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Cardosins: A New and Efficient Plant Enzymatic Tool to Dissociate Neuronal Cells for the Establishment of Cell Cultures

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ABSTRACT: In the present work, we examined the feasibility of using cardosins, plant aspartic-proteinases from *Cynara cardunculus* L., to isolate cells from rat embryonic brain. Using morphological and functional assays, we compared cell cultures obtained with cardosins with those prepared with a well-established trypsin protocol. Cardosins and trypsin dissociation produced cells with similar yield, viability, and GABA release in response to a depolarizing stimulus. However, cardosins-dissociated cells appeared to recover faster in culture, as assessed by the MTT-test and by the number and length of neurtites, suggesting that cardosins are less aggressive to neurons than trypsin. This feature might be helpful for research and medical purposes requiring fast manipulations of cells.
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KEYWORDS: cardosins; tissue dissociation; cell viability; neuronal cultures; trypsin

Introduction

The establishment of primary cell cultures is a widely used tool in Molecular Biology, Toxicology, and Biotechnology. Cell culture requires that the tissue be dispersed into a cell suspension, which is usually achieved by a combined mechanical and enzymatic procedure. Trypsin is by far the most common enzyme used in tissue disaggregation, since it is well tolerated by many cells and has been demonstrated to be effective for many tissues (Andersen, 2002; Agostinho and Oliveira, 2003; Brewer, 1997; Hung et al., 2005; Svenningsen et al., 2003). The purer the trypsin, the less toxic it becomes. On the other hand, the cruder the trypsin, the more effective it may be due to other proteases present in the mixture. Nevertheless, it is important to minimize the exposure of cells to active enzymes to preserve maximum viability.

We have been working with cardosins, plant aspartic proteinases (AP) extracted from pistils of *Cynara cardunculus*, L., which show caseinolytic and collagenolytic activity towards specific peptide bonds (Duarte et al., 2005; Egas et al., 2000; Frazão et al., 1999; Pereira et al., 2005; Sarmento et al., 2003, 2006; Veríssimo et al., 1996), contrasting to trypsin that exhibit a wide specificity (Czapinska and Otlewski, 1999). The collagenase-like specificity of the enzyme, combined with its stability and ease of preparation, raised the possibility of its use for the establishment of primary cell cultures. Additionally, a new biotechnology project concerning large-scale production of cardosins for commercialization is being developed in Biocant (www.biocant.pt).

In the present study, we have developed a new protocol for embryonic brain dissociation using cardosins followed by the culture of neuronal cells in same commercially available defined media and the same plating substrates. Furthermore, cardosins appear to be less aggressive to

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neuronal cells when compared to trypsin, allowing a faster cell recovery, a feature that might be important for research or medical purposes requiring fast processing of cells.

Materials and Methods

Purification of Cardosins

Cardosins were purified according to Sarmento et al. (2004), by a method that allows the purification of high amounts of protein. Briefly, stigmas from fresh flowers were homogenized in a mortar and pestle in sodium citrate 100 mM, pH 3.5, and centrifuged. The supernatant was applied to a Hiload Superdex 75 semi prep (Amersham, GE Healthcare, Buckinghamshire, UK) equilibrated and eluted with 25 mM Tris-HCl pH 7.6 at a flow rate of 3 mL/min. Purity of cardosins was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970). Cardosins solutions were concentrated by lyophilization. Dried protein was stored at -80°C.

Cell Isolation and Culture

Primary cultures of brain cortical cells were prepared from Wistar rat embryos (E15–E16) as described by Agostinho and Oliveira (2003) with minor modifications. Animals were handled following the approved guidelines of National Ethical Requirements for Animal Research and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Briefly, pregnant females were sacrificed by cervical dislocation, the uterus removed under sterile conditions, and the embryos dissected on a 100-mm Petri dish with cold Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (CMFHBSS), pH 7.4. The cortecis were carefully removed and placed in CMF-HBSS containing 0.3% of BSA in a 35-mm Petri dish for subsequent removal of the meninges. After slightly mincing, the cortecis were digested with trypsin (0.1%) or cardosins (concentrations indicated in the figure legends) in CMF-HBSS containing 0.008% DNAse I (Sigma-Aldrich Chemical Co., St. Louis, MO), for 10 min at 37°C. The digestion was stopped by adding 10% fetal calf serum (FCS) and washing. The digested tissue was mechanically dissociated by gentle forcing through a 5 mL glass pipette. After centrifugation (140*g*, 5 min), the cells were resuspended in Neurobasal Medium supplemented with 2% B27 (GIBCO, Invitrogen, Carlsbad, CA), 0.5 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell viability was assessed by Tripan blue exclusion and counting on a hemocytometer. The cells were plated on poly-D-lysine (0.1 mg/mL) coated multi-well plates, at a density of 0.5×10^6 cells/cm^2, or 10-mm glass coverslips at a density of 0.25×10^6 cells/cm^2. The cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

MTT Assay of Cell Viability

The viability of cultured cells was quantitatively assessed by the MTT (3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide) reduction test. Living cells convert MTT to a dark blue, water-insoluble formazan product formed by the reduction of the tetrazolium ring of MTT by the mitochondrial succinate dehydrogenase. The cultures were incubated with 300 μL of MTT solution (0.5 mg/mL in Krebs buffer) at 37°C for 2 h. After washing, the cultures were incubated with 0.08 N HCl in isopropanol to dissolve the blue formazan product, and the optical density read at 570 nm in a microplate reader with background subtraction at 620 nm. Results were expressed as percentage of the optical density in controls.

Immunocytochemistry

Cell identity and morphology were evaluated after the immunocytochemical labelling of the neurons with an anti-MAP-2 (microtubule-associated protein 2) antibody, and the astrocytes with an anti-GFAP (glial fibrillary acidic protein) antibody (Sigma-Aldrich Chemical Co). After removing the culture medium and washing with phosphate buffered saline (PBS), the cells were fixed with 4% paraformaldehyde in PBS for 10 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min. After blocking for 90 min with 0.2% gelatin in PBS, the cells were incubated with the anti-MAP-2 (1:500) and anti-GFAP (1:200) antibodies for 90 min. After extensive washes in PBS, the cells were incubated for 60 min with the secondary antibodies: anti-mouse IgG conjugated to Alexa Fluor 594 (2 μg/mL) and anti-rabbit IgG conjugated to Alexa Fluor 498 (1 μg/mL) (Molecular Probes, Invitrogen). To assess the specificity of the immunostaining, the primary antibodies were omitted in some coverslips. Finally, the coverslips were washed thoroughly and mounted on glass slides in fluorescent mounting medium (DAKO Corporation, Copenhagen, Denmark). The preparations were observed in a Carl Zeiss fluorescence microscope, and images were acquired with the AxioVision software (Carl Zeiss Imaging Systems, Maple Grove, MN). The number of neurites and their length were evaluated in four randomized areas, in three independent preparations, with the AxioVision software, and statistically evaluated with Student's *t*-test.

GABA Release

The release of GABA was determined after loading the cells with [^{3}H]GABA (Amersham) as a tracer. After removing the culture medium and washing, the cells were incubated for 45 min at 37°C, in Na^+-medium (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1.2 H_3PO_4, 1 $CaCl_2$, 6 glucose, 10 HEPES-Na, pH 7.4) containing 25 nM GABA and 1 μCi/mL [^{3}H]GABA, in the presence of 10 μM aminooxyacetic acid

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(AOAA, an inhibitor of the GABA transaminase) to prevent GABA degradation. After loading with the radioactive neurotransmitter, the cells were washed three times with Na^+-medium, and further incubated for 15 min at 37°C. In order to depolarize the cells and to stimulate neurotransmitter release, the medium was replaced by medium containing 50 mM KCl (NaCl was partially substituted by KCl to maintain the isoosmolarity), supplemented with 10 mM NNC-711 (1-(2-(((diphenylmethylene(amino)oxy)ethyl)-1,2,5,6-tetrahydro-pyridine-carboxylic acid hydrochloride))) to prevent GABA reuptake. At the end of each experiment, the cells were lysed in 0.2 M HCl, for 5 min, to determine the remaining intracellular [^{3}H]GABA. The radioactivity was measured using Universol scintillation cocktail (ICN) and a Packard 2000 Spectometer provided with dpm correction. GABA release was expressed as the percentage of the initial intracellular content ([^{3}H]GABA left in cells plus [^{3}H]GABA released).

Data Analysis

Data are expressed as means ± SEM. Statistical significance was determined by using Student's *t*-tests, $^*P \leq 0.05$, $^{**}P \leq 0.001$, and $^{***}P \leq 0.001$, for significant, very significant, and extremely significant, respectively, as indicated in the figure legends.

Results and Discussion

Cell Yield and Viability

To study the feasibility of using cardosin to prepare neuronal cell cultures, we determined its ability to dissociate cortical tissue from rat embryonic brain, and we compared the cell yield and the cell viability with those obtained with an established protocol to prepare cortical cultures using trypsin (Agostinho and Oliveira, 2003). Figure 1a shows the cell yield of cortex dissociation with increasing concentrations of our preparation of cardosins containing cardosins A and B. Cortex dissociation with 1 mg/mL cardosins yielded a number of cells, approximately 20×10^6 cells/mg wet tissue, similar to the yield obtained with trypsin at the concentration routinely used in our laboratory to isolate cortical cells from rat embryos. The percentage of viable cells isolated with cardosins (86.49% ± 0.81 of total) was quite similar to the viability of cells dissociated with trypsin (85.56% ± 3.30). Significantly, the viability of cells obtained with cardosins was less variable (Fig. 1b) than those obtained with trypsin. Heretofore, the concentration of cardosins used for the isolation of cortical cells was 1 mg/mL since the cell yield was similar to that obtained with trypsin, and the use of higher concentrations of cardosins did not further increase the cell yield.

Cell viability was also examined upon culturing the cells (Fig. 2). Using the MTT reduction assay, we observed similar viabilities of cells isolated with cardosins or trypsin 3 h after plating, but at 24 h, the viability of cells that have been isolated with cardosins was much higher than the viability of cells isolated with trypsin ($P \leq 0.001$). The difference in viability was no longer significant upon 7 days in culture. The increase in cell viability observed upon culturing is due to cell recovery after the isolation and to the death and detachment of nonviable cells, leading to the increase in the percentage of cells capable of reducing MTT. The faster increase in cell viability observed for the cells isolated with cardosins as compared to cells isolated with trypsin suggests that cardosins are less harmful to neuronal cells.

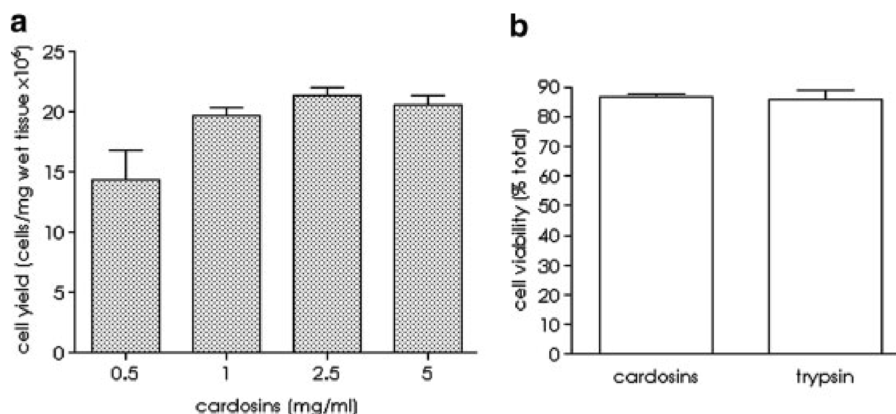

Figure 1. Cell yield of brain cortex dissociation with cardosins or trypsin. **a**: Concentration dependence of cell dissociation with cardosins. Cortices from 15 or 16 days-old rat embryos were weighed, minced, and incubated at 37°C with the indicated concentrations of cardosins. At the end of the isolation procedure, the cells were counted in a hemocytometer. The results are the means ± SEM of at least three preparations. **b**: Comparison of the viability of freshly isolated cells with cardosins (1 mg/mL) or trypsin (1 mg/mL). At the end of the isolation procedure, the cells were diluted in Na^+ medium containing Trypan blue, and observed in the hemocytometer. Cell viability was expressed as the percentage of cells that excluded the dye. The results are the means ± SEM of eight independent preparations performed in parallel with cardosins and trypsin.

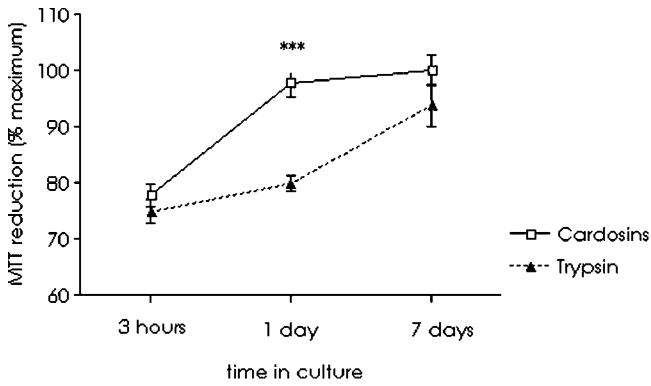

Figure 2. Viability of cell cultures prepared by tissue dissociation with cardosins (□) or trypsin (▲), both at 1 mg/mL. Cell viability was assessed with the MTT reduction assay upon 3 h, 24 h, and 7 days in culture. Data were expressed as percentage of the maximal reduction observed for cells isolated with cardosins upon 7 days in culture. The results are the means ± SEM of at least three determinations performed in triplicate in independent preparations.

Immunocytochemical and Morphometric Analysis of Cell Cultures

In order to identify the cells present in the cultures and to follow their development, we immunolabeled the cultures for a neuron-specific protein, MAP-2, and for a glial cell marker, GFAP. The immunofluorescence images (Fig. 3) show that neurons, isolated with cardosins and subsequently cultured, exhibited a normal morphology, with neurites extending from the cell bodies, demonstrating a normal cellular growth, as a result of re-expression of neuritogenesis, apparently without damage. Actually, longer neurites were observed in cultures prepared with cardosins, 24 h after plating, as compared to the cultures of cells dissociated with trypsin (Fig. 3a and b), but the differences were no longer apparent in cells cultured for longer periods (Fig. 3c and e). Quantitative analysis showed that neurons isolated with cardosins exhibited more and longer neurites (Fig. 4). The number of neurites extending longer than 25 μm was 68.0 ± 5.0 in cells that had been isolated using cardosins,

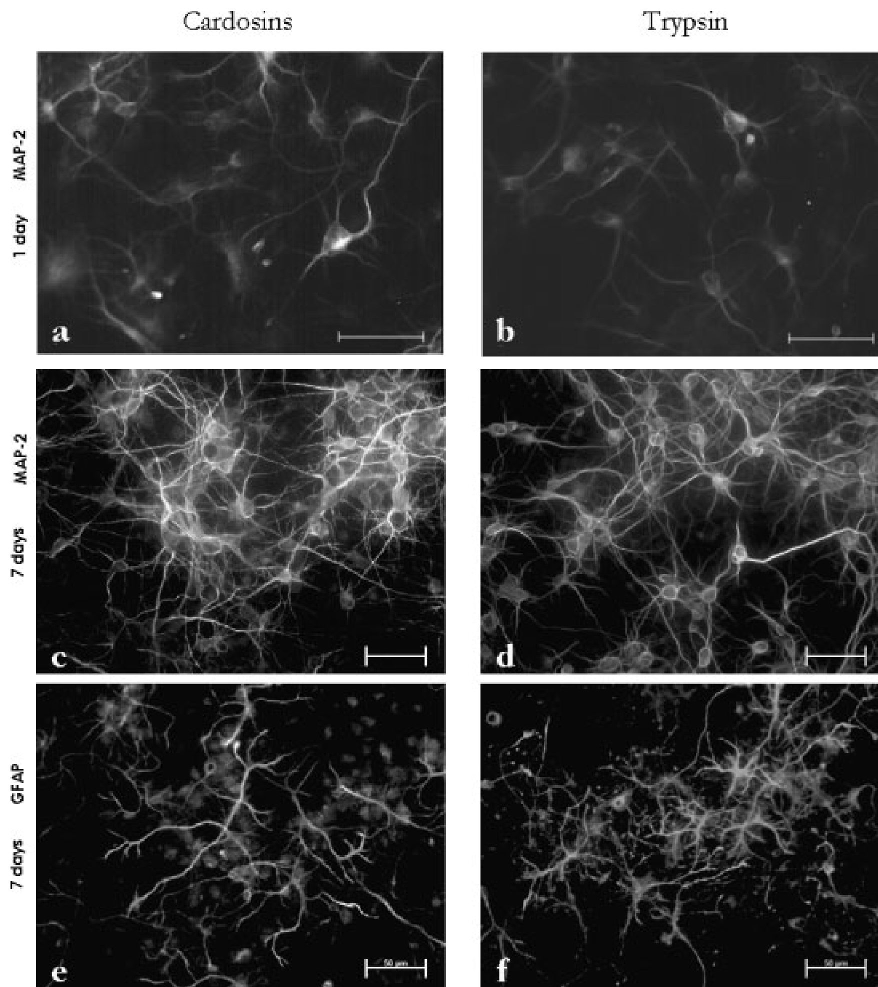

Figure 3. Immunocytochemical characterization of cell cultures prepared by cardosins or trypsin dissociation of embryonic brain cortex. The cells were cultured for 1 day (**a**, **b**), or for 7 days (**c–f**) before fixation and immunolabeling for MAP-2 (a–d), a neuronal marker, and for GFAP (e, f), a glial marker. Fluorescence microphotographies (c) and (e), and (d) and (f) are from the same fields. (Scale bar = 50 μm).

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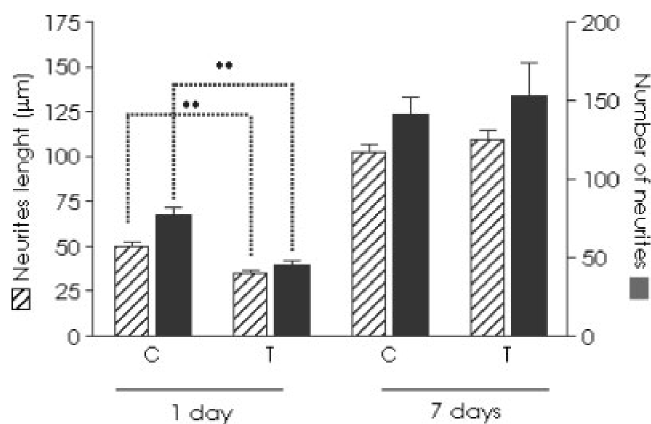

Figure 4. Morphometric analysis of neurons cultured for 1 or 7 days after cell isolation with cardosins (C) or trypsin (T). The cultures were immunostained for MAP-2, and the neurite length and the number of neurites extending longer then 25 μm were determined in four randomly chosen fields. Data shown are the means $\pm$ SEM of determinations in three independent preparations.

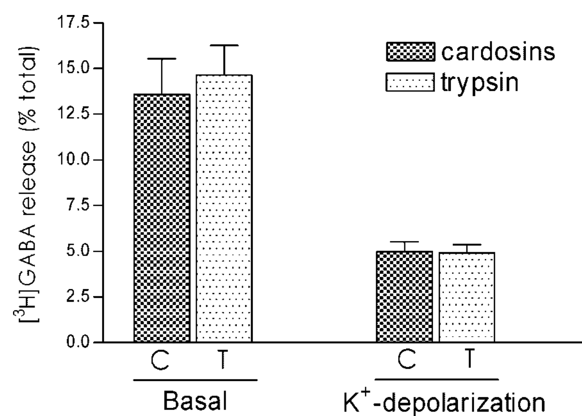

Figure 5. Comparison of GABA release from cultures prepared with cardosins or trypsin. After cell loading with [^{3}H]GABA, GABA release was determined in Na^+ medium (Basal) or in medium containing 50 mM K^+ (prepared from Na^+ medium by replacing isosmotically NaCl by KCl). GABA release evoked by K^+ depolarization was calculated by subtracting release in Na^+ medium from the release in medium containing 50 mM K^+. Values are expressed as the percentage of total cell content. Data shown are the means $\pm$ SEM of three independent determinations performed in triplicate.

which is significantly larger than in trypsin isolated cells (39.5 ± 4.0, $P \leq 0.01$). Their maximal length was 35.0 ± 4.0 μm in trypsin-generated cultures, and 50 ± 6.0 μm ($P \leq 0.01$) in cultures obtained with cardosins. No differences were perceptible after 7 days in culture. These morphological data are in agreement with cell viability data shown in Figure 2.

Immunostaining for GFAP was not observed at 1 day in culture (data not shown). Therefore, the differences in the neuronal development cannot be accounted for by differences in astrocyte support. Moreover, 7 days-old cultures showed a similar and marked astrocytic development, both in cardosin and trypsin cultures. The astrocyte growth was not unexpected since the Neurobasal medium with the B27 supplement does not hinder the proliferation of nonneuronal cells.

Functional Analysis of Cell Cultures

To examine whether the cell cultures prepared with cardosins exhibit physiological responses, we performed an assay of neurotransmitter release in response to membrane depolarization (Fig. 5). The neurotransmitter GABA was chosen because of its abundance in the cortex and because its release from cultured cells is well characterized (Santos et al., 1998). GABA release in the absence of stimulation was quite high, about 15% of the total cell content, but similar in cardosin- and trypsin-prepared cultures. This elevated release of neurotransmitters in the absence of stimulation is often observed in embryonic cultures due to high levels of spontaneous activity (van den Pol et al., 1998). Upon depolarization induced by increasing K^+ concentration in the extracellular medium, we observed an additional release of GABA, about 5% of the cell content, showing that the cells were capable of responding to a depolarizing stimulus, as expected from cells keeping a membrane potential.

In summary, we show that cardosins, aspartic proteases of plant origin, can be used to dissociate neuronal tissue to prepare cell cultures. Cell yield and viability, and the composition in neuronal and glial cells were comparable to those obtained with trypsin digestion, but the improved cell viability at early stages of culture suggest that cardosins are less aggressive than trypsin. This feature is, most likely, associated to the more restrict specificity of cardosins, demonstrated for their collagenenase-like activity (Duarte et al., 2005).

This report raises the possibility of the application of cardosins for the isolation of other types of cells, taking advantage of their collagenolytic activity, which could be especially useful for tissues with a high content of collagen. Trypsin has been shown to be less efficient in these situations and collagenases are more expensive since they require a multi-step purification method (Dioszegi et al., 1995). In contrast, the cardosins mix is obtained by a single-step purification method yielding, approximately, 10 mg of enzyme (Sarmento et al., 2004), which corresponds to five isolation procedures. Therefore, these APs, by themselves or in combination with other enzymes, constitute a promising tool, opening new avenues for cell isolation in tissues when other enzymes are not efficient. Finally, we propose that cardosins can be an alternative enzyme for subcultivation procedures, on the establishment of finite cultures and continuous cell lines, since it has been demonstrated at this point that the use of cardosins induces less cellular damage. This might prove useful for research and medical purposes whenever fast manipulation of cells is required.

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