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Short Communication

Human μ-Calpain: Simple Isolation from Erythrocytes and Characterization of Autolysis Fragments

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Heterodimeric μ-calpain, consisting of the large (80 kDa) and the small (30 kDa) subunit, was isolated and purified from human erythrocytes by a highly reproducible four-step purification procedure. Obtained material is more than 95% pure and has a specific activity of 6–7 mU/mg. Presence of contaminating proteins could not be detected by HPLC and sequence analysis. During storage at –80 °C the enzyme remains fully activatable by Ca^{2+}, although the small subunit is partially processed to a 22 kDa fragment. This novel autolysis product of the small subunit starts with the sequence ^{60}RILG and is further processed to the known 18 kDa fragment. Active forms and typical transient and stable autolysis products of the large subunit were identified by protein sequencing. In casein-zymograms only the activatable forms 80 kDa+30 kDa, 80 kDa+22 kDa and 80 kDa+18 kDa displayed caseinolysis.

Key words: Autolysis / Calcium activation / Calpain / Cysteine proteases / Purification.

Calpains are intracellular calcium-dependent cysteine proteinases with enzymatic activity at neutral pH. Two ubiquitous types are well characterized, μ- und m-Calpain (Calpain I and II). These multidomain proteins consist of homologous large, catalytic subunits (80 kDa) and identical small, regulatory subunits (30 kDa) (see Johnson and Guttmann, 1997; Sorimachi *et al*., 1997; Carafoli and Molinari, 1998; Ono *et al*. 1999, and for a review Wang *et al*., 1999). The cDNAs of several isoforms are known (Emori *et al*., 1986; Sorimachi *et al*., 1993; Graham-Siegenthaler *et al*., 1994; Sorimachi *et al*., 1996; Dear *et al*., 1997). Calpains have pathophysiological roles in human disorders such as muscle dystrophy, traumatic brain injury, spinal cord injury, cerebral ischemia, myocardial infarction and cataract formation. However, their physiological functions are not yet well understood, and the literature presents a lot of conflicting data on activation, autolysis and protein interactions of calpains (for reviews see Wang and Yuen, 1994; Krampfl *et al*., 1997; Carafoli and Molinari, 1998; Wang and Yuen, 1999). The calcium-activated proteases were first described in the sixties (Guroff, 1964), purified in the eighties (Yoshimura *et al*., 1983; Kitahara *et al*., 1984; Zimmermann and Schlaepfer, 1984; Crawford *et al*., 1987) and isolated by improved affinity chromatography in the nineties (Molinari *et al*., 1995; Anagli *et al*., 1996). But even today isolation of μ-calpain is a difficult task (Croall, 2000; Thompson and Goll, 2000). Frequently, SDS polyacrylamide gel electrophoresis reveals more bands than the expected 80 kDa and 30 kDa forms. Here, we describe an optimized isolation procedure for μ-calpain from human erythrocytes which yields a pure, highly active and relatively stable enzyme.

Purification of human μ-calpain was performed at 4 °C. Erythrocyte concentrate (600 ml corresponding to 1 liter of whole blood) was washed twice with washing solution (0.15 M NaCl, 2 mM EDTA, pH 7.5) and centrifuged (1000 *g*), lysed for 15 min at room temperature with lysing buffer (15 mM Tris-HCl, 2 mM EDTA, pH 7.5, 0.5 mM Pefabloc) and centrifuged again (1 h, 20 000 *g*). The supernatant was diluted 1:1 (v/v) with buffer A (20 mM Tris/HCl, 2 mM EDTA, pH 7.5) and applied to an equilibrated DEAE-Sepharose column (Pharmacia XK 50/30). The column was washed with buffer A until E_{280} <0.010 and equilibrated with a mixture of 87:13 parts (v/v) of buffer A and buffer B (20 mM Tris/HCl, 2 mM EDTA, pH 7.5, 0.5 M NaCl). Calpain/calpastatin complexes were eluted with buffer B, adjusted to 1.2 M NaCl and applied to a Phenyl-Sepharose column (Pharmacia XK 50/20) equilibrated with 1.2 M NaCl in buffer C (20 mM Tris/HCl, 2 mM EDTA, pH 7.3). Elution of μ-calpain was performed by a descending linear gradient from 1.2 to 0 M NaCl in buffer C.

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Active fractions were collected (positive in the casein-Coomassie test; Buroker-Kilgore and Wang, 1993), concentrated by ultrafiltration to 10 ml (Amicon 10 kDa cut-off membrane) and further purified on Superdex 200 (Pharmacia 100/3) equilibrated with 0.4 M NaCl in buffer C. The eluted active fractions were applied to a Blue-Sepharose column (Pharmacia Blue Sepharose Hi Trap 5 ml prepacked), washed with 0.25 M NaCl in buffer C and eluted by a stepwise gradient from 0.25 to 0 M NaCl in buffer C. Finally, isolated μ-calpain was concentrated and stored in aliquots (1 – 2 mg/ml).

We routinely obtained approx. 1.5 to 2 mg of highly purified μ-calpain from 600 ml of human erythrocyte concentrate. The isolation procedure includes 4 main steps: ion exchange chromatography, hydrophobic interaction chromatography, gel filtration and affinity chromatography in a very reproducible manner. In fresh preparations two single protein bands were obtained after SDS-PAGE (Laemmli, 1970) corresponding to apparent molecular masses of 80 kDa and 30 kDa (Figure 1). These two bands were primarily identified by anti-domain II and anti-30 kDA monoclonal antibodies as the large and small subunit of μ-calpain (data not shown). Direct Edman degradation of eluted bands failed because the N-termini of both subunits are blocked, but endoproteinase Lys-C cleavage, peptide mapping and partial peptide sequencing of two selected peptides yielded internal sequences of the corresponding subunits.

Fresh material was analyzed by reversed-phase HPLC (Figure 2). Three fractions were separated: the latest eluting fraction C consists of pure heterodimeric μ-calpain (80 kDA und 30 kDA); its composition was determined by endoproteinase Lys-C cleavage, peptide mapping and protein sequencing (^{21}Q-R-A-R-E-L-G- for the 80 kDa-form and ^{126}V-V-T-R-H- for the 30 kDa-form). Fraction B (about 20% by weight of total) consists of pure homogeneous 30 kDa subunit (^{164}R-W-Q-A-I-Y-). The small fraction A (about 20% by weight of total) is a mixture of three heterogenously cleaved 30 kDa-forms, (-M-R-I-)↓^{62}L-G-G-V-I-S-, (-G-V-I-)↓^{68}S-A-I-S-E-A-A- and (-S-A-I-)↓^{71}S-E-A-A-A-Q-. These three minor fractions represent 'autolysed' fragments that have not been described so far.

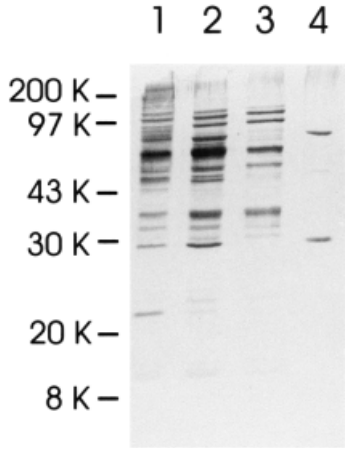

Fig. 1 SDS-PAGE Documenting the Purification of μ-Calpain.
Lane 1, μ-calpain fraction obtained after phenyl-Sepharose; lane 2, μ-calpain fraction after gel filtration; lane 3, material not retained by the Blue-Sepharose column; lane 4, purified μ-calpain obtained after Blue-Sepharose chromatography.

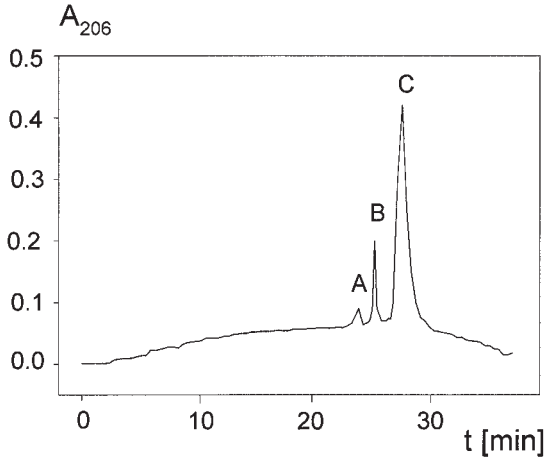

Fig. 2 Separation of Freshly Purified μ-Calpain by HPLC.
Purified calpain (20 μg) was analyzed by reversed-phase HPLC with Aquapore RP 300 (Applied Biosystems GmbH, Weiterstadt, Germany) and a linear gradient of 0 – 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and 30% (v/v) isopropanol at a flow rate of 1 ml/min. Absorption was recorded at 206 nm. Samples were analyzed by SDS-PAGE and sequenced after transfer to PVDF membranes using a gas-phase sequenator 473A (Applied Biosystems GmbH) according the instructions of the manufacturer.

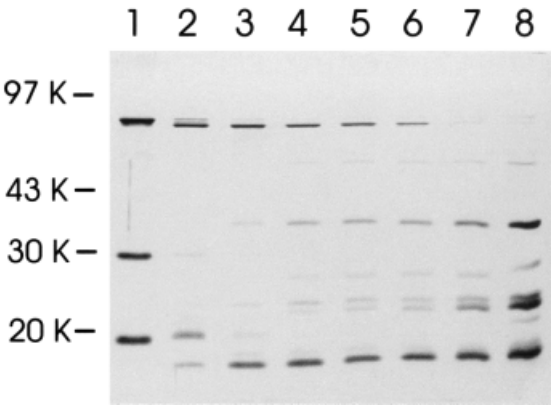

Fig. 3 *In vitro* Autolysis of Human μ-Calpain Followed by SDS-PAGE.
Lane 1, purified μ-calpain after storage for 2 weeks at – 80 °C; lanes 2 – 8, autolytic fragments 0 min (2), 1 min (3), 5 min (4), 15 min (5), 30 min (6), 60 min (7) and 120 min (8) after addition of 0.5 mM free Ca^{2+} (final concentration) to purified μ-calpain (27.5 μg) in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1 mM DTT at room temperature. After the indicated time intervals aliquots were taken (1.1 μg), EDTA was added (25 mM final concentration) and the material was applied to the gel.

Unexpectedly, the purified calpain shows a new prominent band at 22 kDA in SDS-PAGE after short storage at 4 °C and after longer storage at – 80 °C (Figure 3), al-

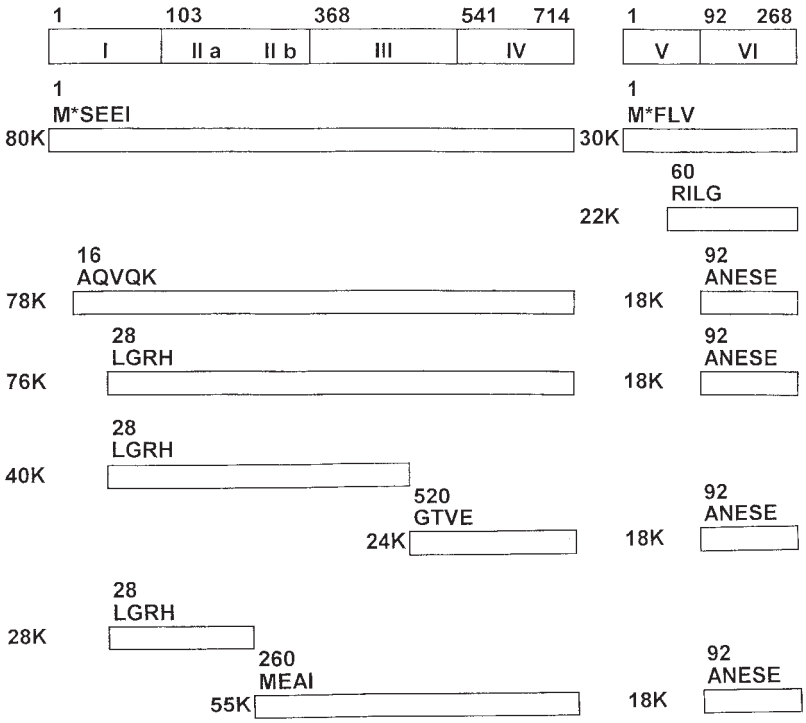

Fig. 4 Scheme of Observed Autolysis Fragments of Purified μ-Calpain after Calcium Activation. Cleavage points are identified by their N-terminal sequences.

though the material was kept in 2 mM EDTA. The N-terminal sequence of the 22 kDa fragment, ^{60}R-I-L-E-, is located in domain V 26 or 32 amino acids upstream of the well-known 21 kDa (^{86}H-Y-S-N-I-E-) or 18 kDa (^{92}A-N-E-S-E-) autolysis products of the 30 kDa form (Emori *et al*., 1986; McClelland *et al*., 1989; Nishimura and Goll, 1991; Figure 4). The cleavage leading to this novel fragment may be due to (i) an undetected contaminating protease, or (ii) to a minor 'dormant' activity of purified μ-calpain itself. It can be hypothesized further that the cleavage site may be located close to the broad active-site cleft of calpain (Hosfield *et al*., 1999; Strobl *et al*., 2000). A comparable 23 kDa autolysis product of the small subunit (^{64}G-V-I-S-A-, starting 4 amino acid residues upstream of ^{60}R-I-L-G-A-Q-) was described by McClelland and coworkers after Ca^{2+}-activation (McClelland *et al*., 1989). Heterogeneity of EDTA-stored μ-calpain, due to the formation of the 22 kDa fragment, may explain the fact that extended crystallization experiments with our purified enzyme were not successful (W. Bode, personal communication). Figure 4 summarizes the complex pattern of transient (80 kDa, 78 kDa, 76 kDa, 55 kDa, 30 kDa, 21 kDa) and stable (40 kDa, 28 kDa, 24 kDa, 18 kDa) autolysis products of our purified μ-calpain.

Enzymatic activity of μ-calpain was measured in 'continuous' fluorimetric assays using Suc-Leu-Tyr-NH-Mec (250 μM) as substrate at 12 °C, pH 7.5, with 200 μM $CaCl_2$ as described in detail previously (Garcia-Diaz *et al*., 2001). The purified enzyme had a specific activity between 5 and 7 mU/mg under the conditions given above. After extrapolation to saturating substrate concentrations, this value (123 mU/mg) corresponds well to the specific activity of porcine calpain (76.3 mU/mg) reported by Sasaki *et al*. (1984).

Fresh and stored purified calpains were analyzed by casein zymography before and after activation using non-denaturing gel electrophoresis (Raser *et al*., 1995). As little as 10 ng fresh μ-calpain (80 kDa and 30 kDa) was detected as a single band. If 5 μg were applied, two additional quicker migrating tiny bands could be detected (Figure 5; line μ#). With stored μ-calpain we found two strong caseinolytic activities which represent the 80

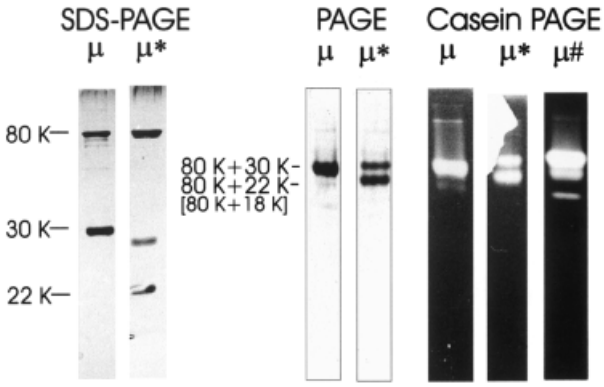

Fig. 5 Comparison of Purified μ-Calpain from Human Erythrocytes in SDS-PAGE, Native PAGE and PAGE Containing Casein (Casein-PAGE).
μ, purified μ-calpain (1 μg) immediately after isolation; μ*, purified μ-calpain (1 μg) after storage for 2 weeks at – 80 °C; μ#, purified μ-calpain (5 μg) immediately after isolation.

kDa+30 kDa and 80 kDa+21 kDa forms (Figure 5; line μ*). No caseinolytic activity was found when already activated μ-calpain forms (78 kDa+18 kDa, 76 kDa+, 18 kDa) were applied to the zymograms (data not shown). This may be explained by further autolysis of the activated forms during electrophoresis leading to inactivation. All caseinolytic bands were completely inhibited by the recombinant calpastatin domain I and by the inhibitor Z-LLY-CHN_2.

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