

## Localization of proteasomes in plant cells

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**Summary.** Proteasomes, also known as multicatalytic proteinase complexes, were localized in suspension cells of potato (*Solanum tuberosum*) by direct immunofluorescence using polyclonal antibodies labelled with fluorescein isothiocyanate. The method used allows an estimate of relative amounts of proteasomal antigens in different cell components. Proteasomes are present in the nuclei and the cytoplasm. The nucleoplasm contains small areas of weak fluorescence. The peripheral cytoplasm and possibly elements of the cytoskeleton show higher fluorescence than other parts of the cytoplasm. This indicates a localization of proteasomes similar to that known from animal cells.

**Keywords:** Cytoskeleton; Immunofluorescence; Multicatalytic proteinase; Nucleus; Plant cell; Proteasome.

**Abbreviations:** DMSO dimethylsulfoxide; EGTA ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetra acetic acid; FITC fluorescein isothiocyanate; PBS phosphate buffered saline; PIPES piperazine-1,4-bis-(2-ethanesulfonic acid).

### Introduction

Proteasomes, also known as multicatalytic proteinase complexes (MCP or MPC) and isolated under a variety of different names (prosome, macropain, ingensin) constitute a major proteolytic system in cells (for reviews, see Orłowski 1990, Rivett 1993, Rivett and Knecht 1993). They are known from animal cells (Wilk and Orłowski 1983, Tanaka et al. 1986), yeast (Achsletter et al. 1984, Arrigo et al. 1987) and cells of higher plants (Kremp et al. 1986, Schliephacke et al. 1991, Skoda and Malek 1992, Ozaki et al. 1992). The plant cytoplasmic particles described by Shelton et al. (1970) are probably identical with proteasomes too. The par-

ticles are evolutionarily highly conserved (Genschick et al. 1992, Rivett and Knecht 1993) with broadly similar properties irrespective of their source (Orłowski 1990, Rivett 1993).

Proteasomes from eukaryotes are cylindrical particles of 20–23 S, about 700 kDa, composed of a set of at least 15 proteins with molecular weights of 19–35 kDa. Their proteolytic activities can be attributed to several catalytic components. Proteasomes associate with other proteins, which apparently modulate their function and therefore may be classified as regulatory factors necessary for in vivo activity (Driscoll et al. 1992, Hoffman et al. 1992, Li and Etlinger 1992). In association, a 26 S proteinase system is formed which degrades ubiquitinated proteins and probably participates in ubiquitin-independent protein degradation (Goldberg 1992, Hershko and Ciechanover 1992, Richter-Ruoff et al. 1992, Rivett 1993).

From yeast mutants we know that some proteasome subunits are essential for cell viability and growth, suggesting important functions in cellular protein turnover. Yeast mutants also allowed a function in stress induced proteolysis necessary for cell survival to be determined (Heinemeyer et al. 1991, Hilt et al. 1993).

Localization of proteasomes was investigated in various types of animal cells using different methods (reviewed in Rivett and Knecht 1993). The particles were found in the cytoplasm and nuclei of cells of vertebrates and invertebrates. The occurrence of particles, which we now know to be proteasomes, in the cytoplasm and nucleus of *Xenopus* was first described by Hügler et al. (1983). Allocation between the two compartments varies according to cell type (Haass et al. 1989) and further

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changes during development and the cell cycle (Rivett 1993).

Proteasomes have been identified in higher plants several times since 1986, but their cytolocalization was not observed. We describe the localization of proteasomes in cells of a suspension culture of *Solanum tuberosum*, revealed by direct immunofluorescence with polyclonal antibodies which allows an estimate of the relative amounts of proteasomal antigens in cellular compartments.

## Materials and methods

### *Establishment of a cell suspension culture*

A source callus culture from tuber cells of a dihaploid clone (HH 258) of *Solanum tuberosum* was grown at 23 °C in the dark on Linsmaier-Skoog medium "M 240" with 2,4-dichlorophenoxyacetic acid added according to Behnke (1975) and subcultured every three weeks. Cell suspension cultures were established after three weeks of growth by passing callus tissue through a screen to obtain small cell groups, which were cultivated with continuous shaking in the same medium without agar. The suspension culture was subcultured every 7 days. Starting with about 5 g (fresh-weight) callus tissue, 5–10 g of cells from the suspension culture were available every week. The yield was highest when suspension cells formed clusters of 3–6 cells. When separating the callus through fine-mesh nets so that single cells prevailed in the suspension, their growth was much slower.

### *Antibody isolation and loading with FITC*

Antibodies against potato proteasomes were raised in rabbits as described by Schliephacke et al. (1991). From the antiserum, the antibodies were isolated as described (Nowotny 1969) by precipitation with ammonium sulfate, centrifugation, suspending in phosphate buffer (pH 7.4) and dialyzation against the buffer (4 °C). The antibodies were quickly frozen and stored at –20 °C until labelling took place.

For labelling with FITC, according to Clark and Shepard (1963), the antibodies were precipitated with ammonium sulfate, suspended in Na<sub>2</sub>CO<sub>3</sub>-buffer (pH 9.9), dialyzed (24 h, 4 °C) against this buffer and then against the FITC containing buffer (24 h, 4 °C). The obtained antibody-FITC conjugates were separated from free FITC by gelfiltration (Sephadex PD 10). As a control, immunoglobulins from a pre-immune rabbit serum were prepared by the same method.

### *Immunofluorescence microscopy*

Cells of the suspension culture were harvested after six days by centrifugation and washed by resuspending in MS buffer (0.1 M PIPES, pH 6.9, and 3 mM EGTA). To obtain predominantly single cells the culture was passed through nylon-tissue (mesh width 100 µm). Cell fixation and incubation with FITC loaded antibodies was accomplished following Schroeder et al. (1985) and Iwasaki et al. (1988).

After washing with MS buffer cells were incubated for 60 min in a fixation and extraction medium containing formaldehyde (3.7%, v/v) as a fixative and DMSO (10%) to stabilize the cytoskeletal network. This was necessary due to reports suggesting linkage between animal cell proteasomes and cytoskeleton elements (Grossi de Sa et al. 1988 b, Briane et al. 1992). After fixation the cells were washed three times for 10 min in MS buffer.

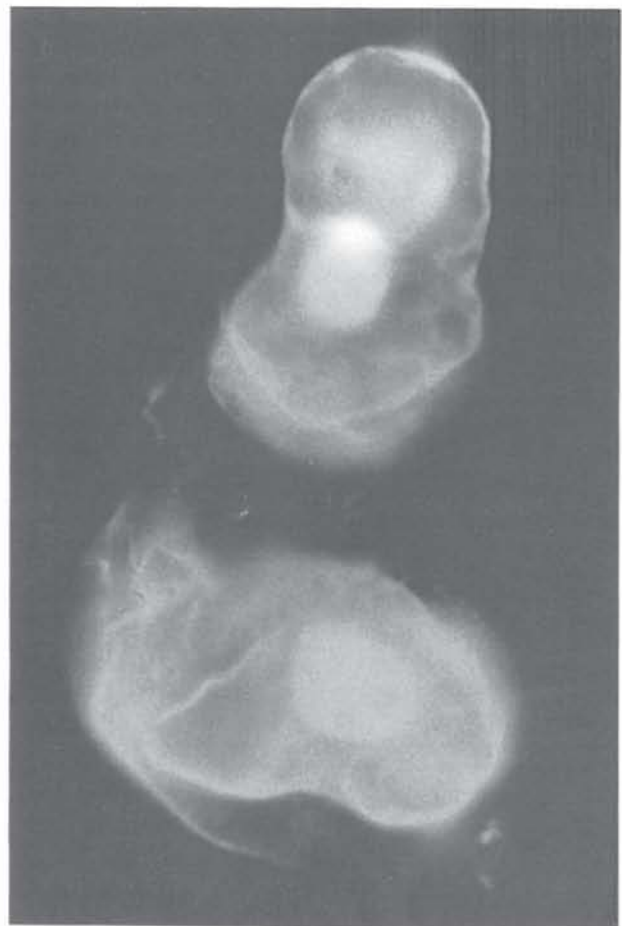
To achieve uniform permeabilization of the plasmalemma it is nec-

essary to degrade the cell walls. To achieve this, cells were treated with enzyme solution (cellulase, pectinase) in MS buffer and then rinsed with MS buffer alone.

Carefully cleaned cover-slips were incubated in poly-L-lysine (Sigma) (15 min) and dried (1 h, 60 °C). Cells were allowed to settle on the cover-slips. To remove cellular components showing non-specific fluorescence, adhered protoplasts were incubated in MS buffer (30 min, 27 °C) containing 1% (w/v) Triton X-100 and 10% (v/v) DMSO (Iwasaki et al. 1988). By this procedure, the plasmalemma was permeabilized for antibodies. After two rinses of the adhered protoplasts with MS buffer incubation with FITC loaded antibodies was accomplished (60 min, 37 °C). Superfluous free antibodies were removed by repeated careful rinsing with 0.05% Tween 20 in PBS. For microscopy, cells on the cover-slips were mounted in glycerol/PBS (1 : 1) containing 0.1% phenylene diamine (Falconer and Seagull 1985). Immunofluorescence was observed with a Zeiss epifluorescence microscope and photographed on colour slide film (400 ASA). As a control, FITC loaded pre-immune serum was used in the same procedure.

## Results

Cells incubated with FITC-labelled antibodies showed distinct fluorescence, not only in the cytoplasm but also



**Fig. 1.** Cytolocalization of proteasomal antigens in suspension cells of *Solanum tuberosum* after incubation with FITC labelled antibodies.  $\times 650$

in the nuclei (Fig. 1). This indicates the presence of proteasomes in both cell compartments. In the controls, incubated with pre-immune serum, no fluorescence was observed. In many cases the peripheral area of the cytoplasm showed more intense fluorescence than other parts. However, in most cells fluorescence intensity was highest in the nuclei. The nucleoplasm contains small areas with very weak fluorescence; these are probably the nucleoli not containing proteasomal antigens. In some cases, in cells which have lost a portion of their cytoplasm during the permeabilization procedure, a network with a diverse fluorescence is observed in the cytoplasmic space, which is perhaps formed by cytoskeletal elements. If this interpretation is correct, proteasomes may perhaps also be connected to the cytoskeleton of plant cells.

### Discussion

In cells of a suspension culture of *Solanum tuberosum* proteasomes are localized in the cytoplasm and, in comparatively higher concentrations, in the nucleus. This cytolocalization, shown here for the first time for plant cells, is similar to findings for animal cells from several investigations, beginning with Hügle et al. (1983) and reviewed by Rivett (1993). The proportion of proteasomes in the cytoplasm compared to the nucleus varies with cell type (Haass et al. 1989) and changes during development (Arrigo et al. 1988, Grainger and Winkler 1989, and others). A similar distribution for particles, named prosomes, now known to be identical with proteasomes (Grainger and Winkler 1989, Nothwang et al. 1992), was detected independently by Martins de Sa et al. (1986) and Kloetzel et al. (1987), while Grossi de Sa et al. (1988 a) showed that their distribution is dependent on differentiation processes. Connection between the proteasomes and the cytoskeleton has been demonstrated in animal cells (Grossi de Sa et al. 1988 b). By immunofluorescence methods using monoclonal antibodies against three different proteasomal proteins these authors showed that the particles were associated with cytokeratin filaments. In addition they observed free proteasomes. Essentially similar results were obtained by Briane et al. (1992).

The rather high concentration of proteasomes which we observed near the cell periphery is similar to that sometimes found for animal cells. These findings were discussed by Rivett and Knecht (1993) who postulate that the metabolism of proteins of the cytoskeletal network and its anchorage in the membrane is regulated by proteasomes accumulated near the cell surface. Such

a regulatory function is of great interest in relation to the cytoskeleton-cell wall continuum (Wyatt and Carpita 1993) in plants. Stress during cell separation may lead to the accumulation of proteasomes near the plasma membrane.

In many potato cells proteasome density was higher in the nucleus than in the cytoplasm. This is also true for many animal cells (reviewed in Rivett 1993). It is not surprising if the presumed substrates of proteasomal protein degradation in the nuclear compartment are considered. According to Rivett and Knecht (1993) these seem to be short-lived regulatory proteins, as, e.g., transcription factors. Our observations thus confirm conclusions drawn from proteasome distribution in animal cells. The important role of these particles in non-lysosomal protein degradation requires stringent control (Hershko and Ciechanover 1992) due to its significance comparable to that of regulation of protein synthesis, but not known yet.

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