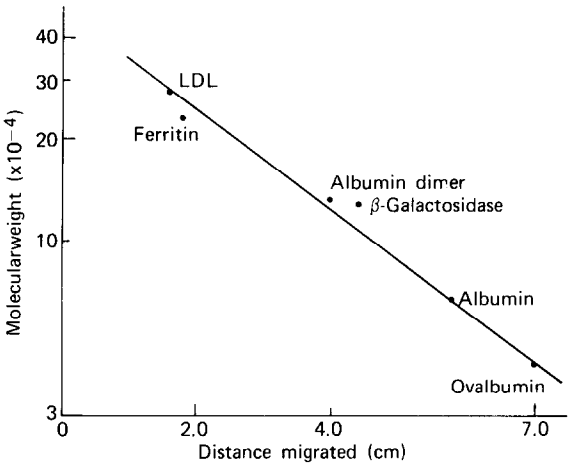

Fig. 2. Molecular weight determination of the LDL_2 subunit. Several markers of known molecular weight, as shown above, were run on different gels. In each case, 10 μg of the sample was applied. Other conditions were the same as for fig. 1.

and extrapolating the plot (fig. 2) a subunit molecular weight of 250000 – 270000 was obtained for the primary pattern LDL_2. This value is in agreement with the one reported recently by Smith et al. [5] for the subunit of human apo-LDL.

3.2. *Degradation of primary pattern*

If the native LDL that exhibits a 'primary pattern' in SDS-gel electrophoresis was stored at 4°C (in presence of sodium azide, 0.2%) for 5 days or longer, and then subjected to SDS-gel electrophoresis, a pattern completely different from that of the original was obtained; instead of a single band, six to seven bands of different migration velocities could be seen. This transformation was found to be gradual (fig. 3) with the time of storage of the preparation. We term this pattern as the 'secondary pattern'. The apparent breakdown of the B-protein of molecular weight around 250000 to smaller units with migration in SDS-polyacrylamide electrophoresis corresponding to other proteins with molecular weights ranging from 70000 – 150000 was found to be faster at the beginning of storage and slow down as the time of storage increases. The degradation of primary to the secondary

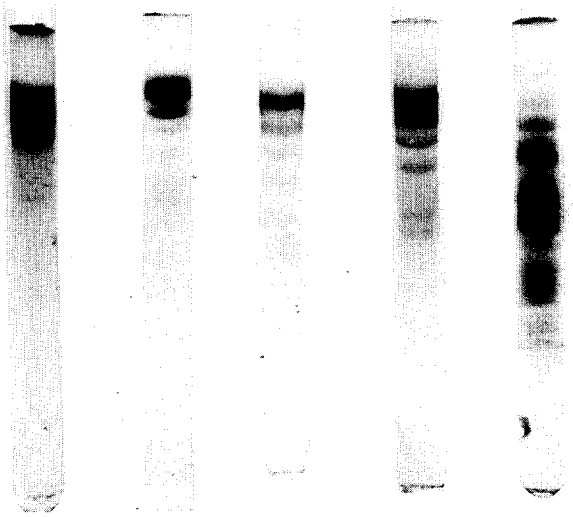

Fig. 3. Degradation of native LDL_2 into smaller subunits. Samples of LDL_2 after various periods of storage at 4°C were drawn and frozen. Electrophoresis in the presence of SDS using 3.75% gels was done after the final sample was drawn. The five gels above represent 0, 2, 4, 8 and 15 days of storage at 4°C.

pattern was also found to be temperature dependent.

In contrast to SDS-gel electrophoresis, analytical gel electrophoresis without SDS showed no significant difference in patterns between the fresh LDL_2 and the stored LDL_2.

3.3. *Inhibition by PMSF*

It was thought that the degradation of primary to the secondary pattern was probably due to a proteolytic enzyme contained in the LDL_2 preparation. This possibility was tested by storing LDL_2, under the same conditions as described, but in the presence of 10^{-3} M of phenylmethyl-sulfonyl fluoride (PMSF). As can be seen in fig. 4, the presence of PMSF inhibited the formation of secondary from the primary pattern, whereas in the case of the untreated LDL_2 preparation the usual degradation occurred. This prevention of degradation of LDL_2 as could be demonstrated by SDS-gel electrophoresis could also be brought about by diisopropyl fuorophosphate at concentrations of 5×10^{-4} M.

3.4. *Determination of protease activity of LDL_2-preparations*

The presence of a protease-like activity in human LDL_2-preparations was further demonstrated by an increase in the number of free amino groups. In this experiment the fluorescence obtained with fluorescamine was measured in the samples of LDL_2 stored at 4°C for various periods of time (fig. 5).

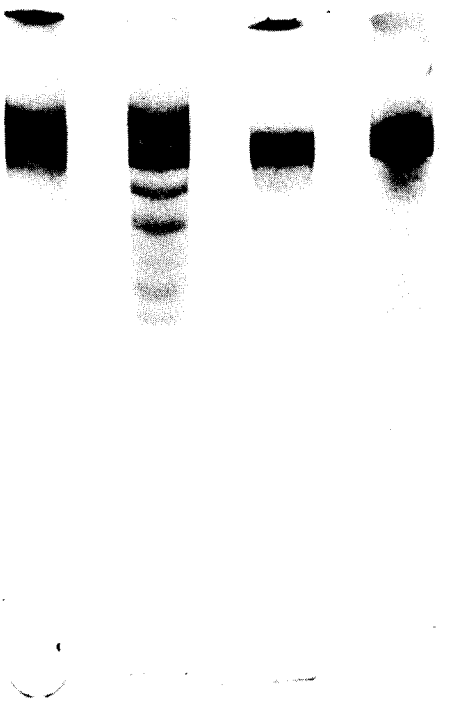

Fig. 4. Inhibition of the degradation of native LDL_2 by PMSF. PMSF (in *n*-propanol) was added to fresh native LDL_2 at a final concentration of 10^{-3} M and the sample was stored at 4°C. The four gels above represent, in order, the fresh sample, sample stored for 8 days, sample stored for 8 days with PMSF and the sample stored for 15 days with PMSF.

4. Discussion

The results presented in this report suggest the presence of a protease-like enzyme in human LDL_2-preparations. The delipidisation of fresh or stored LDL_2 gave in the SDS-electrophoresis the primary and secondary patterns respectively. This rules out the possibility that the formation of secondary pattern was due to the gradual leakage of lipid from LDL_2. The gradual degradation of the primary pattern was shown not to be due to bacterial contamination by storing the preparations in presence of antibiotics, in addition to the sodium azide. A likely reason for the observed degradation could be the presence of plasmin in the preparations; this possibility was ruled out since neither antiplasmin antiserum nor benzamidine hydrochloride proved inhibitory.

The native and 'degraded' LDL_2 showed essentially similar patterns in analytical gel electrophoresis in the absence of SDS. This suggests that the described proteolysis leaves the overall lipoprotein complex of the LDL_2 unaffected. Whether the single band obtained as the primary pattern with the fresh LDL-preparations is a single polypeptide chain or composed of several aggregates is yet to be demonstrated. It also ought to be shown whether the proteolytic activity present in the LDL_2-preparations is due to an exogenous or endogenous enzyme.

We feel that the observations described in this pa-

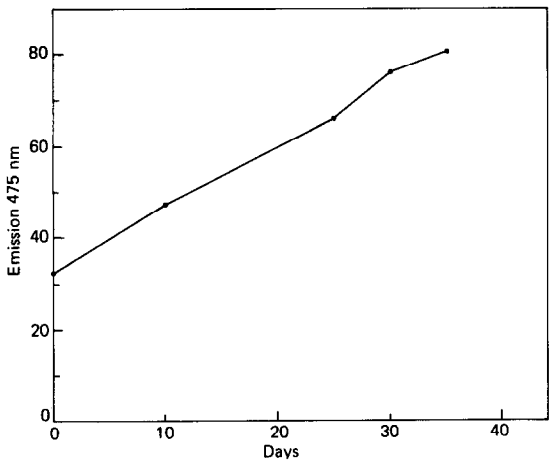

Fig. 5. Assay of proteolytic activity with fluorescamine. Samples of LDL, drawn at various periods of storage at 4°C, were delipidised with chloroform:methanol (2:1) and incubated with SDS at 37°C for 2 hr. After this period, 2 ml of 0.01 M potassium phosphate buffer, pH 6.8 were added, followed by 1 ml of fluorescamine (0.1 mg/ml acetone). The emission at 475 nm was measured and the values were calculated for 100 μg of protein and plotted.

per may offer a possible explanation for the varying degrees of dissociation into subunits that have been observed in many laboratories employing various methods. Further studies are needed to elicit the nature of this enzymic action and its possible physiological significance.

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