The calcium-dependent proteolytic system calpain–calpastatin in *Drosophila melanogaster*

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Ca²⁺-dependent proteolytic activity was detected at pH 7.5 in head extracts of the fruit fly *Drosophila melanogaster*. This activity was abolished by iodoacetate, but was unaffected by phenylmethanesulphonyl fluoride. These properties resemble those of the Ca²⁺-dependent thiol-proteinase calpain. The activity appeared at *M_r* 280 000 on Sepharose CL-6B gel chromatography. DEAE-cellulose chromatography revealed two activity peaks, with elution positions corresponding to vertebrate calpains I and II. The fly head enzymes were inhibited by a heat-stable and trypsin-sensitive component of the fly head extract, which also inhibited calpains from rat kidney. The inhibitor emerged from Sepharose CL-6B columns at *M_r* 310 000 and from DEAE-cellulose at a position corresponding to the protein inhibitor calpastatin from other sources. It is concluded that *Drosophila* heads comprise the Ca²⁺-dependent calpain–calpastatin proteolytic system.

INTRODUCTION

Calpain (EC 3.4.22.17), the Ca²⁺-activated neutral thiol proteinase, is apparently intermediate between the papain family and some other thiol proteinases, as judged from amino acid sequences around the active-site cysteine (Metrione, 1986). Since its discovery (Guroff, 1964), calpain has received ever-increasing attention. It exists as two isoenzymes (Mellgren, 1980), and has a high-*M_r* specific inhibitor protein (Waxman & Krebs, 1978; Nishiura *et al.*, 1978) named calpastatin, and a low-*M_r* activator protein (DeMartino & Blumenthal, 1982; Takeyama *et al.*, 1986). It appears that these components constitute a complex proteolytic regulatory system.

The physiological function of calpains is not clear. They have been shown to be active on a number of proteins, including enzymes, *in vitro*. Thus calpain activates phosphorylase kinase (Huston & Krebs, 1968) and phosphorylase phosphatase (Mellgren *et al.*, 1979), tryptophan hydroxylase (Hamon & Bourgoïn, 1979), adenylate cyclase (Adnot *et al.*, 1982), protein kinase C (Kishimoto *et al.*, 1983), polycation-stimulated protein phosphatase (Waelkens *et al.*, 1985), glycogen synthase (Hiraga & Tsuiki, 1986) and transglutaminase (Ando *et al.*, 1987). Calpain degrades several membrane, cytoskeletal and myofibrillar proteins (for a review, see Zimmerman & Schlaepfer, 1984a). The many actions mentioned above suggest that the calpain system plays a role in various intracellular processes.

Calpains and calpastatin are ubiquitous proteins in vertebrates: they have been found in many tissues (Murachi *et al.*, 1981a; Clark *et al.*, 1986); they are particularly abundant in nervous tissue (Nelson & Traub, 1982; Baudry *et al.*, 1986). In contrast, they have been identified only in a few invertebrates: the marine worm *Myxicola infundibulum* (Gilbert *et al.*, 1975), the Chilean squid *Dosidicus gigas* (Orrego, 1971), the crayfish *Procambarus clarkii* (Nosek & Crosland, 1983), the Bermuda land crab *Gecarcinus lateralis* and the lobster *Homarus americanus* (Mykles & Skinner, 1983, 1986).

We decided to examine whether the calpain system is present in another invertebrate, the fruit fly *Drosophila melanogaster*, in view of the advantages offered by this organism in genetic manipulations, namely that, by inducing mutants defective in various members of this system, insight could be gained into the physiological functions of calpain. In the present paper we report the detection of calpains I and II, as well as of calpastatin, in heads of *Drosophila melanogaster*.

MATERIALS AND METHODS

Materials

Drosophila melanogaster of the Canton-S wild-type strain was used. Flies were maintained and heads were separated as described by Dévay *et al.* (1986).

The sources of materials were as follows: Hammarsten casein and trypsin inhibitor (Serva); leupeptin, pepstatin A, PMSF and tosyl-L-phenylalanylchloromethane (Sigma); DEAE-cellulose DE-52 (Whatman); Sepharose CL-6B (Pharmacia); trypsin, B grade (Calbiochem). The other chemicals were of the highest grade commercially available.

Fly head extract

Canton-S fly heads were homogenized in 0.82 vol. of 20 mM-Hepes/KOH buffer, pH 7.5, containing 5 mM-EGTA, 5 mM-EDTA, 250 mM-sucrose and 0.5 mM-DTE at 0 °C in a glass/glass homogenizer with 13 strokes. The homogenate was centrifuged at 105 000 *g* for 60 min at 4 °C. The protein concentration of the supernatant was about 10 mg/ml.

Crude rat kidney calpain

Rat kidneys were freed from connective tissue, minced and homogenized in 4 vol. of the buffer used for fly heads. The supernatant (105 000 *g*, 60 min, 4 °C) was adjusted to pH 4.5 by addition of acetic acid. This treatment precipitates calpain II (Suzuki *et al.*, 1979). After centrifugation (10 000 *g*, 10 min, 6 °C) the pre-

Table 1. Calpain activities in *Drosophila* heads and rat kidney

Results are means of three independent experiments; the s.d. was $\sim 12\%$. Fly head extract was subjected to fractionation at pH 4.5 as described for crude rat kidney calpain in the Materials and methods section.

Sample	Protein concn. (mg/ml)	Specific calpain activity (nmol of tyrosine/h per mg of protein)	
		In sample	Related to original extract*
Fly head extract after fractionation at pH 4.5	0.76	4.6	0.36
Crude rat kidney calpain	3.5	65	28

* Calpain activity of sample related to the protein concentration of original extract, which was 10 mg/ml for both fly heads and rat kidney.

cipitate was suspended in 80 mM-Hepes/KOH buffer, pH 7.5, containing 1 mM-EDTA and 0.5 mM-DTE; the final volume was identical with that of the original kidney supernatant. The insoluble material was removed by centrifugation (10000 g, 15 min, 6 °C). This preparation (crude rat kidney calpain, ~ 3.5 mg of protein/ml) could be stored for prolonged times at -20 °C after addition of neutralized glycerol to 50 % (v/v) final concn. (rat calpain stock solution).

Ca²⁺-dependent proteinase assay

Calpain activity was measured with alkali-denatured casein as substrate (Guroff, 1964). The reaction mixture contained 100 μ l of sample, 5 μ l of casein (28 mg/ml) and 100 μ l of assay buffer (80 mM-Hepes/KOH, pH 7.5, 1 mM-EDTA and 0.5 mM-DTE). The reaction was started by addition of 21 μ l of 80 mM-CaCl₂, and the mixture was incubated at 30 °C for various periods of time. In control experiments CaCl₂ was replaced by water. The reaction was terminated by addition of 0.1 vol. of 30 % (w/v) trichloroacetic acid, and the mixture was left at 6 °C for at least 30 min. After centrifugation (14 500 g, 2 min), the acid-soluble phenols formed were determined spectrophotometrically with α -nitroso- β -naphthol (Udenfriend & Cooper, 1952). The calibration curve for this reaction had a slope of $\Delta A_{450} = 0.07$ for 10 μ M-tyrosine generated in the reaction mixture. Calpain activity is expressed as the Ca²⁺-dependent increase in tyrosine concentration in the reaction mixture over a given time. Incubation times were kept in the range where the time course of the enzyme reaction was linear. For the measurement of fly head calpastatin activity, either the crude rat kidney calpain or stock solution was used. The reaction mixture was as above, but the assay buffer was replaced by the appropriately diluted kidney calpain preparation.

Protein assay

Protein was determined as described by Read & Northcote (1981) with dye reagent 2, with bovine serum albumin as standard.

RESULTS

Calpain isoenzymes in fly heads

In the unfractionated fly head extract there is no

Fig. 1. Gel-chromatographic analysis of Ca²⁺-dependent-proteinase activity of fly head extract

A Sepharose CL-6B column (0.65 cm $\times$ 95.3 cm) was equilibrated with 10 mM-Hepes/KOH buffer, pH 7.5, containing 1 mM-EDTA, 135 mM-KCl and 0.5 mM-DTE, and 1.3 ml of fly head extract was loaded on the column. Flow rate was 4.2 ml/h, and 0.27 ml fractions were collected. The following proteins were used for M_r calibration: lactate dehydrogenase from pig muscle (140000), aldolase from rabbit muscle (158000), catalase from bovine liver (232000), ferritin from horse spleen (440000) and thyroglobulin from bovine thyroid (669000). The arrow indicates $M_r = 280000$. Symbols: proteolytic activity in the presence (■) and absence (□) of Ca²⁺, in the presence of Ca²⁺ and 0.88 mM-iodoacetate (▲), and in the presence of Ca²⁺ and 0.88 mM-PMSF (×). Reaction time was 13 h.

Fig. 2. DEAE-cellulose DE-52 chromatographic analysis of Ca^{2+} -dependent-proteinase activity of fly head extract

Homogenization was performed in the presence of 2 mM-PMSF. The extract was dialysed overnight against two changes (1 litre each) of 10 mM-Hepes/KOH buffer, pH 7.5, containing 1 mM-EDTA and 0.05 mM-DTE. The DE-52 column (0.65 cm $\times$ 16 cm) was equilibrated with the same buffer. The dialysed extract (25 mg of protein) was applied to the column. After washing with the equilibration buffer until $A < 0.05$, a linear gradient (----) of 0–0.4 M-NaCl in the equilibration buffer was run. Flow rate was 13.3 ml/h, and 1.14 ml fractions were collected. Reaction time was 15 h. Symbols: proteolytic activity in the presence (■) and absence (□) of Ca^{2+} .

measurable Ca^{2+} -dependent proteolytic activity. However, activity becomes detectable after dialysis (against 10 mM-Hepes/KOH buffer, pH 7.5, containing 1 mM-EDTA and 50 μM -DTE) or after fractionation at pH 4.5. In Table 1 the calpain activities of fly heads and rat kidney are compared after fractionation at pH 4.5. The calpain specific activity in samples of rat kidney is about 14 times as high as in samples of fly heads. The difference is even greater if the activities are normalized to the protein concentration of the original extracts. If it is assumed that during the pH 4.5 fractionation calpain activity losses are similar, then rat kidney contains about 80 times as much calpain activity as do *Drosophila* heads, on a protein basis. The unfractionated rat kidney extract, like the fly head extract, exhibits no measurable calpain activity.

On Sepharose CL-6B gel chromatography of fly head extract, Ca^{2+} -dependent proteolytic activity was eluted in a single peak at apparent $M_r = 280000$ (Fig. 1). This activity was unaffected by PMSF, but was completely abolished by iodoacetate, as expected for a thiol proteinase. DEAE-cellulose chromatography of fly head

extract revealed three proteinase activity peaks (Fig. 2). The predominant one was Ca^{2+} -activated and was eluted at 0.26 M-NaCl, which is characteristic of vertebrate calpain II (Guroff, 1964; Zimmerman & Schlaepfer, 1984a). The middle peak was mixed, containing both Ca^{2+} -activated and Ca^{2+} -inhibited fractions, whereas the third peak, eluted at around 0.05 M-NaCl, was Ca^{2+} -independent. The fractions eluted between 0.09 M- and 0.15 M-NaCl, which correspond to the elution position of calpain I of vertebrates (Mellgren, 1980), were pooled and loaded on a Sepharose CL-6B column. In the eluate a Ca^{2+} -activated proteinase peak appeared at $M_r = 280000$ (Fig. 3), which can then be attributed to calpain I. Gel chromatography of the calpain II peak from Fig. 2 also yielded apparent $M_r = 280000$ (Fig. 4). Although the progress curve of calpain II was linear up to at least 15 h under the conditions applied, that of calpain I was anomalous: tyrosine release started only after a lag phase of about 10 h, and then it was linear with time for at least 6 h. The slopes gave activity values of about 20 μM - and 60 μM -tyrosine released/h per g of fly head for calpains I and II respectively.

Fig. 3. Gel-chromatographic analysis of fractions eluted from DE-52 between 0.09 M- and 0.15 M-NaCl

Fractions 23–29 from Fig. 2 were pooled and concentrated by ultrafiltration to 1.5 ml over an Amicon UM 10 membrane. A 1 ml sample (1.5 mg of protein) was applied to a Sepharose CL-6B column (0.65 cm × 94 cm). Reaction time of the proteinase assay was 17.5 h. The arrow indicates $M_r = 280000$. For other details see Fig. 1. Symbols: proteolytic activity in the presence (■) and absence (□) of Ca^{2+} .

Fig. 4. Sepharose CL-6B gel chromatography of calpain II separated by DE-52 chromatography

Fractions 38–46 from Fig. 2 were pooled and concentrated over an Amicon UM 10 membrane (0.54 mg of protein in 0.8 ml), and this sample was applied to the column (0.65 cm × 94 cm). Reaction time was 13 h. The arrow indicates $M_r = 280000$. For other details see Fig. 1. Symbols: proteolytic activity in the presence (■) and absence (□) of Ca^{2+} and in the presence of Ca^{2+} and calpastatin (100 μl of pooled fractions 14–17 from Fig. 5) (●). The pooled peak fractions represent an about 80-fold purification of calpain activity.

The Ca^{2+} -dependence of proteolytic activity for fractions of calpains I and II is shown in Fig. 5. The value for 50% activation of calpain II is 1.4 mM- Ca^{2+} , which is close to that found for calpain II purified from carp muscle (Toyohara *et al.*, 1985). With the calpain I fraction 50% activation was observed at 1.0 mM- Ca^{2+} , but this activation curve is anomalous, exhibiting strong sigmoidicity. Although the reason of this sigmoidicity is unknown, we think that it is due to some Ca^{2+} -binding species other than calpain in the fraction. In fact, the specific activity of the calpain I fraction was about one-tenth of that of the calpain II fraction, and the former exhibited several protein bands on SDS/polyacrylamide-gel electrophoresis (results not shown). To measure the true Ca^{2+} -sensitivity of *Drosophila* calpain I thus needs further purification of the enzyme. In any case, a greater Ca^{2+} -sensitivity of calpain I, as compared with calpain II, is suggested by the fact that calpain I activity approaches a maximum at a significantly lower Ca^{2+} concentration than does calpain II activity. A sharp threshold in Ca^{2+} -dependence, similar to that of impure calpain I, has previously been observed with phosphorylase kinase in the rabbit muscle glycogen particle; this threshold disappeared and half-activation was obtained at a lower Ca^{2+} concentration after purification of the enzyme (Heilmeyer *et al.*, 1970).

Fig. 5. Ca^{2+} -dependence of proteinase activity of partially purified calpain I (□, from Fig. 3) and calpain II (●, from Fig. 4)

Activities were measured at various CaCl_2 concentrations, and Ca^{2+} values were calculated as Ca^{2+} not complexed by EDTA at pH 7.5 ($\text{p}K_D = 7.78$).

Fig. 6. Detection of *Drosophila* calpastatin by DE-52 chromatography

Fly heads were homogenized in 4 vol. of buffer (see the Materials and methods section), the supernatant was adjusted to pH 4.5 and the acidic precipitate was removed by centrifugation as described for crude rat kidney calpain in the Materials and methods section. The supernatant was neutralized with 0.5 M-KOH and kept in a boiling-water bath for 10 min. After centrifugation (10000 g, 10 min, 6 °C), 15 ml of supernatant (6 mg of protein) was loaded on a DE-52 column (0.65 cm × 7 cm). The column was equilibrated and developed as described in Fig. 2. The inhibitor was detected on the basis of inhibition of crude rat kidney calpain. The reaction mixtures contained 0.18 vol. of kidney calpain; the slope of the calpain progress curves was 5 μM-tyrosine released/min. Reaction time was 30 min. Symbols: rat calpain activity in the presence of DE-52 fractions with (■) and without (□) Ca²⁺; ———, NaCl gradient. The pooled trough fractions represent an about 160-fold purification of calpastatin activity.

Calpastatin in fly heads

Comparison of Figs. 2 and 3 suggests that there are some inhibitor(s) of calpain in the fly head extract. To check this, the fly head extract was fractionated at pH 4.5, the precipitate was removed by centrifugation, and the neutralized supernatant, which for vertebrates is expected to contain calpastatin (Ishiura *et al.*, 1982; Brooks *et al.*, 1983), was heat-treated and applied to a DEAE-cellulose DE-52 column. The column was developed with a linear NaCl gradient. To the fractions equal amounts of crude rat kidney calpain were added, and assayed for calpain activity. As seen in Fig. 6, a deep trough of Ca²⁺-dependent proteinase activity appeared in the elution profile around 0.06 M-NaCl. This is the elution range observed for mammalian calpastatin (Waxman, 1981; Murachi *et al.*, 1981b; Mellgren & Carr, 1983). To check whether the inhibitor from fly heads is protein in nature, the pooled fractions from the inhibitor trough of Fig. 6 were treated with trypsin (0.15 mg/ml) at 30 °C for 30 min, and then trypsin action

Fig. 7. Effect of *Drosophila* calpastatin, prepared as described in Fig. 6, on the activities of calpain II (●) and papain (□; 4.7 μg in the reaction mixture)

Calpain II was purified by precipitation at pH 4.5 and DE-52 chromatography; its activity was 1.45 μM-tyrosine released/h in the reaction mixture.

was terminated by addition of soya-bean trypsin inhibitor in 3-fold molar excess. The fraction thus treated had no calpain-inhibitor activity.

The action of pooled inhibitor fraction from the DE-52 column was also examined against the separated fruit-fly calpain I and II. As shown in Fig. 4, the calpain II activity peak was completely abolished by this fraction, and the same held for calpain I (results not shown). Calpain II was inhibited by calpastatin in a dose-dependent manner, whereas papain activity was unaffected by the same amounts of calpastatin (Fig. 7).

The M_r of calpain inhibitor from fly heads was estimated by Sepharose CL-6B gel chromatography. In the elution profile (Fig. 8) there is an inhibition trough at $M_r = 310000$, and a broad range of inhibition toward lower M_r values. The M_r -310000 trough could only be observed if the fly head extract was made in buffer supplemented with proteinase inhibitors (cf. Fig. 8); in the absence of the latter, only inhibition towards the lower- M_r range was detectable, which probably originates from endogenous Ca²⁺ chelators and inhibitory peptides. The high- M_r fractions were pooled, heat-treated, dialysed and loaded on to a DE-52 column as described in Fig. 6. The calpain inhibitor was eluted at 0.10 M-NaCl (Fig. 8 insert), i.e. in the elution range of calpastatin from other sources. We conclude that fly heads contain the high- M_r inhibitor protein calpastatin.

DISCUSSION

Ca²⁺-activated neutral proteinases (calpains) have been detected in heads of *Drosophila melanogaster* according to the following criteria. (i) There was Ca²⁺-activated proteinase activity at pH 7.5 in fly head extracts

Fig. 8. Detection of *Drosophila* calpastatin by Sepharose CL-6B gel chromatography

The buffer used for homogenization of fly heads also contained the following proteinase inhibitors: 1.9 mM-PMSF, 4.8 mM-benzamidine, 2.3 mM-*o*-phenanthroline, 1.2 μ M-trypsin inhibitor, 0.23 mM-tosyl-L-phenylalanylchloromethane, 0.23 mg of leupeptin/ml and 0.1 mg of pepstatin A/ml. A 1 ml portion of extract was applied to the column (0.65 cm $\times$ 97.3 cm); 310 μ l fractions were collected, and calpastatin activity was detected with rat calpain stock solution. The reaction mixture contained 0.88 vol. of stock solution; the slope of calpain progress curve was 2.7 μ M-tyrosine released/min. The endogenous proteolytic activity of fractions was taken into account. The arrow indicates $M_r = 310000$. For other details see Fig. 1. Symbols: rat calpain activity in the presence of Sepharose CL-6B fractions with (■) and without (□) Ca^{2+} . Insert: DE-52 column (0.65 cm $\times$ 7 cm) chromatography of high- M_r calpastatin (fractions 70–81 in the main Figure). The pooled fractions were heat-treated as described in Fig. 6. The supernatant was dialysed and chromatographed (0.1 mg of protein) as in Fig. 2; 750 μ l fractions were collected and assayed for calpastatin activity with rat calpain stock solution. The reaction mixtures contained 0.88 vol. of stock solution; the slope of the calpain progress curve was 1.75 μ M-tyrosine released/min. Symbols are as in Fig. 6.

fractionated by precipitation at pH 4.5, or by gel chromatography or by ion-exchange chromatography. (ii) This activity was sensitive to iodoacetate, but was unaffected by PMSF, characteristic of thiol proteinases. (iii) Two Ca^{2+} -dependent proteinase activity peaks were separated by DE-52 chromatography, with elution positions corresponding to calpains I and II from vertebrates (Mellgren, 1980; Zimmerman & Schlaepfer, 1984a, and references therein); the two activities had different Ca^{2+} -dependences, although the apparent Ca^{2+} -sensitivity of the first activity is likely to be fallaciously low. Altogether, we assign the two activities from fly heads tentatively to calpains I and II.

In the unfractionated fly head extract we could not detect any calpain activity, probably owing to the presence of endogenous inhibitor(s). After fractionation at pH 4.5, the amount of calpain activity from fly heads was about 1/80 of that from rat kidney, on the basis of protein content of the original extract (Table 1). The apparent scarcity of *Drosophila* calpains in fly heads necessitated unusually long incubation times in the activity assays.

The apparent M_r of *Drosophila* calpains was found by gel chromatography to be 280000, whether analysed directly from head extract (Fig. 1) or after resolution by DE-52 chromatography and calpain I and II were run separately (Figs. 3 and 4). The M_r value of 280000 is considerably higher than that found for calpains from most other sources, which is 110000–150000 (cf. Zimmerman & Schlaepfer, 1984a). Detailed structural

analysis has revealed that mammalian and avian calpains are heterodimers consisting of a large, $M_r \approx 80000$, catalytic subunit, and a small, $M_r \approx 30000$, subunit (Ohno *et al.*, 1984; Sakihama *et al.*, 1985; Aoki *et al.*, 1986). At present we cannot offer any clear-cut explanation for the unusually high apparent M_r of *Drosophila* calpains. It may well be that some other component of fly heads associates with calpains tightly, so that these associations survive chromatographic separations. In fact, the association of some protein kinases with rat muscle calpain has been described (Zimmerman & Schlaepfer, 1984b). Furthermore, crustacean calpains have been reported to range in M_r between 310000 and 59000 (Mykles & Skinner, 1986).

In fly head extracts we could detect a high- M_r component that inhibited rat kidney calpain, as well as *Drosophila* calpains I and II. The inhibitor was heat-stable but sensitive to trypsin, which are properties characteristic of calpastatin from other sources (Nishiura *et al.*, 1978; Murachi *et al.*, 1981b). The elution position on DEAE-cellulose of this inhibitor also agreed with that of vertebrate calpastatin (Waxman, 1981) as did its apparent M_r (310000), which is close to the range $M_r = 270000$ –300000 observed for mammalian calpastatins (Murachi *et al.*, 1981b). It is concluded that the specific protein inhibitor of calpains, calpastatin, is also present in *Drosophila* heads.

The anomalous progress curve of *Drosophila* calpain I, showing a prolonged lag phase, needs comment. We attribute this phenomenon to the co-purification of

calpain I and calpastatin on DEAE-cellulose (cf. also Waxman, 1981), and partially also on Sepharose CL-6B. Calpastatin in vertebrates has been shown to be a substrate of calpains, and some of the degradation products were also inhibitory (Shigeta *et al.*, 1984; Mellgren *et al.*, 1986). After extensive digestion, calpain may eliminate the inhibitory effect of calpastatin and then attack the casein substrate; this would explain the long latency. The fact we did not observe such a lag phase after direct gel chromatography (Fig. 1) can be rationalized by our observation (not shown here) that the M_r -310000 inhibitor could only be detected if the extract was protected against proteolysis (cf. Fig. 8).

The identification of calpains and calpastatin in *Drosophila melanogaster* paves the way toward the isolation of fly populations deficient in various components of this proteolytic system, in the hope of getting meaningful answers about the physiological function of calpains.

We thank Miss Anikó Rozgonyi for the skillful technical assistance. This work was supported in part by grants Tt 175/1986 and AKA 1-3-86-223.

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Received 20 July 1987/8 March 1988; accepted 29 March 1988