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PURIFICATION AND CHARACTERIZATION OF A CALCIUM DEPENDENT SULFHYDRYL PROTEASE FROM HUMAN PLATELETS

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SUMMARY: A calcium dependent sulfhydryl protease (CDSP) responsible for over 95% of the neutral protease activity of human platelets has been purified. CDSP is composed of two different polypeptide subunits of molecular weights estimated to be 80,000 and 30,000 daltons. It requires millimolar calcium, reduced sulfhydryl groups and neutral pH for optimal activity. It is inhibited by Leupeptin and an endogenous inhibitor which is removed during purification. CDSP specifically cleaves the platelet's Actin Binding Protein, crosslinker of F-actin microfilaments, into two high molecular weight fragments, the heavier of which still retains its ability to bind to, but not crosslink F-actin into cytoskeletal arrays.

Calcium dependent neutral proteases, similar to if not identical to the human platelet CDSP described here, have been identified in various tissues (1-12). We and others (1,2,13,14) have observed that disrupting a platelet's cellular integrity and allowing calcium to rise above 10^{-4} M, leads to a rapid proteolysis of several platelet proteins including ABP. Platelet cytoskeletons are composed of F-actin filaments crosslinked by the high MW ABP (15,16). It has been demonstrated (15,16,17) that ABP is involved in the regulation and assembly of actin microfilaments. Therefore, its rapid and specific proteolysis by endogenous CDSP may indeed be related to the irreversible disruption of microfilament assembly in the platelet cytoskeleton.

Remarkably, nearly all of the platelet's neutral protease activity requires a calcium concentration in excess of 10^{-4} molar. This activity can

ABBREVIATIONS: CDSP, Calcium Dependent Sulfhydryl Protease; ABP, Actin Binding Protein; EGTA, Ethylene Glycol bis (2-Amino ethyl ether) N,N'-tetraacetic acid; PEG-6000, Polyethylene glycol; TCA, Trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, 2-((tris-(hydroxymethyl) methyl) amino) ethane sulfonic acid; MES, 4-morpholine ethanesulfonic acid; 2-MSH, 2-mercaptoethanol; MW, Molecular Weight.

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be attributed to a single calcium dependent sulfhydryl protease, whose purification is described herein.

MATERIALS AND METHODS

Assays for Proteolytic Activity: CDSP activity is detected by visualization on SDS-gels of the proteolytic cleavage of high MW polypeptides. Assaying by gel analysis is used to demonstrate the specificity of CDSP activity on certain proteins and to determine proteolytic cleavage products. A sensitive, quantitative method for assaying of CDSP activity was developed by measuring the hydrolysis of (^{14}C)-radiolabled casein. (^{14}C)-methylation of casein (18, 19) was produced at a concentration of 8 mg/ml and yielding 2.5 x 10^6 cpm/mg.

Standard Assay Procedure: 50 ul of sample is mixed with 200 ug (5 x 10^5 cpm; 25 ul) of (^{14}C)-casein and 400 ul of buffer having the appropriate final concentrations of solutes and conditions for the assay. The reaction is stopped and the proteins precipitated by adding 50 ul of 50% TCA. 50 ul of 6 mg/ml cold casein is added to aid in protein precipitation. The mixture is centrifuged at 12,000 x g for 2 min to pellet the precipitate. 450 ul of the supernatant, containing the non-TCA-precipitable (^{14}C)-peptides, is pipetted into 6 ml of Aquasol-2 (N.E. Nuclear) scintillation cocktail and counted to less than 1% error. Standard buffer, to which varying concentrations of $CaCl_2$, EGTA, 2-MSH, Leupeptin (20), and other constituents are added, is 40 mM KCl, 25 mM TES, pH 7.4, 1 mM NaN_3. Standard conditions for determining CDSP activity are 4 mM $CaCl_2$ and 10 mM 2-MSH in standard buffer incubated with sample and 200 ug (^{14}C)-casein for 30 min at 22°C.

Reversible sulfhydryl group (-SH) blocking (21,22) of CDSP is accomplished by incubating the CDSP fraction in 1 mM potassium tetrathionate, 25 mM TES, pH 7.9, 1 mM NaN_3 for 15 min at 4°C; excess tetrathionate is dialyzed away.

Non-Denaturing (Native) and SDS-PAGE: 5.5% polyacrylamide gels with a 25 mM Tris-glycine, pH 8.3 buffering system was used for both Non-Denaturing and SDS PAGE. Non-Denaturing gels used a 25 mM Tris-glycine, pH 8.3, (except second series native gels: pH 7.5 used), 1 mM EDTA, 5 mM 2-MSH anode buffer, and were electrophoresed at 10°C with 3.5 watts constant power. Samples for the Non-Denaturing gels were prepared in 25 mM TES, 1 mM EDTA, 5 mM 2-MSH, pH 7.6, 100 ul loaded. Protein samples for SDS-PAGE were prepared by heating at 100°C, for 5 min in buffer (25 mM Tris-SO_4, pH 6.7, 1% SDS, 1% 2-MSH, 0.002% bromophenol blue and 2% glycerol). Up to 50 ul of SDS-sample was loaded and electrophoresed with an anode buffer of 0.1% SDS, 25 mM Tris-glycine, pH 8.3 at 3.5 mAmp/gel. Gels were stained in 0.1% Coomassie Blue.

Purification of Human Platelet Calcium Dependent Sulfhydryl Protease: Fresh human platelets were isolated from platelet concentrates prepared for clinical transfusion and washed 3 times in a buffer containing 10 mM $NaPO_4$, pH 7.4, 0.3 mM EDTA, 126 mM NaCl, and 5 mM KCl. These platelets were found to be free of contaminants and viable as measured by aggregation and secretion (23).

Step I--Solubilization of Platelets and Removal of ABP-Actin Cytoskeletal Complex: Platelets were solubilized, complex removed and Triton X-100--EGTA Supernatant produced as described by Lucas (15,16). The supernatant, containing the CDSP Activity, was then processed at 4°C during all following steps.

Step II--Processing of the TX-100--EGTA Supernatant: Supernatant (40 ml) was made 10 mM in EDTA and centrifuged at 25,000 x g for 20 min to clarify and remove Actomyosin. Supernatant was then made 5 mM in 2-MSH and adjusted to pH 7.8. Alternatively, for use in larger CDSP preparations, supernatant can

be fractionated by the addition of 50% PEG-6000. The precipitate of the 10-20% PEG-6000 fraction collected by centrifugation is brought into 3 ml of Buffer S (25 mM TES, 1 mM EDTA, 1 mM NaN_3, pH 7.4) and stored frozen at -20°C for later use (retains 90% of its activity after 1 year).

Step III--DEAE Ion-Exchange Chromatography: Processed Supernatant (40 ml) was applied to a DEAE-Sephacel (Pharmacia, 1.5 x 10 cm) column equilibrated with Buffer S. The column was eluted with a linear 60-500 mM KCl gradient in Buffer S (50 ml). CDSP Activity was eluted between 190-290 mM KCl. Fractions from the peak of activity (8 ml) were pooled and adjusted to pH 7.9.

Step IV--Activated Thiol-Sepharose (Affinity) Chromatography: DEAE fractions (8 ml) were loaded onto an Activated Thiol-Sepharose 4B (Pharmacia,0.7 x 7 cm) column equilibrated with deaerated Buffer S, adjusted to pH 7.9. The column was washed free of non-binding protein, then eluted with Buffer S, pH 7.9 containing 10 mM 2-MSH. The peak CDSP Activity was eluted in 4 ml.

Step V--PEG-6000 Concentration Dialysis: Pooled Thiol-Sepharose fractions (4 ml) were placed in a dialysis sac and dialyzed against 150 ml of a 50% PEG-6000 solution until its volume was reduced to 1.1 ml.

Step VI--Non-Denaturing PAGE (Native gel) Separation: 100 ul of concentrated CDSP sample (from Step V) was loaded onto 5.5% gels. Gels were electrophoresed at 3.5 watts constant power for 1.75 hrs at 10°C. One gel was stained to visualize the protein bands. The corresponding parallel gels were sliced into 2 mm slices. Those slices containing the CDSP activity were isolated and used for further analysis. CDSP is extracted from gel slices by electrophoresis in 25 mM Tris-Glycine, 1 mM EDTA, pH 8.3 buffer using an ISCO electrophoretic concentrator (3 watts, 15 min). In lieu of Non-Denaturing PAGE separation, for large scale CDSP preparations, an Ultrogel AcA 44 (LKB, 1.6 x 40 cm) column was found to be effective in producing purified CDSP.

Step VII--Determination of CDSP Purity by SDS-PAGE and Non-Denaturing Re-Electrophoresis: Gel slices from Step VI, corresponding to a single band on the parallel native gel and identical to gel slices demonstrated to have met all criteria of CDSP activity, were placed directly on top of 5.5% SDS gels. 40 ul of SDS-gel sample buffer with 1 mM EDTA added was loaded on top of each gel, electrophoresed and then stained to visualize the polypeptide bands of the purified CDSP. To further verify the purity and subunit consistency of CDSP, a second series of native and SDS gels were run on the protein produced by the first series of native gels. Gel slices from the first series (Step VI) of parallel native gels, demonstrated to have met all criteria of CDSP activity, were loaded directly on top of a second series of Non-Denaturing gels. Gels were electrophoresed as in Step VI, except for a change in pH (7.5 vs 8.3). This second series of native gels was treated as described above for the first series: The gel slices identical to those having CDSP Activity were electrophoresed on SDS-gels. The subunit polypeptide bands of this re-purified CDSP were visualized on the SDS-gels to confirm homogeneity.

RESULTS AND DISCUSSION

Intact platelets dispersed in hot SDS sample buffer and electrophoresed contain several high molecular weight polypeptides: Figure 1,"TP". The three prominant high MW polypeptide bands are ABP (ca.270,000 daltons, subunit MW), Band-2 (unknown type, ca.240,000), and myosin (ca.200,000). When platelets

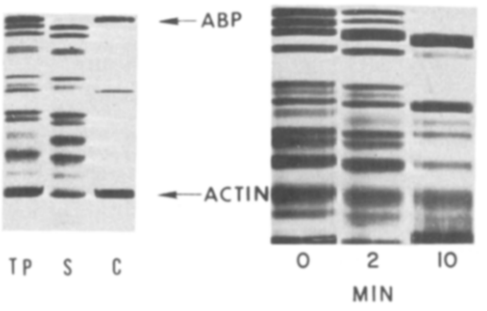

FIGURE 1 SDS-PAGE of TP: Total platelet proteins; S: Triton X-100--EGTA Supernatant Proteins; C: Actin Binding Protein(ABP)-Actin Complex Proteins. Cleavage of High MW Polypeptides is demonstrated in the last three gels (an enlargement, cut off above the actin band): platelets immersed in TX-100 solution devoid of calcium chelator and incubated at 0°C for 0, 2, and 10 min.

are suspended in a Triton X-100--EGTA solution, an ABP-actin cytoskeletal complex is isolated by low speed centrifugation; this complex is shown in Figure 1,"C" (15,16,17). The remaining proteins are in the TX-100--EGTA supernatant: Figure 1,"S". Platelets lysed without calcium chelator show rapid proteolysis of the high MW polypeptides of ABP and Band-2 (1,2). The last three gels in Figure 1 show the time course for their rapid disappearance. The upper two bands of this high MW polypeptide triplet are specifically and completely cleaved in less than 10 min at 0°C. Concomitant with this disappearance, the bottom band of the triplet, corresponding to myosin, appears to increase. This was later shown to be a composite band containing myosin and the 190,000 dalton cleavage product of ABP (Heavy Fragment-1). CDSP is responsible for this cleavage and accounts for over 95% of the platelet's neutral protease activity, as determined by the hydrolysis of added (^{14}C)-casein substrate (2,13).

Table I summarizes the purification data for CDSP and Figure 2 shows the results of the gel electrophoresis separation step. A 2256-fold increase in specific activity was obtained by these procedures. The increase in total yield of activity, 129%, is likely the result of removal of a high MW endogenous inhibitor eluted in the void fraction during DEAE chromatography. Similar endogenous inhibitors have been found by other investigators (24,25,26).

TABLE I

PURIFICATION OF HUMAN PLATELET CALCIUM DEPENDENT SULFHYDRYL PROTEASE

Procedure	Volume (ml)	Total Units of Activity* (UA)	Total Protein (mg)	Specific Activity (UA /mg)	Yield+ of Procedure %	Increase in Specific Activity
I-Total Platelet+	1.8	($3.4x10^6$)	(207)	($1.6x10^4$)	--	--
II-TX-100 Supernatant	40	$3.4x10^6$	140	$2.4x10^4$	100	1.5
III- DEAE-Sephacel	8	$8.1x10^6$	13.6	$6.0x10^5$	235	36
IV-Activated Thiol-Sepharose	4	$7.8x10^6$	1.2	$6.5x10^6$	96	396
V-PEG-6000 Concentration Dialysis	1.0	$7.6x10^6$	1.1	$6.9x10^6$	97	421
VI- Non-Denaturing PAGE Separation	1.0	$4.4x10^6$	0.12	$3.7x10^7$	58	2256

TABLE I: Data presented represents the average of four separate preparations.

+ Data inside brackets is extrapolated and calculated, based on the starting material of 1.8 ml of packed, intact platelets, resulting in 40 ml of Triton X-100--EGTA supernatant and the ABP-actin complex removed.

* A Unit of Activity (UA) is defined as one cpm above control of Non-TCA precipitable (^{14}C)-casein peptides released when 50 ul of sample is incubated for 30 min at 22°C with 200 ug ($5x10^5$ cpm) of (^{14}C)-casein under standard assay conditions (4 mM $CaCl_2$, 10 mM 2-MSH, ph 7.4, EGTA inhibitable: see text).

ǂ The percent yield of the procedure directly reflects the ratio of the total activity recovered by this procedure relative to that recovered by the previous step. Note that the DEAE-ion-exchange chromatography used in Step III removes an endogenous inhibitor of CDSP, resulting in an absolute increase in the total units of activity recovered (see text).

In Figure 3, SDS-gels A, B, and C are loaded to depict the major polypeptides found in their respective fractions containing peak CDSP Activity. The Non-Denaturing gel (Fig. 3D) shows one major band, corresponding to the peak CDSP Activity, and several minor bands; migration of these proteins is related to time of electrophoresis. The SDS-gel in Fig. 3E shows that the resulting purified CDSP has two polypeptide subunits estimated to be 80,000 and 30,000 daltons. The mass ratio of these subunits was found to be approximately 3:1

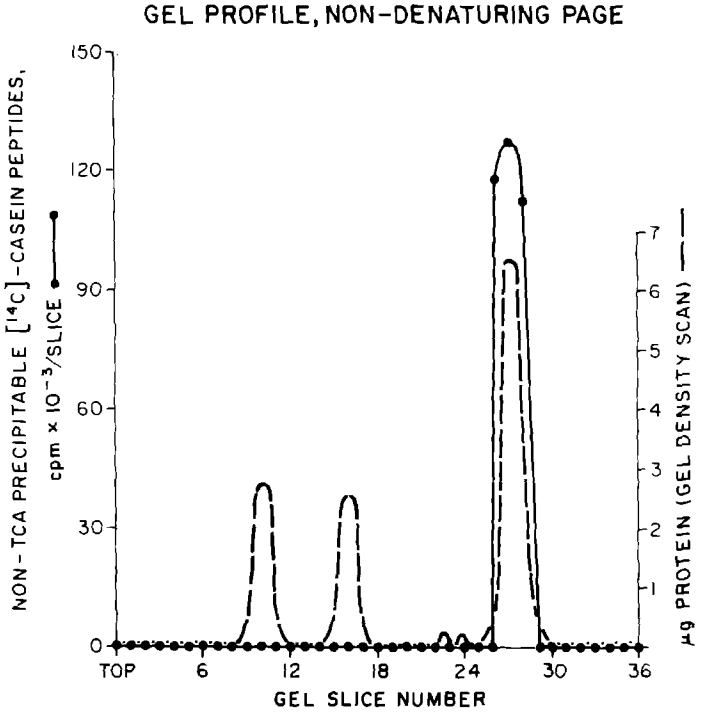

FIGURE 2: Separation of CDSP by Non-Denaturing (Native) polyacrylamide gel electrophoresis (see Methods).

by gel scanning; yielding a molar ratio of approximately 1:1. These results are similar to those found for CDSP from other mammalian sources (4,9,10).

ABP (Fig. 3F) cleaved by CDSP results in two major fragments: Heavy Fragment-1 (ca.190,000) and Heavy Fragment-2 (ca.90,000) as shown in Fig. 3G. ABP recombined with F-Actin forms a crosslinked, cytoskeletal-like complex precipitable by low speed centrifugation (16,17). When the mixture of HF-1 and HF-2 (Fig. 3G) is recombined with F-Actin no crosslinked complex is formed. Yet when this mixture is ultracentrifuged at g forces sufficient to sediment F-Actin, HF-1 (190,000 daltons) is found bound to F-Actin. Thus, once ABP is cleaved it irreversibly loses its ability to crosslink F-Actin microfilaments, but its subfragment retains the ability to interact with Actin.

The conditions and criteria defining human platelet CDSP activity are detailed in Table II. In summary, CDSP has the ability to proteolyze ABP,

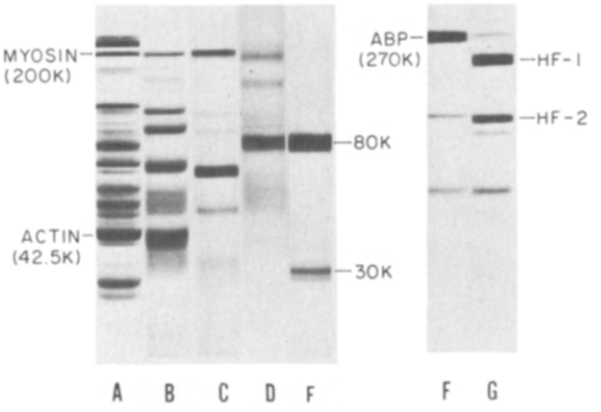

FIGURE 3. Stained 5.5% Polyacrylamide Gels: A: Triton X-100--EGTA Supernatant, starting material; B: Fraction from DEAE-Sephacel column having CDSP activity; C: Fraction from Activated Thiol-Sepharose column having CDSP activity; D: Non-Denaturing (Native) gel (first series), separating CDSP; E: Calcium Dependent Sulfhydryl Protease (SDS gel of purified CDSP from first series native gel)--note 80,000 and 30,000 dalton subunits. Myosin, 200,000, and Actin, 42,500 daltons, are marked for comparison. Gel loads are qualitative: loaded to show major proteins in each fraction.
F: Partially Purified ABP (270,000 daltons, subunit MW); G: ABP, shown in "F", incubated with a small quantity of CDSP (non-detectable on gels) under standard activity conditions. CDSP cleaves ABP into two major fragments: Heavy Fragment-1 (ca. 190,000 subunit MW) and Heavy Fragment-2 (ca. 90,000 subunit MW). Proteolysis was stopped to leave a trace of uncleaved ABP as reference band. If proteolysis is allowed to go beyond completion, the same products result; no furthur breakdown is detected.

TABLE II

ACTIVITY PARAMETERS OF HUMAN PLATELET CDSP

Assay Conditions	Non-TCA Precipitable (^{14}C)-Casein Peptides CPM x 10^{-3}
CDSP + 10 mM 2-MSH + 4 mM Ca^{2+}	112
CDSP + 10 mM 2-MSH + 1 mM Ca^{2+}	114
CDSP + 10 mM 2-MSH + 0.3 mM Ca^{2+}	54
CDSP + 10 mM 2-MSH + 0.1 mM Ca^{2+}	6
CDSP + 10 mM 2-MSH + 4 mM Ca^{2+} + 10 mM EGTA	5
CDSP + 10 mM 2-MSH + 4 mM Ca^{2+} + 50 ug/ml Leupeptin	5
CDSP + 10 mM 2-MSH + 4 mM Ca^{2+}, pH 6.1	53
CDSP + 10 mM 2-MSH + 4 mM Ca^{2+}, pH 8.5	50
(Sulfhydryl Blocked-CDSP) + 4 mM Ca^{2+} - 2-MSH	7
(Sulfhydryl Blocked-CDSP) + 4 mM Ca^{2+} + 10 mM 2-MSH	104
(-CDSP) + 4 mM Ca^{2+} + 10 mM 2-MSH (Background Control)	5

TABLE II: Standard assay conditions (see methods) are used to quantify CDSP Activity parameters: 50 ul of CDSP sample was incubated for 30 minutes at 22°C with a buffer containing 200 ug (5 x 10^5 cpm) (^{14}C)-casein, 40 mM KCl, 25 mM TES-pH 7.4 (or MES-pH 6.1 or Tris-pH 8.5), and having the appropriate final concentrations of $CaCl_2$, 2-MSH, EGTA and Leupeptin as noted in the table. Additional assays to those illustrated above have shown that CDSP incubated under standard conditions with or without 10 mM 2-MSH and in the absence of added Ca^{++}; or with 4 mM Mg^{++} alone; or with 2 mM EGTA alone; result in the background control level (5 x 10^3 cpm) of Non-TCA precipitable radioactivity.

Band-2 and hydrolyze (^{14}C)-casein; it requires 1 mM calcium, reduced sulfhydryls and pH 7.4 for optimal activity; it is inhibited by excess calcium chelator (EGTA), sulfhydryl blockers, and the protease inhibitor Leupeptin.

Although we have noted the major effects and characteristics of platelet CDSP, what physiological role it plays in platelet function remains obscure and is the object of further investigation.

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