Characterisation and partial purification of an acid endopeptidase from human erythrocytes

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Summary: An acid endopeptidase was partially purified from normal human red blood cells by ammonium sulphate precipitation, gel filtration chromatography, ion exchange chromatography and HPLC. The enzyme had a molecular mass of around 45-54 kD as measured by SDS-PAGE. The endopeptidase does not belong to serine and metallo protease families and seems to be a cathepsin D-like enzyme.

Introduction

Proteolytic enzymes play a key role in the normal turnover of proteins. They provide a level of quality control for the cellular protein pool by eliminating abnormal and potentially toxic proteins. They are capable of processing misfolded and denatured proteins [1-3]. Study of RBC proteases have been the subject of extensive research due to their potential importance in the normal physiology of RBC and diseases characterised by haemolytic anaemia.

In both physiological and pathophysiological processes for example red cell ageing, heating, oxidant challenge due to reactive oxygen metabolites, haemolytic anaemia etc. RBC proteinases catalyse the degradation of submembraneous cytoskeleton proteins, transmembrane proteins, denatured alpha and beta globin chains that have been deprived of heme group and many other cytosolic denatured proteins [4-6]. Physiological consequences of these degradation processes due to proteolytic activity may be related to changes in red cell shape, deformability, and lateral movement of transmembrane proteins that are correlated with a number of well-known functions, including removal of senescent or damaged erythrocytes from the blood circulation [7-9].

Red blood cells contain many types of proteolytic systems including cathepsin D [10], cathepsin E [7], calcium activated neutral protease (calpain II) [11-13], molecular mass multi-catalytic proteosome [14], membrane bound serine protease [15], metallo proteases such as dipeptidylamino-peptidase and aminopeptidase [16], an alkaline protease with kininogenase activity [17].

Among aspartic proteases of erythrocyte cathepsin E is the well-characterised enzyme. Its purification, and properties for example molecular mass, kinetic parameters, N-terminal amino acid sequences were described by various workers [7]. On the contrary to this, cathepsin D, in spite of its potentially important contribution in intracellular protein degradation in both normal and diseased conditions, has not been studied in detail in human erythrocyte. Cathepsin D (EC 3.4.23.5) 42 kD, an intracellular aspartic proteinase with low pH optimum, function primarily in normal degradation of cellular proteins [18]. Cathepsin D are bilobal, monomeric molecules and synthesised as proenzyme form [19]. The proenzyme is catalytically inactive and auto-activation of procathepsin D is accomplished by a proteolytic removal of the N-terminal pro-part which is usually 44-50 amino acid

* This paper is dedicated in the memory of Prof. Zafar H. Zaidi, who passed away on January 7, 2001.

long [20,21]. It is a glycoprotein that resolves into several forms of similar molecular masses and different isoelectric points upon purification [22]. Among different isoforms 100 kD, 50 kD, 46 kD forms have been reported [23]. It has limited action against native proteins but considerable activity against denatured proteins at pH 3.5-5 [24]. It has been proposed that cathepsin D plays a role in pathological degradation of proteins in many diseases characterised by tissue degradation [25].

Due to increasing interest in role(s) of aspartic proteases (in conditions such as, cell ageing, oxidant challenge, and heating etc.) [7], we undertook the study on isolation and characterisation of cathepsin D from erythrocytes involved in intracellular protein degradation. This paper describes partial purification and some characterisation of a cathepsin D-like enzyme from human red blood cells.

Results And Discussion

For preparation of hemolysate, packed cells were washed, then lysed for obtaining hemolysate at neutral pH in the presence of PMSF and EDTA for the prevention of proteolysis of endogenous proteins. Metallo proteases such as aminopeptidase, dipeptidylaminopeptidase, erythrocyte-calpain II and serine type proteases reported to exist in red blood cells [6, 15-17] were inhibited by EDTA and PMSF. Cathepsin E, a major aspartic protease from erythrocyte [7] is inactive above pH 6, thus no proteolytic activities were observed as the isolation was carried out at pH 6.8.

Autolysis of hemolysate was performed at pH 3.5, which did not in itself increase acid endopeptidase activity, but it improved the result of subsequent ammonium sulphate fractionation by precipitation of the large amount of haemoglobin, which was removed from hemolysate by centrifugation. The pH of hemolysate was readjusted at 6.8 after autolysis. Autolyzed hemolysate was then used for ammonium sulphate fractionation up to 80% saturation. 80% pellets were then dissolved in 0.1 M ammonium acetate pH 6.8 and subjected to Sephadex G-75 GFC. The elution profile is shown in figure 1. Gel permeation chromatography of dissolved 80% pellets separated the contents in three peaks (fig. 1). Enzyme assay determinations of different fractions of GFC revealed that acid proteolytic activities are present in the fractions of major peak II co-eluting

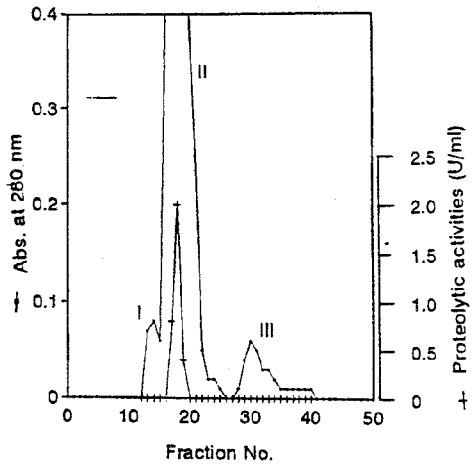

Fig. 1: Gel filtration chromatography of dissolved 80% ammonium sulphate pellets from hemolysate on Sephadex G-75 columns. Conditions as described in methods

with haemoglobin. Active fractions peak II of Sephadex G-75 GFC were collected for further purification of the enzyme.

Peak II of GFC was rechromatographed by DEAE Sephacel as shown in figure 2. In this step haemoglobin separated from endopeptidase by elution from the column, as unbounded fraction at pH 6.8. No proteolytic activities were detected in this fraction. Absorbed material was eluted from column with linear gradient of NaCl ranging from 0-0.5 M and yielded three peaks.

Endopeptidase assays of different fractions of anion exchange chromatography showed that activity were observed in earlier fractions of peak II with a peak of activity at the concentration of 0.1 M NaCl (fig 2).

SDS PAGE of the active pooled fractions of DEAE chromatography revealed multiple bands of apparently 34-66 kD molecular mass range (data not shown). Enzyme was further purified by high performance gel filtration chromatography using TSK G 2000 SW column, which separated the contents in three peaks. Peak II of HPLC showed enzymatic activity (fig 3). SDS PAGE of peak II was performed for determination of purity and molecular mass of the protein. After silver staining two intense bands (designated as B1 and B2) appeared on 12.5 %

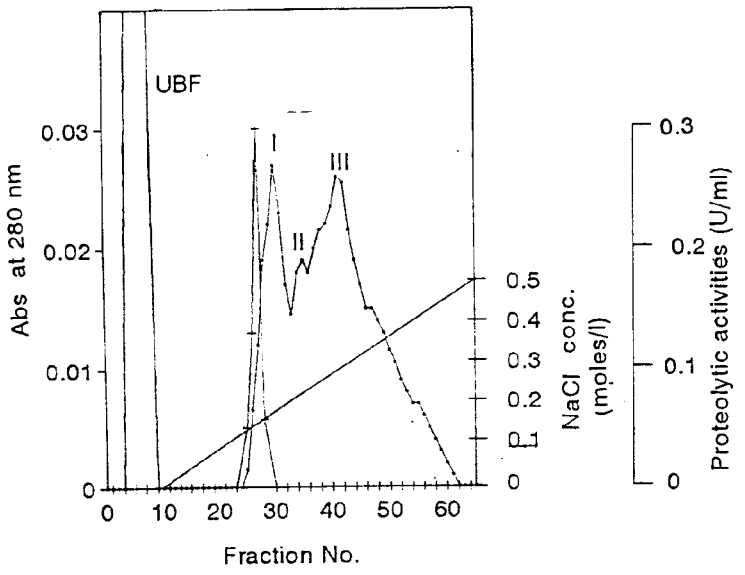

Fig. 2: Anion-exchange chromatography of Peak II obtained from GFC on a DEAE Sephacel column. Conditions are described in methods. UBF = Unbound fraction

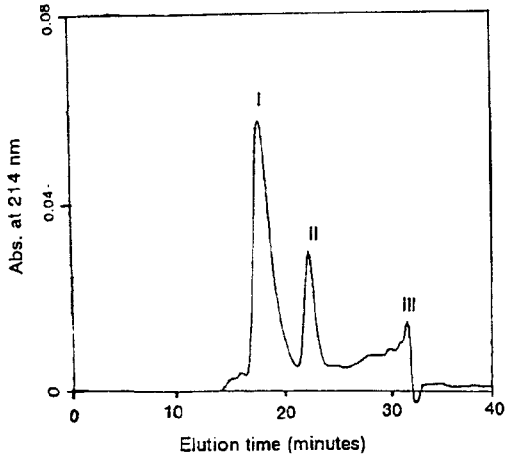

Fig. 3: High performance gel filtration chromatography of active pooled fractions of DEAE Sephacel ion exchange chromatography on TSK 2000 SW column in 0.1 M ammonium acetate, pH 6.8. Flow rate = 0.5ml/min. Peak II showed proteolytic activity.

gel (fig. 4). Molecular masses of B1 and B2 are 54 kD and 45 kD respectively. One of the bands corresponds to acid endopeptidase.

The present study describes the partial purification and some characterisation of an acid endopeptidase from human red blood cells. Enzyme was purified by ammonium sulphate precipitation, sephadex gel filtration chromatography followed by DEAE anion exchange chromatography and high performance gel filtration chromatography (HPGFC). Enzyme could not be purified to homogeneity and the electrophoresis of enzyme's peak of final step of purification revealed two bands of 54 and 45 kD.

The intracellular, soluble endopeptidase is optimally active at acidic pH region (data not shown). It is suspected by electrophoretic pattern (fig. 4) that the endopeptidase exists in monomeric form of 45 or 54 kD molecular mass. Enzyme could not be inhibited by PMSF (serine protease inhibitor) and EDTA (chelator sensitive protease inhibitor). These inhibitors are used in preparations during purification. All these evidences suggest that the endopeptidase corresponds to cathepsin D; Shuzo and Hiroshi [10] has also deduced its presence in the erythrocytes by analogy.

On the basis of these observations, we are justified in presuming that the 54 kD band-1 in figure 4 is the band of proendopeptidase and 45 kD band 2

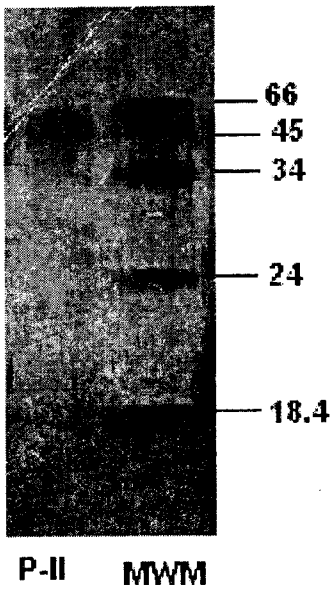

Fig. 4: SDS-PAGE of Peak-II of high performance gel filtration chromatography. Conditions are described in the methods. The marker proteins used were bovine albumin (66 kD), egg albumin (45 kD), pepsin (34 kD), trypsinogen (24 kD) and beta-lactoglobulin (18.4 kD).
MWM=Molecular wt. marker
P-II=Peak II
P-II=54 kD band-1
B2=45 kD band-2

corresponds to the active form of cathepsin D-like enzyme as removal of N-terminal pro-part converts it to the active form [20,21]. The appearance of two bands in the electrophoretic pattern of the active peak of HPLC is due to presence of both active and non-active forms. Further studies are in progress to purify, elucidate its structure and study its biological role in detail.

Experimental

Materials

10 days old normal human blood was obtained from the blood bank of Aga Khan University Hospital, Karachi, Pakistan. PMSF obtained from Sigma Chemical Company, BSA and EDTA obtained from Fluka, TCA from Aldrich chemical company, DEAE Sephacel and Sephadex G-75 from Pharmacia. All other chemicals were of analytical grade.

Acid endopeptidase assay

Acid endopeptidase activity was assayed at pH 3.5 in 0.1M sodium acetate buffer in a total volume of 400 µl of reaction mixture containing 150 µl of enzyme solution and 250 µl of substrate solution (1.4% BSA). After 1 hour of incubation at 37°C the enzymatic reaction was stopped by the addition of 10% TCA solution. The absorbance of liberated peptides was measured at 280 nm, with reference to the blank. One unit of endoprotease activity is defined to be the amount of enzyme required producing an absorbance change of 1.0 in a 1-cm cuvette, under the conditions of the assay.

Preparation of hemolysate

Human blood (50 ml) was centrifuged at 1500 xg for 15 min. to remove plasma. Packed cells were then washed three times in 5 volumes of 150 mM NaCl containing 20 mM sodium phosphate buffer pH 6.8 and centrifuged at 1500 x g to remove remaining plasma and buffy coat. Washed erythrocytes were than lysed in 5 volumes of 20 mM sodium phosphate buffer pH 6.8 containing 1mM EDTA and 0.2 mM PMSF. Endopeptidase assay of hemolysate was performed.

Autolysis

The pH of hemolysate obtained by above was adjusted to pH 3.5 with 1M sodium phosphate-citric acid buffer pH 3.5 and incubated overnight at 37°C to allow autolysis to occur. After autolysis pH was readjusted to pH 6.8 and denatured haemoglobin removed by centrifugation at 1500 xg for 15 minutes.

Ammonium sulphate precipitation

Ammonium sulphate was added to autolyzed hemolysate to give 40 % saturation whilst stirring for one hour. Then centrifuged at 10,000 rpm for 20 min. to remove the pellet. 40% pellets were dissolved in 20 mM sodium phosphate buffer pH 6.8. 60% and 80% saturation pellets were prepared by using supernatant of 40% pellets by above-mentioned procedure. Acid endopeptidase activities of 40%, 60% and 80% pellets were performed after removing ammonium sulphate by dialysis. Pellets of 80% saturation showed proteolytic activity.

Gel filtration chromatography

Pellets of 80% ammonium sulphate saturation dialysed against 0.1 M ammonium acetate pH 6.8.

The dialysed sample was subjected to gel filtration chromatography on Sephadex G-75 (0.8/65 cm) in 0.1 M ammonium acetate solution pH 6.8. Material was eluted with the same eluent at a flow rate of 6 ml/hr. Fractions of 2 ml each were collected and the absorbance was recorded at 280 nm. Acid endopeptidase activities of fractions (nos. 12-40) of GFC were performed.

Anion exchange chromatography

Active fractions from gel filtration column were pooled, concentrated and dialysed against 20 mM sodium phosphate buffer pH 6.8 and subjected to anion exchange chromatography on DEAE Sephacel column (0.8/10 cm), previously equilibrated with the same buffer.

The absorbed material was eluted from the column at a flow rate of 12 ml/hour with the linear gradient of NaCl ranging from 0-0.5 M. Fractions of 2 ml each collected and absorbance was recorded at 280 nm. Acid endopeptidase assay of fractions collected was determined.

High performance gel filtration chromatography

Active fractions from anion exchange chromatography were pooled, concentrated and subjected to run on a high performance gel filtration column of TSK G2000SW. Proteolytic activity of each peak was performed.

Electrophoresis

Polyacrylamide gel electrophoresis under denaturating conditions (SDS-PAGE) of the active fractions of anion exchange chromatography and of high performance gel filtration chromatography was performed using 10% and 12.5% respectively, by the method of Laemmli [26] and silver staining was employed. Molecular masses of proteins were measured by plotting relative mobility versus $\log_{10}$ molecular masses of standard proteins.

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