

Identification of proteasome (prosome) Associated endonuclease activity in plants

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Summary:

We have identified proteasomes of sunflower hypocotyles. They elute with a molecular mass of 600 kDa from gel filtration columns and contain about 16 different protein subunits. These proteasomes hydrolyze Tobacco Mosaic Virus RNA with a cleavage pattern homologous to RNase activity of calf liver proteasomes. This is the first evidence that proteasome endonuclease activity exists in plant cells too. Thus like proteasome endopeptidase activity, proteasome RNase activity was highly conserved during evolution and the possible involvement of proteasomes in plant resistance to pathogens is discussed.

Keywords: sunflower, proteasomes, endonuclease activity, plant resistance.

Résumé :

Nous avons identifié le protéasome à partir d'hypocotyles de *tournesol* (*Helianthus annuus* L.). Sa masse moléculaire déterminée par chromatographie sur colonne de gel filtration est de 600 kDa. Il se compose de 16 sous-unités différentes. Les protéasomes ainsi purifiés sont capables d'hydrolyser l'ARN du virus de la mosaïque du tabac avec un profil de digestion homologue à celui obtenu avec les protéasomes de foie de veau. C'est la première fois qu'une activité endonucléasique portée par le protéasome est mise en évidence chez les plantes. Ainsi, comme l'est son activité peptidasique, l'activité endoribonucléasique du protéasome est très conservée au cours de l'évolution. Aussi, discuterons-nous de la possible implication du protéasome dans la résistance des plantes aux pathogènes.

Mots clés : tournesol, protéasomes, activité endoribonucléasique, résistance des plantes.

Introduction

The subribosomal fractions of eukaryotic cells contain a number of protein complexes with a relatively high sedimentation rate. Most abundant are the 20S proteasomes (for review, Coux et al., 1996). In human and animal cell systems the 20S proteasome is involved in the control of physiological processes as diverse as the cell cycle (Gordon et al., 1993), apoptosis (Drexler 1997), oncoprotein degradation (Ciechanover 1994), gene expression (Orian et al., 1995), antigen presentation (Ortiz-Navarette et al., 1991), the inflammation response (Orian et al., 1995), and the degradation of damaged proteins due to oxidative stress (Conconi and Friguet, 1997). Furthermore, it has recently been demonstrated that 20S proteasomes isolated from calf liver possess, in addition to proteolytic activity, an endonuclease activity that is particularly effective in the degradation of viral mRNAs (Petit et al., 1997) and cytokine mRNAs (Chen and Shyu, 1995). Thus proteasomes are considered to play an important role in cellular surveillance (Tanaka and Tsurumi, 1997). The 20S proteasome forms a cylindrical structure. The cylinder is made up of 28 subunits (α 7, β 7, β 7, α 7) with molecular weights ranging from 19 to 35 kDa and contains internal chambers harboring at least five different endopeptidase activities (Wilk and Orłowski, 1983). These proteolytic activities are associated with the β type subunits (Rivett et al., 1995) while the RNase activity coeluates with the α type subunits zeta and iota (Petit et al., 1997).

The 20S proteasome is highly conserved from yeast to human (Arrigo et al., 1988). However only little information exists about the structure and function of 20S proteasomes of the plant kingdom.

Originally discovered by Schmid and co-workers in tobacco leaves (Kremp et al., 1986), potatoes and mungbean (Schliephacke et al., 1991), "plant 20S proteasomes" were also purified from pea (Skoda and Malek, 1992) and spinach (Ozaki et al., 1992). Structural, immunological and biochemical investigations have shown that plant proteasomes are very similar to those of other eukaryotes (Ozaki et al., 1992 ; Shirley and Goodman, 1993 ; Fujinami et al., 1994). A great homology was reported for the amino acid sequences of the proteasome subunits from Arabidopsis (Parmentier et al., 1997), human and yeast (Tanaka and Tsurumi, 1997).

However, very poor information exists about the relationships between plant proteasomes and RNAs. In the present work we demonstrate for the first time that sunflower proteasomes harbor an endonuclease activity that is homologous to the nuclease activity of calf liver proteasomes.

Methods

Culture of plants:

Sunflower seeds (*Helianthus annuus* L.) from the hybrid EL64 (INRA Clermont-Ferrand, France) were cultivated in plastic pans containing soilless compost at 18°C, 70% humidity and total darkness. Eight days after sowing, the hypocotyles were harvested and immediately processed.

Isolation and purification of proteasomes:

The isolation and purification of proteasomes were carried out as described by Kremp et al., (1986).

Proteins gel electrophoresis:

Proteins were analyzed by electrophoresis on one-dimensional 12.5 % SDS-PAGE according to Laemmli (1970).

Two-dimensional gel electrophoresis was performed according to O'Farrell et al., (1977).

Assay for TMV RNA:

Purified TMV-RNA of *Nicotiana rustica* was purchased from DSM-Braunschweig (Germany), incubated with proteasomes and the digest was analyzed by gel filtration or RNA gel electrophoresis as described by Pouch et al, 1995.

Results

Identification and initial characterization of sunflower proteasomes:

The objective of this work was to elucidate the relationships between « plant » proteasomes and RNAs. Following exactly the purification protocol as described by Schliephacke et al. (1991), sunflower proteasomes eluted from Superose 6 gel filtration column as one sharp peak of absorbance, corresponding to a molecular mass of approximately 600 kDa somewhat smaller than calf-liver proteasomes. Subsequently eluted particles were analyzed by Laemmli PAGE and two-dimensional protein gel electrophoresis. All proteins visualized by Coomassie Blue banded in a range of 19-35 kDa quite similar to the characteristic subunit pattern of calf-liver proteasomes as well as the two-dimensional analysis (Fig. 1).

Sunflower proteasomes harbor endonuclease activity creating the same RNA fragments as calf liver proteasomes:

To test whether proteasomes of sunflower hydrolyze RNA we incubated TMV RNA with plant proteasomes. After 30 min. of incubation in TBK 240, 50% of the RNA was degraded comparing the peaks of absorbance before and after digestion. In addition there was no visible erosion of the sharp peak of absorbance of TMV RNA which would be typical for a random degradation in the presence of exonucleases or a non specific RNase activity. Our experiments revealed that this RNase activity eluted exactly with the peak of absorbance of highly pure proteasomes (Results not shown). Analysis of the digests of TMV RNA by electrophoresis on MOPS-agarose gels showed that both calf liver proteasomes and sunflower proteasomes created well defined fragments which were not smaller than 100 nucleotides (Fig. 3) as was reported by Pouch et al (1995). The specific proteasome endonuclease activity described for calf liver proteasomes is also an integral part of sunflower proteasomes.

Discussion

In this report we initially have characterized the proteasomes of sunflower. We have shown that the protein composition of these 600 kDa complexes is quite similar to proteasomes isolated of other plant cell systems. Our results clearly demonstrate that sunflower proteasomes hydrolyse RNAs. Degradation of TMV RNA was not random, releasing no mononucleotides or very small oligonucleotides between 100 and 500 nucleotides, which suggest that these proteasomes harbor an endonuclease activity. This is the first experimental evidence that proteasome associated endonuclease activity exists in plant cells too. Proteasome RNase activity was highly conserved during evolution. Actually we have not yet identified the cellular RNA substrates of plant proteasomes. Very recent

investigations in our laboratory indicate that short lived cytoplasmic mRNAs of human and animal cell systems containing ARE elements in their 3'UTR are potential RNA substrates for proteasome associated RNase activity (Jarrousse et al., 1999).

Furthermore, these substrates for proteasome associated endonuclease activity could be viral mRNAs, as it was reported for HeLa proteasomes which inhibit the translation of mRNAs from adenovirus infected cells but not the translation of the bulk mRNAs of uninfected cells (Homma et al., 1994). Indeed earlier experiments of Schmid and coworkers have shown that proteasomes isolated from potatoes sprouts or tobacco leaves inhibited the translation of tobacco mosaic virus RNA but not the protein synthesis of globin mRNA or HeLa cellular RNA (unpublished data). In all these cases translation inhibition was due to a destabilization of the viral RNAs while globin mRNA was not degraded by proteasomes under the same conditions.

In plant cells there is only little evidence about the nature and function of intracellular RNases. In animal and human cell systems, the 20S proteasome is considered to be the central player of an intracellular surveillance system.

It is interesting to mention, that published data clearly demonstrate a relationship between proteasomes and plant resistance to pathogens (Petitot et al., 1997). Thus fungi elicitor, cryptogein induces the early expression of a tobacco gene encoding a β -type proteasome subunit. These results suggest that the synthesis of the total 20S complex is enhanced by these components since it has been never shown that single β -subunits occur free in cytoplasm.

Abbreviations used :

TMV : Tobacco Mosaic Virus ;

PAGE : Polyacrylamide Gel Electrophoresis ;

MOPS : 4 Morpholine propane sulfonic acid ;

FPLC : Fast Protein Liquid Chromatography ;

ARE : AUUUA rich region within the 3' untranslated region.

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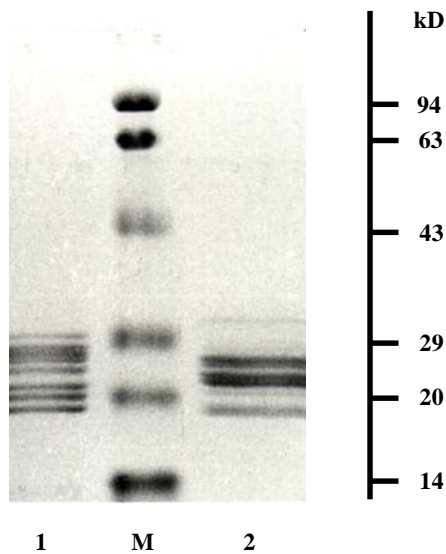


Fig 1 : Mono - dimensional protein gel electrophoresis

Proteasomal proteins of calf liver and sunflower were separated by 12.5 % SDS-PAGE. Proteins were stained with Coomassie Blue ; lane 1 : proteasomes of calf liver, lane 2 : proteasomes of sunflower, M: Marker proteins.

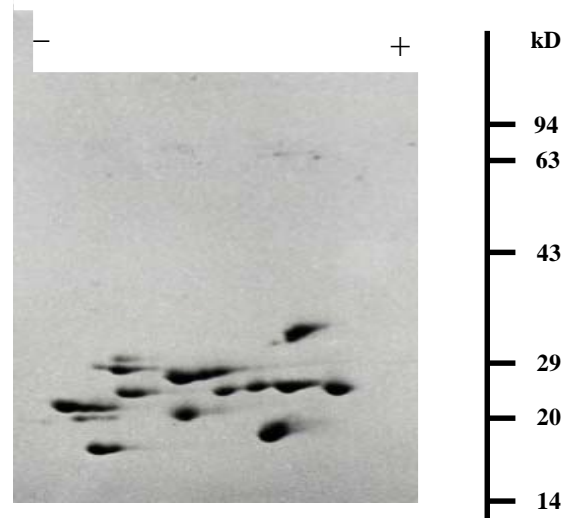


Fig.2 : Two-dimensional protein gel electrophoresis

50 g of purified proteasomes were separated by two dimensional PAGE according to O' Farrell (1977). Proteins were visualized by Coomassie Blue stain.

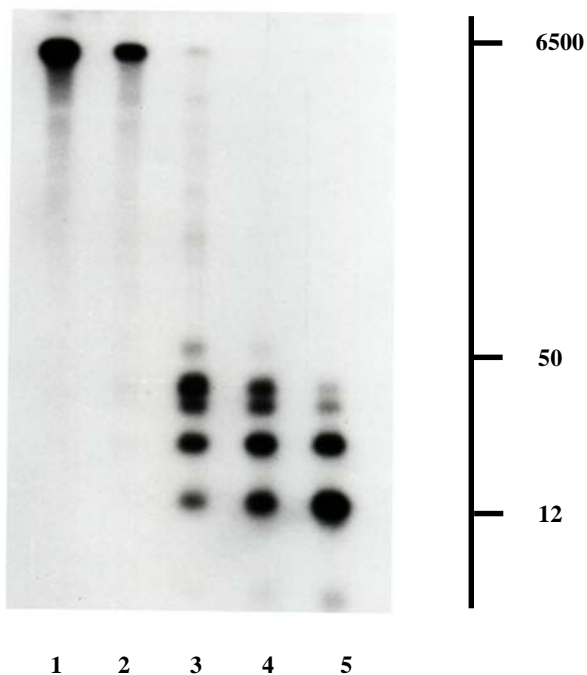


Fig. 3 : Analysis of TMV - RNA digests by electrophoresis on agarose gels

lane 1 : control : TMV RNA incubated in buffer TBK 240 for 30 min at 37°C ; lane 2 : control : TMV RNA incubated with 20 g of proteasomes preincubated at 100° C for 10 min ; lane 3 : TMV RNA incubated with 20 g of calf liver proteasomes in TBK 240 for 15 min at 37°C ; lane 4 and 5 : TMV RNA incubated with 20 g of sunflower proteasomes in TBK 240 for 15 min and 30 min at 37°C .