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Dry Pea Seed Proteasome[1]

Purification and Enzymic Activities

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ABSTRACT

Proteasomes were isolated from mature, dry pea seeds (*Pisum sativum* L.). They appear to be similar to proteasomes from other sources in that they are cylindrical (shown by negative staining), have a molecular mass greater than 600 kilodaltons (by gel permeation chromatography), and consist of several subunits between 25 and 31 kilodaltons. The seed proteasomes possess three characteristic partial activities (trypsin-like, chymotrypsin-like, and peptidyl glutamyl peptidase) as determined with fluorogenic peptide substrates. Activation and inhibition by various effectors, and particularly sensitivity to porphyrins, also match characteristics of proteasomes described for other organisms. The potential role of the proteasome in seed biology is discussed.

As early as 1970, a cytoplasmic particle with a sedimentation coefficient of 19S called prosome was described in, among other sources, young pea seedlings (*Pisum sativum* L.) (22). This particle was ultimately shown in eukaryotes other than plants to be homologous with a large proteinase complex currently called a proteasome or multicatalytic proteinase complex (reviewed in refs. 9 and 18).

In mammals and other eukaryotes the proteasome participates in, among other reactions, the hydrolysis of protein-ubiquitin conjugates. In this case, the 19S core particle associates with another large 500-kD proteinase, multipain, to form a 26S (1.3 MD) complex tentatively termed ubiquitin conjugate-degrading enzyme (5, 9, 11). The precise regulation of the targeting of cytoplasmic proteins for the ubiquitination pathway is not understood. Ubiquitin was shown to be one of the heat-shock proteins (2, 7, 17, 19) and ubiquitination tends to increase following environmental stress (7, 23, 25). Damage to, or redundancy of, target proteins are generally considered to be signals for ubiquitin conjugation (18). An additional link to stress response was found in *Drosophila*, in which the proteasome was shown to contain heat-shock protein 23 (21).

Plant proteasome was recently rediscovered as a structural entity in several plant tissues, including cotyledons of germinated mung bean (20). We (26) presented preliminary evidence for the enzyme activity of putative proteasome from dry peas. Here we describe many similarities of the dry seed proteasome with those of proteasomes from other biological sources and confirm the identity of the seed particle as a proteasome.

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Sigma (St. Louis, MO) except where noted otherwise. Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA).

Proteasome Purification

Dry pea seeds (*Pisum sativum* L. cv Spring, treated with Captan; Asgrow Seeds, Bradford, Ontario, Canada) were washed in absolute methanol, dried, and ground in a manual mill. Seed powder (100 g) was extracted with 400 mL of 50 mM Tris-HCl (pH 8), 15 g polyvinylpolypyrrolidone, and 0.02% NaN_3. Ammonium sulfate precipitation, dialysis, and DEAE Bio-Gel A (Bio-Rad) anion exchange followed the "nonreducing" protocol of Yang and Malek (26). Active fractions (about 25 mL) were dialyzed overnight in No. 4 dialysis tubing (Spectrapor) against 4 L of 5 mM Tris-HCl (pH 8), 0.002% NaN_3 buffer, and loaded onto 1.6- × 12-cm column of arginine-Sepharose (Sigma) equilibrated with 50 mM Tris-HCl (pH 8), 0.02% NaN_3. After unbound proteins passed through, a gradient of 150 mL equilibration buffer plus 150 mL of the same buffer containing 250 mM NaCl was applied. Active fractions eluting early in the gradient were collected and dialyzed overnight as above. Dialysate was concentrated to 2 to 3 mL by ultrafiltration (Diaflo XM300; Amicon, Danvers, MA) and applied on 2.6- × 70-cm column of Sephacryl S-300 HR (Pharmacia, Baie d'Urfe, Quebec, Canada) equilibrated with 50 mM Tris-HCl (pH 8), 0.02% NaN_3 buffer. Fractions (5 mL) were analyzed for activity by nondenaturing gel electrophoresis. Alternate substrate, inhibitor, and activator studies were performed with two fractions (10 mL) of highest activity obtained after this gel filtration step. An additional gel filtration step on a 1.6- × 70-cm column of Sephacryl S-400 HR was performed in the experiments used in producing Figures 1 and 2, to remove a faint diffuse band of large molecular mass (>800 kD) proteins associating with the proteasome preparation. These give bands of >50 kD on denaturing SDS gels and do not appear to affect the hydrolytic activities measured.

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Table I. *Purification of Proteasome from Dry Seeds while Monitoring Caseinolytic Activity*

Purification Step	Protein Yield[a]	Activity Yield	Specific Activity	Purification
	%	%	$ng \cdot \mu g\ enzyme^{-1} \cdot h^{-1}$	-fold
Crude extract	100	100	0.01	1
$(NH_4)_2SO_4$	22	340	0.20	15
Anion exchange	1.5	66	0.58	44
Affinity	0.4	78	2.90	223
Gel filtration	0.1	69	15.00	1154

[a] Total protein decreased from an initial 16.5 g to 9 mg of final product.

EM

Drops (10–20 μL) of proteasome preparation were evaporated at room temperature onto glow-discharged and carbon-coated copper grids and stained with 1% uranyl acetate, rinsed, and dried. The micrographs were produced with a Philips EM300 electron microscope.

Enzyme Assays

[^{3}H]Methyl-labeled casein was prepared according to the method of Means and Feeney (14). Assays for routine fraction analysis contained 10 μL of substrate and 70 μL of enzyme in 50 mM Tris-HCl (pH 8), 0.02% NaN_3. These were incubated at 37°C, stopped with 10 μL of 10 mg/mL of BSA and 70 μL of 10% TCA, kept on ice for 30 min, and collected at maximum speed for 10 min in a bench-top centrifuge. Half of the supernatant volume was carefully collected, dried onto GF/A filters, and counted in 0.1% 2,5-diphenyloxazole in toluene. For inhibitor and activator studies, an additional 10 μL of water or reagent stock was added, and the mixture was preincubated for 30 min at room temperature. An extra 10 μL of 10% TCA was used to stop the reaction.

Fluorogenic substrates *N*-succinyl-Ala-Ala-Phe-MCA[3], Z-Gly-Gly-Arg-MCA, *N*-succinyl-Leu-Leu-Val-Tyr-MCA, and Z-Phe-Leu-Glu-naphtylamide were dissolved in DMSO to 1 mM and 10 μL was used in a reaction mixture also containing 10 μL of enzyme, 20 μL of 0.5 M Tris-HCl (pH 8.5), and water or additive to 100 μL. Incubations at 37°C were stopped at times shown, up to 60 min, with 100 μL of 10% SDS and 2 mL of 0.1 M Tris-HCl (pH 9). Fluorescence emmission at 460 nm was determined following excitation at 360 nm for MCA derivatives and 410 and 335 nm for the naphtylamide derivative (Perkin-Elmer, model 204, Rexdale, Ontario, Canada). Fluorescence emission was compared with that of standard MCA but not naphtylamide.

Electrophoresis

Separation of SDS- and mercaptoethanol-denatured final purification product and of seed protein hydrolysis products was performed according to the method of Laemmli (12) using precast 4 to 20% polyacrylamide gels (Bio-Rad). The buffer system of Davis (4) was used to separate nondenatured proteins. Gels were stained with Coomassie blue and dried using standard protocols.

[3] Abbreviations: MCA, 7-amido-4-methyl coumarin; Z, α-*N*-carbobenzoxy; IAAmide, iodoacetamide; pCMBS, *p*-chloromercury bezoyl sulfonate.

RESULTS AND DISCUSSION

The method of purification of proteasome adopted in these experiments was simplified compared with the previously reported one (26) in that affinity chromatography and gel filtration on Sephacryl S-300 HR were substituted for the laborious preparative electrophoresis/electroelution. In the previous work to achieve protein yield in the milligram range, several large preparative native gels had to be run. Subsequent electroelution in the absence of negatively charged SDS proceeded very slowly even at high voltages, causing overheating. The alternate purification sequence used here was more convenient, resulting in at least 1000-fold purification (Table I) and the presence of a distinct Coomassie blue-staining band on a native gel (Fig. 1a). However, the diffuse band (not shown) of stained material above the proteasome band before chromatography on Sephacryl S-400 HR could be removed by this step. This removed the material dissociating into subunits >40 kD on SDS gels, leaving essentially the characteristic proteasome subunits in the 25- to 31-kD range (Fig. 1b, lane 4). Similar subunit composition has been reported in proteasomes from several diverse nonplant sources (6, 13, 16, 18, 20). The overall molecular mass of the native complex was reexamined on Sephacryl S-300 HR and was determined to be at least 600 kD. On nondenaturing 4 to 20% PAGE, the protein complex

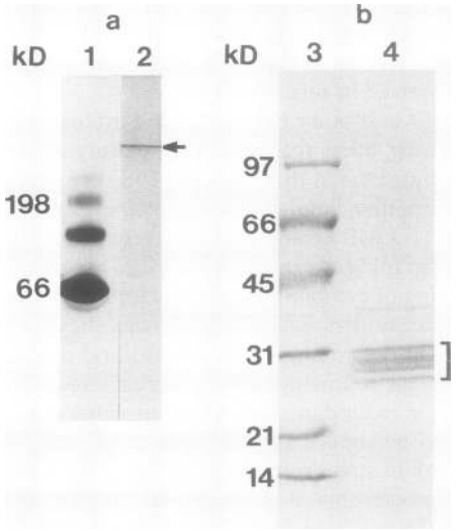

Figure 1. Migration of proteasome (arrow and bracket) on a native 4 to 20% gel (a) and on a reducing SDS 4 to 20% gel (b). Lane 1, BSA 66-kD monomer, 132-kD dimer, and 198-kD trimer; lane 2, purified proteasome (4 μg); lane 3, molecular mass standards: lysozyme (14.4 kD), soybean trypsin inhibitor (21.5 kD), bovine carbonic anhydrase (31 kD), hen ovalbumin (45 kD), BSA (66 kD), rabbit phosphorylase b (97.4 kD); lane 4, proteasome subunits (2 μg of protein).

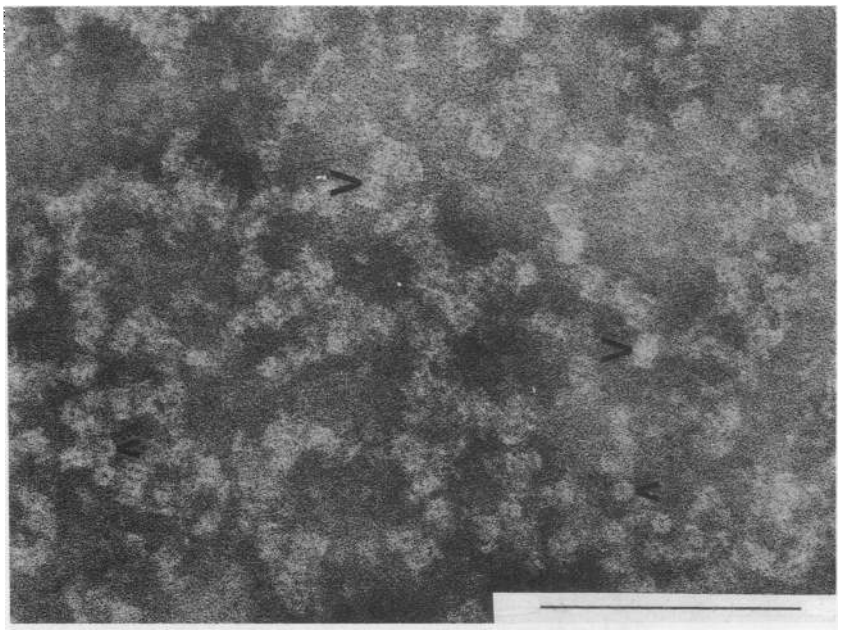

Figure 2. Electron micrograph of negatively stained final preparation of seed proteasome. Small arrows pointing left indicate particles in end-view orientation, and larger arrows pointing right indicate particles in side view. Bar, 100 nm.

migrated slower than the 545-kD urease hexamer used as the largest standard (not shown).

Another modification of the purification procedure in ref. 26 was that only the most active fractions (10–15 mL) were used in the present work rather than the entire activity peak (40–50 mL). The unusually high total activity yields (Table I), apparently exceeding the initial activity in the crude homogenate, are due to the competitive inhibition of the caseinolytic activity by endogenous protein substrates. The arginine-agarose affinity chromatography step was not very effective and will be deleted in future work. We are puzzled by the apparent tenaciousness with which some of the "contaminating" proteins copurify with the proteasome after anion exchange. These proteins may either be substrates or be related to the larger complex into which proteasome appears to assemble (9). This aspect of proteolytic particle assembly in seeds is under active investigation.

Structurally, the pea seed proteasome (Fig. 2) resembles hollow cylindrical particles isolated from nonplant sources (1, 6, 10, 13, 16) and also from wheat germ, tobacco, potato, and mung bean (20). The seed particle was about 15 nm in length and 9 nm in diameter.

The proteasome was active not only against casein but also against the three fluorogenic peptide substrates normally used to demonstrate its multicatalytic nature. The seed enzyme possesses partial chymotrypsin-like activity (cleavage on the carboxyl side of a hydrophobic residue; Fig. 3, solid circles), trypsin-like activity (cleavage on the carboxyl side of a basic residue; Fig. 3, open circles), and peptidylglutamyl-peptidase activity (cleavage on the carboxyl side of a glutamyl residue; Fig. 3, solid triangles) (18). The activity was linear during the 60-min incubation period (Fig. 3) and was not affected by 5 mM MgATP (not shown). The enzyme was also active against *N*-succinyl-Leu-Leu-Val-Tyr-MCA (another substrate for chymotrypsin-like activity, not shown).

The polycations polylysine, histone H1, and Polybrene almost doubled the enzymic activity when used at 200 μg/

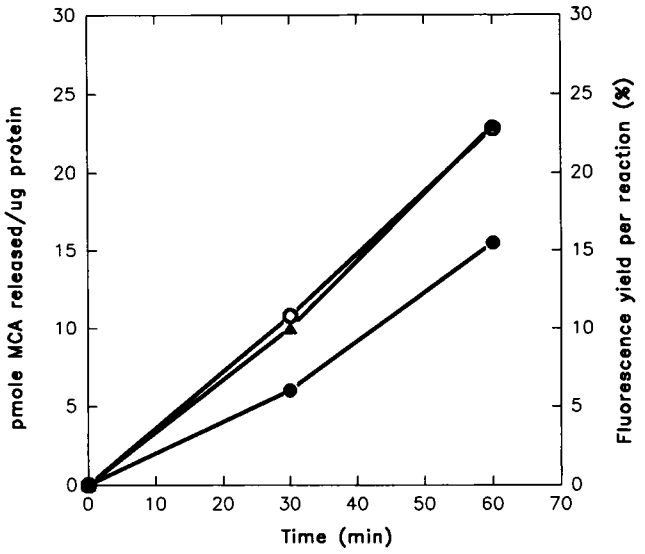

Figure 3. Activity of seed proteasome against fluorogenic peptides *N*-succinyl-Ala-Ala-Phe-MCA (dark symbols) and Z-Gly-Gly-Arg-MCA (open symbols). Replicates of three experiments; SD did not exceed 0.004 pmol/μg of protein. Activity of the same proteasome preparation against Z-Phe-Leu-Glu-naphtylamide (solid triangles, right axis) is expressed as fluorescence yield *versus* time; the SD did not exceed ± 2%.

Table II. *Activation of Proteasome Activity against Casein by Polycations and SDS*

Effector	Concentration	Activity[a]
	μg/mL (except ppm SDS)	*% of control*
Polybrene	50	162
	200	185
	800	172
Histone H1	50	189
	200	229
	800	197
Polylysine (22 kD)	50	156
	200	206
	800	187
SDS	10	113
	30	103
	50	94
	100	53

[a] Average of at least three determinations. SE calculated on the original values did not exceed ±16%.

mL (Table II). This activation was not nearly as effective as the 20-fold increase reported for human erythrocyte proteasome (15). It is possible that the dry seed enzyme is in a partially quiescent and less responsive state compared to the erythrocyte enzyme. SDS at 0.01% increased activity against casein only slightly. This is in contrast to the reported activation by low levels of SDS of proteasomes from rat muscle (3). The small increase may be a reflection of a significant increase of the partial glutamyl-peptidase activity only, as shown for the pituitary proteasome (18).

Of the inhibitors tested, hemin and the porphyrins, Chl *a* and chlorophyllin, were the most effective (Table III). This suggests that we are dealing with proteolytic activity comparable to the porphyrin-sensitive, ubiquitin conjugate-degrading activity from oats and yeast (24). Human brain (13) and crustacean muscle (16) proteasome, among others, were shown to be inhibited by hemin. The specificity and *in vivo* significance of this inhibition has been questioned (24), and a plausible explanation is currently lacking.

With respect to the other inhibitors, the seed proteasome did not respond as strongly to cysteinyl protease inhibitors (IAAmide and pCMBS, Table III) as would be expected based on reports of inhibition in other organisms and tissues (13,

Table III. *Inhibition of Proteasome Activity against Three Substrates*

Inhibitor	Final Concentration	Activity		
		[^{3}H]Methyl-labeled casein	Z-G-G-R-MCA	Succinyl-A-A-P-MCA
			% of control	
Soybean trypsin inhibitor	0.5 mg/mL	55	92	104
	0.05 mg/mL	74	90	113
Egg trypsin inhibitor	0.5 mg/mL	89	120	89
	0.05 mg/mL	95	51	136
Leupeptin	0.5 mM	85	17	104
	0.05 mM	58	21	105
Bestatin	0.25 mM	112	93	113
	0.025 mM	95	86	98
Antipain	1 mM	57	12[a]	63[a]
	0.1 mM	65	15[a]	64[a]
N-Tosyl-L-phenylalanine chloromethyl ketone	0.5 mM	90	71	102
	0.05 mM	70	88	101
IAAmide	5 mM	92	38	110
	0.5 mM	74	71	57
pCMBS	5 mM	45	36	48
	0.5 mM	79	96	53
EDTA	5 mM	120	124	104
	0.5 mM	141	177	133
$CaCl_2$	5 mM	105	40	76
	0.5 mM	121	63	107
$CuSO_4$	1.5 mM	83	33	82
	0.15 mM	85	78	92
Chlorophyllin	1.5 mM	8	10	23
	0.15 mM	19	64	98
Chl *a*	1.5 mM	36	18[a]	28[a]
	0.15 mM	54	74[a]	94[a]
Hemin	1.5 mM	51	13[a]	5[a]
	0.15 mM	86	18[a]	49[a]

[a] Average of at least three replicate assays, except where indicated by asterisk. SE calculated on original data did not exceed ±15%.

16, 18). Interaction between the complex and pCMBS or IAAmide may not be within the active site. Cysteinyl residues are likely involved in the maintenance of the tertiary and quaternary structure of the complex.

The complex was insensitive to bestatin (Table III) and pepstatin (not shown) and was inhibited to various degrees by serine protease inhibitors (Table III). Inhibition of one of the component activities may result in relative activation of another component activity. For example, the inhibition of trypsin-like partial activity by 0.5 mM IAAmide may have been responsible for the increase in chymotrypsin-like activity. This translated into an overall higher activity against the general substrate [^{3}H]methyl-labeled casein in 0.5 mM IAAmide than in 0.05 mM. Similar situations may arise with leupeptin and *N*-tosyl-L-phenylalanine chloromethyl ketone (Table III).

The fluorogenic activities were inhibited by the higher concentrations of divalent cations, and, conversely, EDTA slightly increased these partial activities (Table III). Ionic interactions probably play a role in stabilizing the structure, indirectly affecting catalytic rates, considering the size and subunit complexity of the proteasome. Copper ion (1.5 mM) was also tested to ascertain whether it alone, and not the entire copper-containing chlorophyllin molecule, was responsible for inhibition. It appears that the bound copper of chlorophyllin may contribute slightly to the inhibition by the porphyrin ring (trypsin-like activity at 33% of control, Table III).

The function of the proteasome in the seed and the nature of substrate proteins remain the most important questions to be answered. Proteasome may play a generalized, constitutive role in "mopping up" target proteins made redundant in the normal course of metabolism. A significant body of direct and indirect evidence has accumulated suggesting that environmental stress and resulting damage and metabolic shifts result in increased protein ubiquitination and ultimate removal of such conjugates by the proteasome and related proteolytic particles (7, 8, 19, 21, 23, 25). Seeds represent some of the most environmental stress-tolerant structures in nature and provide an ideal system for the study of the function of proteasome and related reactions and proteins. Experiments on the degradation of ubiquitin conjugates and the role of the enzyme tentatively named ubiquitin complex-degrading enzyme (5, 9, 11) are in progress.

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