Purification and Characterization of a Ca^{2+}-Activated Thiol Protease from *Drosophila melanogaster*†

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ABSTRACT: A Ca^{2+}-activated thiol protease was purified from *Drosophila melanogaster*. The procedure involves Phenyl-Sepharose, Reactive Red-Agarose and Q-Sepharose fast flow (or MonoQ) chromatographic steps. The enzyme eluting from Q-Sepharose fast flow seems to be homogeneous as judged by silver staining on SDS–PAGE: it consists of a single polypeptide chain of $M_{r,app}$ = 94K and pI = 5.46. The proteolytic activity of the purified enzyme is absolutely Ca^{2+}-dependent, characterized by 0.6 mM free Ca^{2+} at half-maximal activity. Ca^{2+} ions cannot be replaced effectively by the divalent cations Mg^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, and Cd^{2+}. The enzyme shows the inhibitor pattern of thiol proteases. Human recombinant calpastatin (domain I) completely inhibits the enzyme at a nearly 1:1 molar ratio. Several of these properties resemble those of vertebrate calpain II. However, various attempts to detect a small subunit of M_r ~30K, common with vertebrate calpains, remained unsuccessful. We suggest that the *Drosophila* enzyme is a novel calpain II-like protease.

The increasing body of evidence indicating the multifarious regulatory roles of Ca^{2+} entering the cytoplasm underlines the importance of Ca^{2+}-regulated target proteins. Among these are enzymes that transmit the Ca^{2+}-signal via reversible and irreversible steps. The latter include the Ca^{2+}-dependent proteolytic system, calpain–calpastatin [for a review, see Mellgren (1987), Murachi (1989), Suzuki et al. (1990), and Johnson (1990)]. There are a number of suggestions for the involvement of the calpain system in various physiological processes, including several long-term or irreversible phenomena (Lynch & Baudry, 1984; Schollmeyer, 1988; Orrenius et al., 1988; Siman et al., 1989; Okita et al., 1989; Watanabe et al., 1989), yet the role in vivo of calpain is ill understood. In addition, there seem to be other Ca^{2+}-activated neutral thiol proteases in animal cells that differ from established calpains in some of their structure–function properties (Mykles & Skinner, 1986; Wolfe et al., 1989; Yoshihara et al., 1990).

We have undertaken to scrutizine the Ca^{2+}-activated neutral protease (CANP) system in the fruit fly, *Drosophila melanogaster*, since this organism is particularly suitable for genetic manipulations, which paves the way to addressing physiological function. We have earlier detected a Ca^{2+}-dependent proteolytic system in *Drosophila* (Pintér & Friedrich, 1988). Now we report the purification and characterization of a calpain II-like enzyme from the same source.

EXPERIMENTAL PROCEDURES

Drosophila melanogaster of Canton-S wild-type strain was grown on standard cornmeal medium. Adult flies were collected and stored in liquid nitrogen until use. Porcine brain was procured from the slaughterhouse in ice and was used within 3 h after killing the animal.

The sources of chemicals were as follows: leupeptin, Phenyl-Sepharose CL-4B and Reactive Red 120-Agarose (type 3000-CL) were from Sigma; MonoQ HR 5/5 and Q-Sepharose fast flow were from Pharmacia; Protein Test mixture 9 was from Serva. [^{3}H]Formaldehyde was a NEN product. Calpeptin was kindly provided by Dr. N. Higuchi (Suntory Research Center, Osaka, Japan). Calpastatin (human recombinant, domain I) was purchased from Calbiochem.

Protease assay: [^{3}H]Casein [prepared according to Rice and Means (1971)], 66 μg/mL, was digested with CANP samples in the presence or absence of 5.3 mM $CaCl_2$. The specific radioactivity of casein in the reaction mixture was 400 Bq/mg. CANP samples (65 μL) were assayed in a total volume of 75 μL at 30 °C in the following reaction mixture: 10 mM HEPES–KOH, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM dithioerythritol, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine (buffer A) plus 0–0.3 M NaCl. The reaction was terminated by adding 58 μL of 30% trichloroacetic acid and 118 μL of 3.7 mg/mL of bovine serum albumin solutions. CANP activity was calculated from the Ca^{2+}-dependent increase in the radioactivity (i.e., Δcpm with Ca^{2+} *minus* Δcpm without Ca^{2+}) of trichloroacetic acid-soluble supernatant in the reaction mixture and was expressed as microgram of casein digested per minute.

Electrophoretic techniques: SDS–PAGE[1] was performed according to the method of Laemmli (1970). Molecular weights were assigned by using a Pharmacia low molecular weight standard. Two-dimensional gel electrophoresis with isoelectric focusing of urea-denaturated protein in the first dimension and SDS–PAGE in the second dimension was made as described by O'Farrell (1975), but samples were focused for 7000 Vh, 400 Vh prefocusing included. Gels were stained with silver (Poehling & Neuhoff, 1981) or with CBB (Neuhoff et al., 1985).

Free Ca^{2+} was determined with a F2112Ca selectrode (Radiometer). The calibration curve was measured in the following mixture: 14 mM HEPES–KOH, pH 7.5, containing 0.28 M KCl with different Ca^{2+} concentrations. When measuring free Ca^{2+}, the reaction mixture did not contain calpain itself.

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue G.

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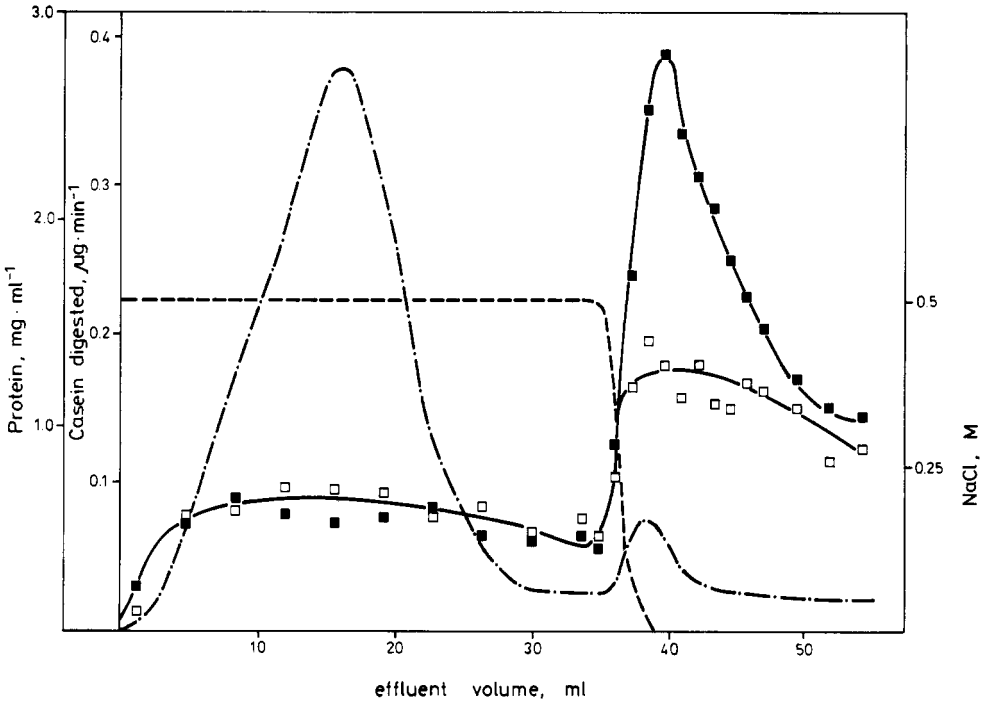

FIGURE 1: Phenyl-Sepharose CL-4B chromatography of the crude *Drosophila* extract. Symbols: proteolytic activity in the presence (■) and absence (□) of Ca^{2+}; (– –), NaCl gradient; (– · –), protein concentration.

Protein was assayed according to Read and Northcote (1981).

Preparation Procedure of Drosophila CANP. Crude extracts: Ten grams of frozen adult flies was homogenized in 40 mL of the following buffer: 20 mM HEPES–KOH, pH 7.5, containing 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, and 0.5 mM dithioerythritol, in a glass–glass homogenizer by 13 strokes. The homogenate was centrifuged at 105,000*g*, for 60 min, at 4 °C. To the supernatant (approximately 35 mL) the same volume of 100% saturated $(NH_4)_2SO_4$ in buffer A was added (50% saturation). The mixture was allowed to stand at 0 °C for at least 0.5 h (conveniently overnight). The precipitate was centrifuged at 20000*g* for 20 min at 4 °C and resuspended in 5 mL of buffer A containing 0.5 M NaCl. The insoluble debris was removed by centrifugation (20000*g*, 30 min, 4 °C), and the clear supernatant was applied onto a Phenyl-Sepharose column.

Phenyl-Sepharose chromatography (Figure 1): The Phenyl-Sepharose CL-4B column (0.65 × 10 cm) was equilibrated with buffer A containing 0.5 M NaCl. The crude extract was loaded onto the column. The unbound material was washed out by the equilibrating buffer. The fractions containing Ca^{2+}-activated protease activity were eluted by buffer A.

Reactive Red-Agarose chromatography (Figure 2): Reactive Red-120 Agarose column (0.65 × 5 cm) was equilibrated with buffer A containing 0.5 M NaCl. The conductivity of pooled CANP fractions from the previous step was adjusted with 5 M NaCl to the same value as that of the equilibrating buffer, and the sample was applied to the column. The resin was washed with the high-salt buffer free of protein. CANP was eluted by buffer A.

MonoQ or Q-Sepharose chromatography (Figure 3): MonoQ HR 5/5 was equilibrated with buffer A. Reactive Red-purified CANP was directly applied onto the column. After a 5-min prewashing (1 mL/min), the gradient-program shown in Figure 3 was started. Alternatively, a small Q-Sepharose fast flow column (0.65 × 3 cm) equilibrated with buffer A was used. The stepwise elution was designed on the basis of the MonoQ elution curve. After loading the crude CANP, the column was washed with about 40 mL of buffer A, then with 40 mL of buffer A containing 0.3 M NaCl; CANP was eluted with buffer A containing 0.5 M NaCl.

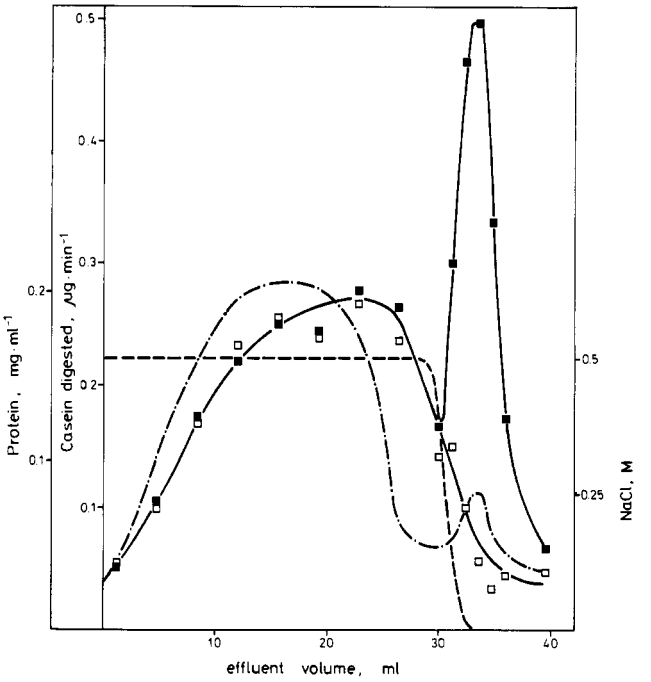

FIGURE 2: Reactive Red-Agarose chromatography of Phenyl-Sepharose-purified CANP. Symbols are as in Figure 1.

RESULTS AND DISCUSSION

Table I summarizes the data of the purification procedure. CANP activity becomes measurable only after the Phenyl-Sepharose step (Figure 1), probably owing to the presence of inhibitor(s) in the earlier steps. The total activity after Phenyl-Sepharose chromatography may come from more than one enzyme, as observed in previous studies on Ca^{2+}-activated proteases (Kubota & Suzuki, 1982). Reactive Red-Agarose

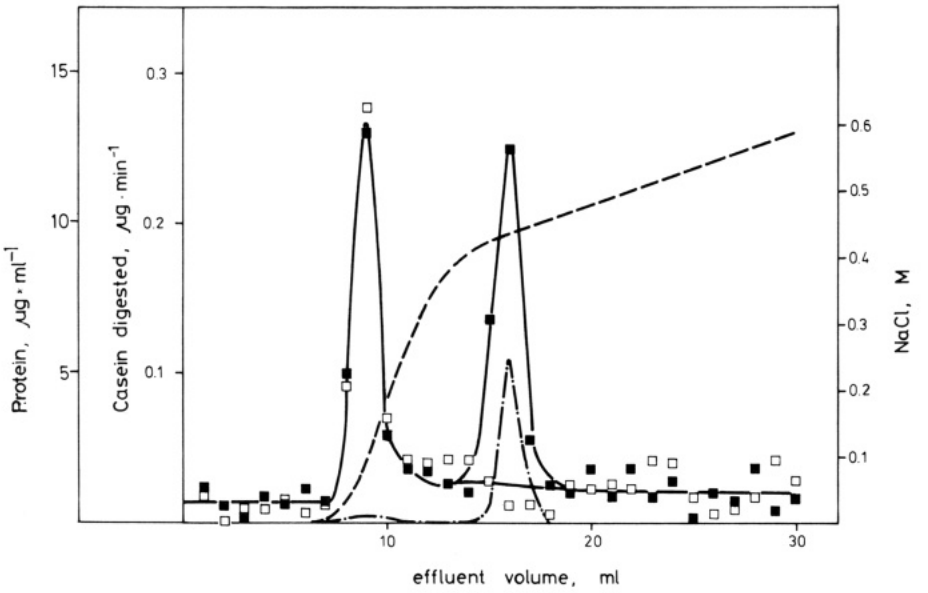

FIGURE 3: MonoQ chromatography of CANP peak from Reactive Red-Agarose. Symbols are as in Figure 1.

Table I: Purification of CANP from *Drosophila*

	total protein, mg	total act., μg of casein digested min^{-1}	sp act., μg of casein digested min^{-1} mg^{-1}	yield, %
100000*g* supernatant	60			
$(NH_4)_2SO_4$ pptn	40			
Phenyl-Sepharose[a]	2	140	(70)	(100)
Reactive Red Agarose	0.12	60	500	43
Q-Sepharose	0.01	5	500	4

[a] Both CANP I and II are bound to the resin. Values in parentheses may arise from two enzymes of text.

chromatography (Figure 2) results in a major purification of the enzyme. It should be mentioned that this affinant binds calpain II, but not calpain I, from mammalian sources (Clark et al., 1986). In the present Reactive Red-Agarose run only one Ca^{2+}-activated protease peak could be observed corresponding to the elution position of mammalian calpain II. Although the Q-Sepharose chromatography (Figure 3) apparently did not increase specific activity, it removed some contaminating minor proteins visible on SDS–PAGE after Reactive Red-Agarose (not shown) along with an unidentified Ca^{2+}-independent protease activity. The lack of specific activity increase at this step was probably due to the partial inactivation of the enzyme caused by the extremely low amount of protein. (In pilot experiments with 10 μg of protein at the Phenyl-Sepharose step the activity loss was likewise about 90%.) The final product gave a single band on SDS–PAGE of $M_{r,app}$ = 94K, whose intensity was roughly proportional to the CANP activity of the sample (Figure 4). On two-dimensional electrophoresis (Figure 5) a major spot (M_r = 94K, p*I* = 5.46) and a minor spot (M_r = 86K, p*I* = 5.65) were discernible. The minor spot must correspond to a degradation product of the major 94-kDa form, because (i) it was hardly present in one-dimensional SDS–PAGE (Figure 4) made right after the Q-Sepharose chromatography and (ii) it appeared after the isoelectric focusing step in relative amounts that varied from one preparation to the other.

The specific activity we measured for the pure enzyme, 500 μg of casein digested min^{-1} (mg of protein)$^{-1}$, is probably a lower estimate, because of the substantial loss in total activity in the Q-Sepharose step. Nevertheless, it can be compared with other Ca^{2+}-activated neutral proteases for which data

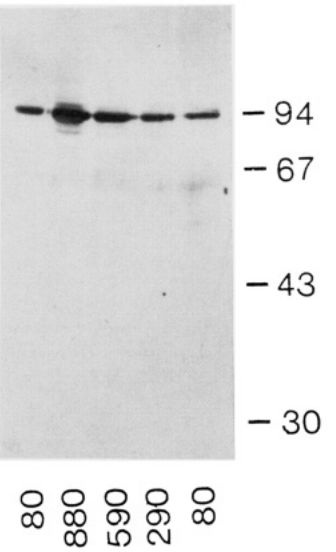

FIGURE 4: Silver-stained SDS–PAGE picture of Q-Sepharose fast flow peak fractions. 19-μL fractions were applied to the gel. Numbers under the lanes indicate CANP activity of the same fractions (ng of casein digested by 100 μL of fraction in 1 h).

are available on methylated casein as substrate. Thus, the specific activity of CANP1, CANP2, and CANP3 from calf brain, expressed in the same units as above, was 690, 1708, and 267, respectively (Malik et al., 1987a), whereas that of human platelet CANP-I was 1105 (Malik et al., 1987b). These activities, however, were measured at 37 °C instead of 30 °C used in the present study. The specific activity of *Drosophila* CANP therefore falls in the range of mammalian Ca^{2+}-activated neutral proteases.

The Ca^{2+}-activation curve of the enzyme (Figure 6) demonstrates that half-maximal activation is attained at about 0.6 mM free Ca^{2+}. This value agrees with the same parameter of mammalian calpain IIs (Zimmerman & Schlaepfer, 1984). Rat kidney calpain I, but not calpain II, could be activated in the absence of Ca^{2+} by Mn^{2+} and Ba^{2+} (Yoshimura et al., 1983). We tested *Drosophila* CANP in the absence of Ca^{2+}: the Mn^{2+} and Ba^{2+} ions were ineffective, just like Mg^{2+}, Zn^{2+}, and Cd^{2+}, all at 4.25 mM concentration. These data with divalent cations again point to the resemblance of *Drosophila* CANP to mammalian calpain II.

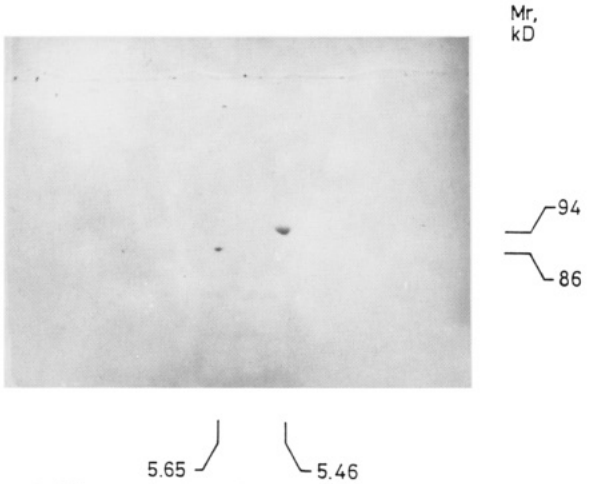

FIGURE 5: Silver-stained two-dimensional gel analysis of about 0.4 μg of purified *Drosophila* CANP. The M_r and p*I* values of spots are indicated.

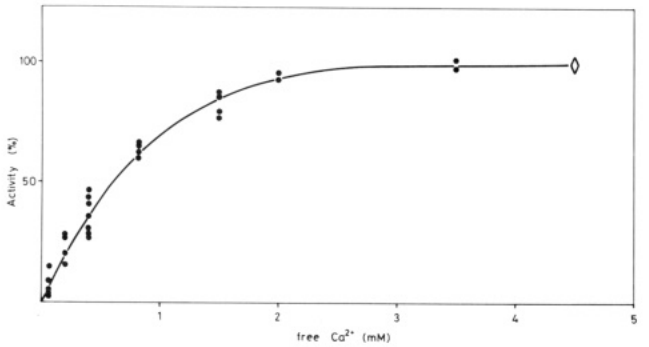

FIGURE 6: Ca^{2+}-activation curve of purified *Drosophila* CANP. Symbol (◊) indicates CANP activity measured under the standard assay conditions. 100% enzyme activity corresponds to 0.3–0.9 μg of casein digested by 100 μL of Q-Sepharose-purified CANP in 1 h.

Table II: Effect of Protease Inhibitors on CANP II from *Drosophila*

inhibitor	remaining act., %
none	100[a]
phenylmethanesulfonyl fluoride (1.3 mM)	100
benzamidine (5 mM)	81
N-tosyl-L-phenylalanine chloromethyl ketone (1 mM)	<5
leupeptin (27 μM)	<5
iodoacetic acid (2 mM)	<5
p-hydroxymercuribenzoate (1 mM)	<5
calpeptin (0.36 uM)	<5

[a] The 100% corresponds to 0.3 μg of casein digested/h in a reaction mixture. Estimated standard error is 10%.

The sensitivity of purified *Drosophila* CANP to various protease inhibitors is given in Table II. Serine protease inhibitors phenylmethanesulfonyl fluoride and benzamidine had hardly any effect, whereas thiol reagents (iodoacetic acid and *p*-hydroxymercuribenzoate), leupeptin, and calpeptin (Tsujinaka et al., 1988) completely abolished activity. These data clearly indicate that *Drosophila* CANP is a thiol protease. Furthermore, human recombinant calpastatin (Calbiochem), which corresponds to domain I of the original molecule consisting of four domains that are independent inhibitory units for calpains (Maki et al., 1987; Emori et al., 1988), fully inhibited *Drosophila* CANP at a nearly 1:1 molar ratio (Figure 7).

The above data suggest that *Drosophila* CANP is related to mammalian calpain II. However, canonical calpains consist

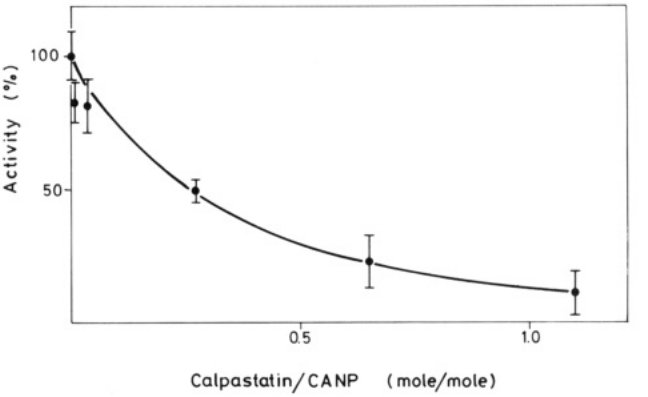

FIGURE 7: Inhibition of *Drosophila* CANP by human recombinant calpastatin domain I. The activity of 1.7 μM purified *Drosophila* CANP was measured in the standard assay (cf. Experimental Procedures) in the presence of 0–1.9 μM calpastatin. The remaining activity is plotted against the inhibitor/enzyme molar ratio. Molarities were calculated with M_r = 94K for CANP. The means ± SD of three experiments are shown.

of two subunits: a catalytic chain of about 80 kDa and a small chain of about 30 kDa. *Drosophila* CANP has an apparent molecular weight, as measured by SDS–PAGE, of 94K, which is significantly higher than would be expected for a calpain large subunit. Recently, however, a putative calpain catalytic chain, p94, has been found by screening mammalian cDNA libraries and sequenced (its calculated M_r = 94 084K) and is specific for skeletal muscle (Sorimachi et al., 1990). The extra mass in this species comes from three unique segments, two inserts and an N-terminal extension, in an otherwise conservative sequence. A similar enlargment is possible in *Drosophila* as well. A more intriguing feature is the apparent lack of a small subunit in *Drosophila* CANP. Since the small subunit is notoriously sensitive to autolysis and attack by other proteases, this is a particularly difficult issue. All our attempts at the detection of a small CANP subunit in *Drosophila* proved unsuccessful. These included the following analyses and measures:

(1) With purified preparations we never observed Coomassie or silver-stained material running with the front in various SDS–PAGE systems, which could have indicated the presence of a nicked small subunit.

(2) Throughout the purification procedure, protease inhibitors were present in the buffer, and Ca^{2+} was chelated. Several earlier purification procedures that did not include protease inhibitors still yielded calpains consisting of both large and small subunits (Waxman, 1981; Cottin et al., 1981; Croall & DeMartino, 1983). In a recent protocol (Edmunds et al., 1991) there was strong protection against proteolysis, but these authors used a very hypotonic extraction buffer, which involves the risk of unleashing lysosomal proteases, in contrast to our and other's (Zimmerman & Schlaepfer, 1988) isotonic medium.

(3) Immunoblotting with antichicken calpain antibodies raised in rabbits (kindly supplied by Dr. K. Suzuki, Tokyo Metropolitan Institute of Medical Science) according to Towbin et al. (1979) using a silver ion enhanced immunogold staining (Danscher, 1981) proved negative with the *Drosophila* enzyme, while it detected mammalian calpain II, both large and small subunits (not shown).

(4) The present purification procedure when applied to porcine brain resulted in a final product after Q-Sepharose which consisted of a major band of $M_{r,app}$ = 80K and a minor band of $M_{r,app}$ = 25 K, but only if protease inhibitors were not included in the extraction buffer (Figure 8). The reason for

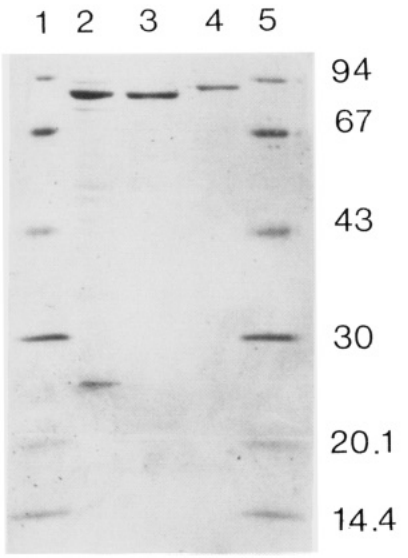

FIGURE 8: SDS–polyacrylamide gel electrophoretic pattern of *Drosophila* and porcine brain CANP preparations in 9–13.5% gradient gel. The enzymes from both sources were prepared according to the standard protocol described under Experimental Procedures, with the modifications indicated below. Lanes 1 and 5: Pharmacia LMW standard; lanes 2 and 3: procine brain CANP; lane 4: *Drosophila* CANP. In the preparations on lanes 2 and 4, benzamidine and PMSF were omitted from the buffer up to the dissolving of the ammonium sulfate precipitate.

this is unknown; we surmise that some proteolysis may help release the membrane-anchored small subunit. However, with *Drosophila* we could detect only a single polypeptide of $M_{r,app}$ = 90K under these conditions (Figure 8); the decrease from 94 to 90 kDa was probably due to the nicking of the polypeptide chain in the early phase of purification.

The above findings support the view that *Drosophila* CANP has no small subunit. Nevertheless, we cannot exclude that, despite all precautions made, an even more protective extraction procedure would not result in the detection of a small subunit. On the other hand, our data clearly show that *Drosophila* CANP does not require a small subunit to have a catalytic efficiency comparable to that of mammalian calpains. In fact, even with authentic calpains, the small subunit does not seem to be a prerequisite of catalytic activity in vitro. DeMartino and Croall (1983) could not find any difference in the specific activities of the 80-kDa large chain and of the heterodimer (80 kDa + 28 kDa) from rat liver. Fukui et al. (1988) demonstrated that a 70-kDa monomer, apparently artificially derived from a larger heterodimer, is practically as active as the heterodimeric form from polymorphonuclear leukocytes. Renaturation experiments (Tsuji & Imahori, 1981; Kikuchi et al., 1984) indeed show a greater recovery of catalytic activity in the presence of the small subunit, but the data also indicate that a great deal of the activity enhancement by the small subunit is due to a protective effect during renaturation rather than to the interaction of the two folded subunits.

In addition, calpain-like enzymes have been described which in the purified state were devoid of a small subunit, yet they were catalytically active. Such is "calpain II" from carp muscle, which is inhibited by rat liver calpastatin (Toyohara et al., 1985); "calpain II" from the muscle of the Nilotic fish Tilapia (Jiang et al., 1991); and a "high *m*-calpain" from chicken skeletal muscle (Wolfe et al., 1989). In PC12 cells a polyclonal antibody against rat calpain II detected only the large subunit, yet enzyme activity was evident (Oshima et al., 1989). According to the recent preliminary characterization of the calpain–calpastatin system in the honeybee, the apparent M_r is 80K as revealed by gel filtration for both calpain I and calpain II-like activities, which leaves hardly any room for a small subunit (Müller & Altfelder, 1991). It may well be though that more thorough analysis will eventually demonstrate a small subunit with most of these enzymes. Nevertheless, the evidence available now strongly argues for the independent catalytic competence of the large subunits. It is pertinent to mention here that Sorimachi et al. (1990) speculated that p94 might not be associated with a small subunit in muscle, since there was an about fivefold excess of p94 mRNA over small subunit mRNA in vivo.

In conclusion, the Ca^{2+}-activated neutral thiol protease we purified from *Drosophila* is in several respects similar to mammalian calpain II, while some of its properties do not fit the canonical calpain image. According to our knowledge, this is the first invertebrate Ca^{2+}-dependent thiol protease resembling mammalian calpains that has been purified to homogeneity. Further studies including cloning, sequencing, and modifying the corresopnding gene bear the promise not only to reveal the precise structure of a Ca^{2+}-activated neutral protease which is evolutionarily far from the commonly studied species but also to address the question of the physiological functions of this intriguing enzyme system.

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REFERENCES

Clark, A. F., DeMartino, G. N., & Croall, D. E. (1986) *Biochem. J. 236*, 279–282.

Cottin, P., Vidalenc, P. L., & Ducastaing, A. (1981) *FEBS Lett. 136*, 221–224.

Croall, D. E., & DeMartino, G. N. (1983) *J. Biol. Chem. 258*, 5660–5665.

Danscher, G. (1981) *Histochemistry 71*, 81–88.

DeMartino, G. N., & Croall, D. E. (1983) *Biochemistry 22*, 6287–6291.

Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F., & Goll, D. E. (1991) *Biochim. Biophys. Acta 1077*, 197–208.

Emori, Y., Kawasaki, H., Imajoh, S., Minami, Y., & Suzuki, K. (1988) *J. Biol. Chem. 263*, 2364–2370.

Fukui, I., Toyohara, H., Ito, K., Hamakubo, T., & Murachi, T. (1988) *Biochemistry 27*, 3260–3267.

Jiang, S.-T., Wang, J.-H., & Chen, C.-S. (1991) *J. Agric. Food Chem. 39*, 237–241.

Johnson, P. (1990) *Int. J. Biochem. 22*, 811–822.

Kikuchi, T., Yumoto, N., Sasaki, T., & Murachi, T. (1984) *Arch. Biochem. Biophys. 234*, 639–645.

Kubota, S., & Suzuki, K. (1982) *Biomed. Res. 3*, 699–702.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lynch, G., & Baudry, M. (1984) *Science 224*, 1057–1063.

Maki, M., Takano, E., Mori, H., Sato, A., Murachi, T., & Hatanaka, M. (1987) *FEBS Lett. 223*, 174–180.

Malik, M. N., Fenko, M. D., Sheikh, A. M., Kascsak, R. J., Tonna-DeMasi, M. S., & Wisniewski, H. M. (1987a) *Biochim. Biophys. Acta 916*, 135–144.

Malik, M. N., Ramaswamy, S., Tuzio, H., Shiekh, A. M., Fenko, M. D., Wisniewski, H. M., & Howard, R. (1987b) *Life Sci. 40*, 593–604.
Mellgren, R. L. (1987) *FASEB J. 1*, 110–115.
Müller, U., & Altfelder, K. (1991) *Insect Biochem. 21*, 473–477.
Murachi, T. (1989) *Biochem. Int. 18*, 263–294.
Mykles, D. L., & Skinner, D. M. (1986) *J. Biol. Chem. 261*, 9865–9871.
Neuhoff, V., Stamm, R., & Eibl, H. (1985) *Electrophoresis 6*, 427–448.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Okita, J. R., Frojmovic, M. M., Kristopeit, S., Wong, T., & Kunicki, T. J. (1989) *Blood 74*, 715–721.
Orrenius, S., McConkey, D. J., Jones, D. P., & Nicotera, P. (1988) *ISI Atlas of Science: Pharmacology*, Vol. 2, pp 319–324, Institute of Science Information, Philadelphia.
Oshima, M., Koizumi, S., Fujita, K., & Guroff, G. (1989) *J. Biol. Chem. 264*, 20811–20816.
Pintér, M., & Friedrich, P. (1988) *Biochem. J. 253*, 467–473.
Poehling, H.-M., & Neuhoff, V. (1981) *Electrophoresis 2*, 141–147.
Read, S. M., & Northcote, D. H. (1981) *Anal. Biochem. 116*, 53–64.
Rice, R. H., & Means, G. E. (1971) *J. Biol. Chem. 246*, 831–832.
Schollmeyer, J. E. (1988) *Science 240*, 911–913.
Siman, R., Noszek, J. C., & Kegerise, C. (1989) *J. Neurosci. 9*, 1579–1590.
Sorimachi, H., Ohmi, S., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Saido, T. C., Ohno, S., Minami, Y., & Suzuki, K. (1990) *J. Biol. Chem. 264*, 20106–20111.
Suzuki, K., Sorimachi, H., Hata, A., Ohno, S., Emori, Y., Kawasaki, H., Saido, T., Imajoh-Ohmi, S., & Akita, Y. (1990) *Neurotoxicity of Excitatory Amino Acids* (Guidott, A., Ed.) pp 79–93, Raven Press, Ltd., New York.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Toyohara, H., Makinodan, Y., Tanaka, K., & Ikeda, S. (1985) *Comp. Biochem. Physiol. 81B*, 573–578.
Tsuji, S., & Imahori, K. (1981) *J. Biochem. 90*, 233–240.
Tsujinaka, T., Kajiwara, Y., Kambayashi, J., Sakon, M., Higuchi, N., Tanaka, T., & Mori, T. (1988) *Biochem. Biophys. Res. Commun. 153*, 1201–1208.
Watanabe, N., Woude, G. F. V., Ikawa, Y., & Sagata, N. (1989) *Nature 342*, 505–518.
Waxman, L. (1981) *Methods Enzymol. 80C*, 664–680.
Wolfe, F. H., Sathe, S. K., Goll, D. E., Kleese, W. C., Edmunds, T., & Duperret, S. M. (1989) *Biochim. Biophys. Acta 998*, 236–250.
Yoshihara, Y., Ueda, H., Fujii, N., Shide, A., Yajima, H., & Satoh, M. (1990) *J. Biol. Chem. 265*, 5809–5815.
Yoshimura, N., Kikuchi, M. T., Sasaki, T., Kitahara, A., Hatanaka, M., & Murachi, T. (1983) *J. Biol. Chem. 258*, 8883–8889.
Zimmerman, U.-J. P., & Schlaepfer, W. W. (1984) *Prog. Neurobiol. 23*, 63–78.
Zimmerman, U.-J. P., & Schlaepfer, W. W. (1988) *Arch. Biochem. Biophys. 266*, 462–469.

Registry No. Ca^{2+}, 7440-70-2; calpain, 78990-62-2; calpastatin, 79079-11-1.