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# The 26S Proteasome in Garlic (*Allium sativum*): Purification and Partial Characterization

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The 26S proteasome (multicatalytic protease complex, MPC) was purified from fresh garlic cloves (*Allium sativum*) to near homogeneity by ion exchange chromatography on DEAE-sephacel, gel filtration on Sepharose-4B, and glycerol density gradient centrifugation. Two α-type (20S proteasome "catalytic core") subunits were identified by the direct sequencing of peptide fragments (mass fingerprint analysis, Mass Spectrometry Lab, Stanford University) or the sequencing of a cloned cDNA generated using a garlic cDNA library as the template; these subunits were found to have a high homology to those from other plants. Polyacrylamide gel electrophoresis under denaturing conditions separated the garlic MPC into multiple polypeptides having molecular masses in the range of 21−35 (components of the 20S catalytic core) and 55−100 kDa (components of the 19S regulatory units). The banding pattern of the garlic MCP is similar to that of spinach and rat liver with minor differences in some components; however, polyclonal antibodies against mammalian proteasomes failed to significantly stain the enzyme from garlic. This is the first work to identify the garlic proteasome.

**KEYWORDS:** ***Allium sativum*; multicatalytic protease; prosome; purification; molecular cloning; mass fingerprint analysis; polymerase chain reaction**

## INTRODUCTION

First isolated from bovine pituitary over 20 years ago (*1*) and described as a neutral endopeptidase, the 26S proteasome was subsequently identified as a MPC that has three distinct peptidase activities: T-L, ChT-L, and PGPH (*2*, *3*).

The 26S proteasome is the principle cytoplasmic enzyme for the removal of cellular proteins that have been mistranslated, misfolded, or oxidized (*4*−*6*). It consists of a ring-shaped 20S "catalytic core" with two 19S regulatory units capping the ends (*7*−*9*). The complete proteasome will degrade ubiquitin-conjugated proteins in an ATP-dependent manner (*10*), while the 20S catalytic core can break down proteins in an ATP- and ubiquitin-independent manner (*11*).

The 26S/20S proteasome has been identified in archaebacteria and eubacteria (*12*) and many species in the animal [*Xenopus* (*7*), human (*10*), rat (*13*), lobster (*14*), and *Drosophila* (*15*)], fungi [yeast (*8*)], and plant [spinach (*16*, *17*), rice, and carrot (*18*)] kingdoms.

We have been involved in studying proteolytic enzymes for some years and have characterized calcium-activated proteases and a high molecular weight glycosylated protease in calf brain (*19*, *20*). As a general interest, we expanded our work to plant proteases. We detected proteolytic activity using an extract of garlic cloves as the source of enzyme and [methyl-$^{14}$C] α-casein as the substrate (unpublished). Our interest in this plant grew due to its presumed therapeutic role in various diseases (*21*). To learn more about this newly discovered protease, we purified and partially characterized it.

The nucleotide sequence for the garlic proteasome α-3 subunit has been deposited in the GenBank database under GenBank Accession Number AY376440. The following GenBank entries exhibit the greatest similarity to the garlic sequence: *Lotus japonicus* (GenBank Accession Number AP00607), *Petunia x hybrida* (GenBank Accession Number AF088914), *Nicotiana tabacum* (GenBank Accession Number AJ291733), *Euphorbia esula* (GenBank Accession Number AF227625), *Arabidopsis thaliana* (GenBank Accession Number AF043521), and *Spinacea oleracea* (GenBank Accession Number X96974). The amino acid sequence for the garlic proteasome α-5 fragments has been deposited in the PIR Protein Databank under Accession Number A59490.

## MATERIALS AND METHODS

**Materials.** The materials used were obtained as follows: Heads of garlic were purchased from a local market; Suc-LLVY-MCA (Peninsula Laboratories, Inc., Belmont, CA), ion exchange and gel filtration media, DEAE-Sephacel and Sepharose-4B and Ready-To-Go PCR Beads (Amersham Biosciences, Inc., Piscataway, NJ), TOPO TA Cloning Kit (Invitrogen/Life Technologies, Carlsbad, CA), and PVP (ICN Biomedicals, Inc., Aurora, OH) were also purchased. All other chemicals were of reagent grade.

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**Assay of Peptidase Activity.** The fluorogenic substrate Suc-LLVY-MCA [used previously (*17*) to illustrate the ChT-L peptidase activity of the 26S proteasome] was incubated with 10 $\mu$L aliquots of column/gradient fractions at a concentration of 0.5 mM in 50 mM Tris-HCl (pH 8.0) for 30 min and 2 h at 37 °C in the absence or presence of 0.02% SDS in a total volume of 100 $\mu$L. The stock substrate was dissolved in DMSO at a concentration of 5 mM and stored at 4 °C. The reaction was stopped by adding 100 $\mu$L of 10% SDS and 2 mL of 0.1 M Tris-HCl (pH 9.0). The fluorescence of the reaction products was measured using an excitation wavelength of 360 nm and an emission wavelength of 480 nm (model LS 50 B Luminescence Spectrometer, Perkin-Elmer, Norwalk, CT).

**Effect of Protease Inhibitors on the 26S Proteasome from Garlic Cloves.** The hydrolysis of [methyl-$^{14}$C] α-casein (13.6 mM, 8.4 Bq), with or without protease inhibitors, was performed as previously described (*19*) except that MES (MES−KOH), pH 5.5 (the observed pH optimum for this substrate), at a final concentration of 23.3 mM was substituted for the tris buffer. Each assay contained 15 $\mu$g of purified proteasome in a reaction volume of 75 $\mu$L.

**Assay of Protein Concentration.** Protein concentrations were determined by the method of Lowry et al. (*22*) with bovine serum albumin as a standard.

**Purification of the 26S Proteasome from Garlic Cloves.** Purification was performed following the procedure for spinach leaves (*17*), with modifications. All purification procedures were performed at 4 °C with standard buffer of 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, 2 mM ATP, 5 mM $MgCl_2$, and 20% (w/v) glycerol (unless otherwise specified), pH 8.0. ATP and glycerol were found to stabilize the 26S proteasome (*23*).

Freshly peeled garlic cloves (101.56 g) were homogenized in 200 mL of standard buffer containing 5% (w/v) PVP in a Waring blender. The homogenate was squeezed through cheesecloth, and the filtrate was centrifuged at 10 000*g* for 30 min. The resulting supernatant was used as the crude extract.

The crude extract was mixed with 100 mL of DEAE-Sephacel equilibrated in standard buffer, without ATP. The excess liquid was decanted, and the ion exchange medium was batch-washed four times, each with 250 mL each of standard buffer. After the last wash, the medium was packed into a 2.5 cm diameter column (settled bed height was approximately 13 cm). The column was washed further with standard buffer until the UV absorbance of the eluate stabilized. Elution of protein was achieved with a 500 mL linear 0−0.5 M KCl gradient, in standard buffer. One hundred fractions of 5 mL each were collected. Peptidase activity was measured using Suc-LLVY-MCA as the substrate, in the absence of SDS. A single peptidase activity peak eluted between fractions 30−70, with a broad leading shoulder covering the first 19 fractions. Fractions 49−70 were pooled and concentrated in a stirred ultrafiltration cell using a YM-10 membrane (Millipore, Bedford, MA). This part of the activity peak eluted at approximately 0.25−0.35 M KCl.

Aliquots (2 mL) of the concentrated pool (∼36 mg of protein) were mixed with 1 mL of glycerol and layered onto a Sepharose-4B gel filtration column (2.5 cm × 95 cm) equilibrated with standard buffer. Fractions (200, 3 mL each) were collected; successive separations were collected in a single set of tubes. Peptidase activity was measured using Suc-LLVY-MCA as the substrate, in the absence of SDS. A single peptidase activity peak eluted between fractions 100 and 135, which preceded a large protein peak that eluted between fractions 136 and 185. Fractions 100−135 were pooled and concentrated to 5−8 mL, final volume, in a stirred ultrafiltration cell using a YM-10 membrane. To remove the glycerol from the concentrate, 100 mL of standard buffer without glycerol was added and the volume was reduced again. This buffer exchange was repeated once.

Final purification was performed by density gradient centrifugation (10−40% glycerol, w/v) at 25 000*g* in a swinging-bucket rotor (SW-27Ti, XL-90 Ultracentrifuge, Beckman-Coulter, Palo Alto, CA) for 22 h at 4 °C (volume/tube was 38 mL). Thirty-four fractions were collected from the bottom of each tube using a disposable syringe (∼1.12 mL/fraction). Peptidase activity using Suc-LLVY-MCA as the substrate, in the absence and presence of 0.02% SDS, was measured in all fractions. The peptidase activity peak in the absence of SDS covered fractions 14−23, with the maximal activity occurring in fraction 19. Fractions 18−20 were combined and concentrated to give the final purified 26S proteasome.

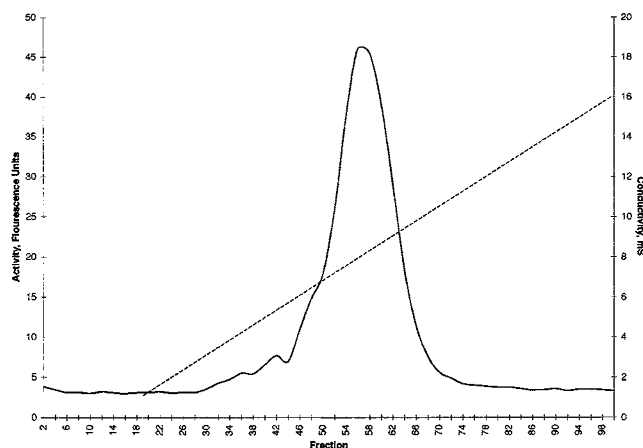


**Figure 1.** Ion exchange chromatography of the garlic 26S proteasome on DEAE-Sephacel. The peptidase activity profile (Suc-LLVY-MCA as substrate, in the absence of SDS) is represented by the solid (—) line (fluorescence units, left vertical axis). The dotted (• • •) line represents the conductivity (mS, right vertical axis) of the KCl gradient. Fractions 49–70 were pooled. See Materials and Methods.

**PAGE.** Routine separation under denaturing conditions (SDS−PAGE) was carried out as previously described (*24*). Silver staining of the proteins was by a modified method of Merril et al. (*25*). The modifications were as follows: (i) after dichromate activation, gels were rinsed quickly with water once and then washed three times with water for 5 min each; (ii) following impregnation with $AgNO_3$, gels were rinsed very quickly with water three times, 30 s to 1 min each; and (iii) a single small-to-medium sized crystal of sodium thiosulfate was added to the developer solution.

**Sequencing of a Peptide Subunit of the 20S Catalytic Core of the 26S Proteasome.** The purified proteasome was subjected to two-dimensional (2D) PAGE [pH 3−11 for the first dimension, nondenaturing IEF, followed by a Tricine-SDS slab gel (*26*) for the second dimension] and stained with Coomassie Brillant Blue to visualize the protein spots (data not shown). A single, well-separated, spot was cut from the gel and sent to the Mass Spectrometry Lab at Stanford University for analysis.

**Molecular Cloning of a Subunit of the 20S Catalytic Core of the 26S Proteasome.** An expression cDNA library was constructed from garlic using the Uni-Zap Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The initial titer of the library (prior to primary amplification) was 8.03 × $10^6$ pfu/mL (1.2 × $10^6$ primary clones). The stored library (after primary amplification, at −70 °C, with DMSO added) had a titer of 1.12 × $10^{10}$ pfu/mL. Attempts to screen the library with several commercially available polyclonal and monoclonal antibodies prepared against mammalian proteasomes were unsuccessful due to poor species cross-reactivity to the garlic proteasome.

We then turned to PCR in an attempt to amplify one or more proteasome subunits using the cDNA library as template. All available nucleotide sequences for proteasome subunits (http://www.ncbi.nlm.nih.gov) were aligned in a search for conserved regions to use for the design of forward and reverse PCR primers. The sequences spanned the plant and animal kingdoms. Initially, five pairs of PCR primers were considered, despite having a 128−512-fold degeneracy. These were redesigned to cut down the degeneracy level by employing plant codon "preferences". This reduced the average degeneracy by more than 96%. One of the primer pairs repeatedly gave a single band of constant size. The forward primer sequence was 5′-CAGGTKGAGTAYGCRATGGAGGC (23 bases, degeneracy = 8, $T_m$ = 63.7 °C), and the reverse primer sequence was 5′-RTYDGCDGYGGCTTTCCA (18 bases, degeneracy = 36, $T_m$ = 53.3 °C). PCR was performed using 1 $\mu$L of the library and 5 pmol of each primer in a reaction volume of

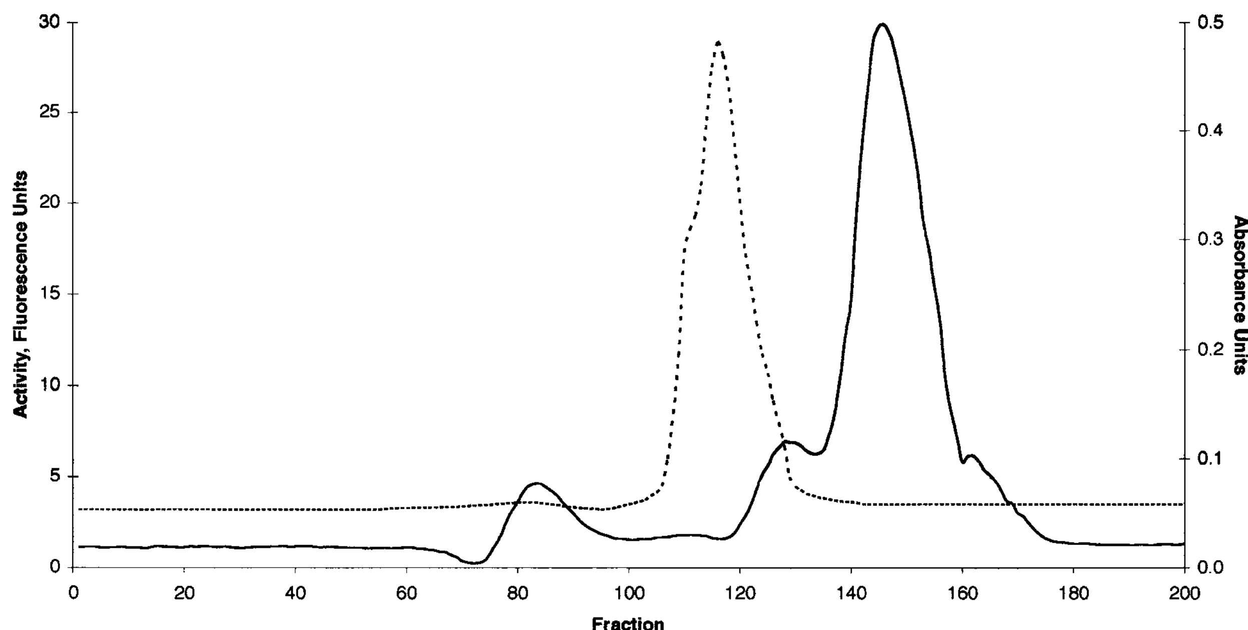


**Figure 2.** Gel filtration of the garlic 26S proteasome on Sepharose-4B. The peptidase activity profile (Suc-LLVY-MCA as substrate, in the absence of SDS) is represented by the dotted (• • •) line (fluorescence units, left vertical axis). The protein elution profile is represented by the solid (—) line (UV absorbance units at 280 nm, right vertical axis). Fractions 100–135 were pooled. See Materials and Methods.

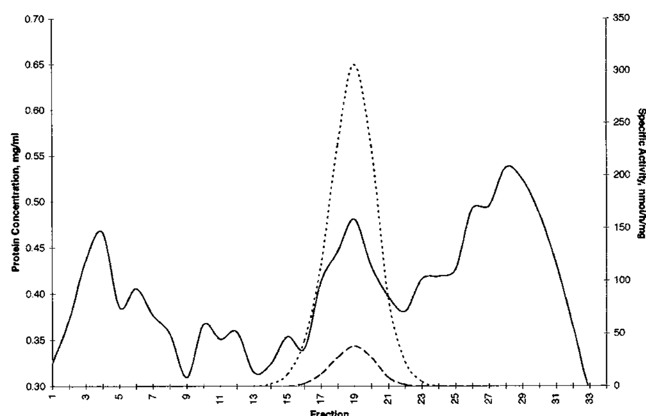


**Figure 3.** Glycerol density gradient (10–40%, w/v) centrifugation of the garlic 26S proteasome. Thirty-four fractions were collected from the bottom of the tube(s) (~1.12 mL each). The protein concentration is represented by the solid (—) line (mg/mL, left vertical axis). The peptidase activities (Suc-LLVY-MCA as substrate) in the absence and presence of 0.02% SDS are represented by the dotted (• • •) and dashed (- - -) lines, respectively (specific activity, nmol/h/mg, right vertical axis). Fractions 18–20 were pooled. See Materials and Methods.

**Table 1.** Purification of the Garlic (*A. sativum*) 26S Proteasome

| purification step | total protein (mg) | total activity[a] | % yield[b] | specific activity (nmol/h/mg) | -fold purification |
|---|---|---|---|---|---|
| crude extract | 790 | 825.6 | | 1.045 | 1 |
| DEAE-sephacel | 164 | 1236.7 | 149.8 | 7.541 | 7.2 |
| sepharose-4B | 12.1 | 875.3 | 70.8 | 72.34 | 69.2 |
| density gradient | 1.83 | 841.3 | 96.1 | 459.7 | 439.9 |

[a] Activity is expressed as nmol of Suc-LLVY-MCA hydrolyzed per hour. [b] Step-to-step yield.

25 μL (Ready-To-Go PCR Beads) in a Biometra Personal Cycler (initial denature at 94 °C, 4 min; 50 cycles: denature at 94 °C, 1 min; annealing at 65 °C, 1 min; extension at 72 °C, 45 s). The reaction product was subcloned (TOPO TA Cloning Kit, pCR 2.1-TOPO) to facilitate the use of standard M13 forward and reverse primers for sequencing on a Beckman-Coulter model CEQ 2000XL DNA Analysis System, using the manufacturer's supplied reagents.

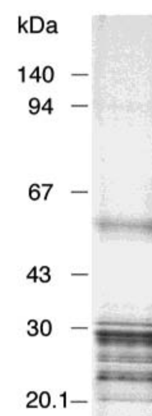


**Figure 4.** Electrophoretic analysis of the garlic 26S proteasome. SDS–PAGE profile of purified 26S proteasome from garlic. Proteins were visualized by silver staining. See Materials and Methods.

## RESULTS

**Purification of the Proteasome.** The first step in the purification was to separate the proteasome activity from the crude extract by DEAE-Sephacel chromatography using a linear salt gradient of 0−0.5 M KCl as shown by the breakdown of the fluorogenic substrate Suc-LLVY-MCA, which indicates the ChT-L peptidase activity (**Figure 1**). This was followed by Sepharose-4B gel filtration (**Figure 2**) and finally by ultracentrifugation on a 10−40% (w/v) glycerol density gradient (**Figure 3**); peptidase activity, in the presence of 0.02% SDS in fraction 19, was only 12.4% of the activity obtained without SDS. It has been revealed that small amounts of SDS promote latent peptidase activity of the 20S catalytic core of the 26S proteasome, that this activity overlaps the trailing edge of the 26S proteasome activity peak in the absence of SDS, and that it would be seen in the later fractions from the glycerol density

| Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Rank | Peptide |
|---|---|---|---|---|---|---|---|
| 532.25 | 1062.49 | 1062.51 | -0.02 | 0 | 51 | 1 | GVNTFSPEGR |
| 712.38 | 1422.75 | 1422.78 | -0.03 | 0 | 60 | 1 | LFQVEYAIEAIK |
| 430.26 | 858.50 | 858.52 | -0.01 | 0 | 45 | 1 | LGSTAIGLK |
| 472.27 | 942.53 | 942.54 | -0.01 | 0 | 51 | 1 | EGVVLAVEK |
| 586.84 | 1171.67 | 1171.68 | -0.01 | 0 | 32 | 1 | TKEGVVLAVEK |
| 693.36 | 1384.70 | 1384.74 | -0.05 | 1 | 47 | 1 | VTSPLLEPSSVEK |
| 771.42 | 1540.82 | 1540.85 | -0.03 | 1 | 45 | 1 | RVTSPLLEPSSVEK |
| 734.65 | 2200.93 | 2201.03 | -0.10 | 1 | (38) | 1 | IMEIDDHIGCAMSGLIADAR |
| 970.92 | 1939.84 | 1939.88 | -0.04 | 1 | 138 | 1 | AIGSGSEGADSSLQEQYNK |
| 535.79 | 1069.56 | 1069.58 | -0.02 | 0 | 26 | 1 | VTPNNVDIAK |

MFLTRTEYDR GVNTFSPEGR LFQVEYAIEA IKLGSTAIGL KTKEGVVLAV EKRITSPLLE

PSSVEKIMEI DEHIGCAMSG LIADARTLVE HARVETQNHR FSYGEPMTVE STTQAICDLA

LRFGEGDEES MSRPFGVSLL IAGHDENGPS LYYTDPSGTF WQCNAKAIGS GSEGADSSLQ

EQFNKELTLQ EAETIALSIL KQVMEEKVTP NNVDIAKVAP AYHLYTPQEV EAVIARL

**Figure 5.** Mass fingerprint data for peptide fragments from an α-5 subunit of the garlic 26S proteasome. A protein spot from the 2D PAGE was reduced with dithiothreitol and alkylated with acrylamide, resulting in propionamide-modified cysteines. LC-MS/MS was run using a peptide trap for online desalting, followed by a 0.075 mm × 100 mm C18 column, and eluted directly into the ESI-quadrupole time-of-flight (Micromass Q-Tof) mass spectrometer (Mass Spectrometry Lab, Stanford University, Stanford, CA). A consensus sequence for the α-5 subunit from four plant species (*Oryza sativa*, *Arabidopsis thaliana*, *Glycine max*, and *Nicotiana tabacum*) is shown with the matching fragments shaded in black (three intervening amino acids that do not match the consensus are shaded in gray). There is a 43% identity within the fragments to the consensus sequence.

gradient (*17*, *18*). This could be due to partial dissociation of the 26S complex during column chromatography. However, such dissociation apparently did not take place during the purification of the 26S proteasome from garlic cloves since only a small latent peptidase activity was detected.

The purification process is summarized in **Table 1**. From 101.56 g of fresh garlic cloves, 1.83 mg of proteasome was obtained with ~440-fold purification. The yield was 0.23%.

**PAGE.** The purified garlic proteasome was electrophoresed under denaturing conditions (SDS−PAGE) to examine the subunit structure (**Figure 4**), which is similar to that of proteasomes from rat liver and spinach (*17*). Repeated attempts to immunostain the subunits with commercially available monoclonal and polyclonal antibodies prepared against mammalian proteasomes, via Western blotting, failed due to very poor species cross-reactivity (data not shown).

**Sequencing of a Proteasome Peptide Subunit from Garlic.** A protein spot was cut from a gel after 2D PAGE and sent for mass fingerprint analysis. The peptide corresponded to the α-5 subunit from four other plant species. It exhibits a 43% identity within the matching fragments (**Figure 5**).

**Molecular Cloning of an α-Type Subunit of the Garlic Proteasome.** A cDNA corresponding to part of an α-3 subunit of the 20S catalytic core (**Figure 6**) was obtained by PCR using forward and reverse primers, which were designed from the alignment of proteasome subunit sequences available in the nucleic acid database at the National Center for Biotechnology Information (http://www.ncbi.nih.nlm.gov). A previously constructed garlic cDNA library was used as a template.

**Effect of Protease Inhibitors on the 26S Proteasome from Garlic Cloves.** Eleven protease inhibitors were tested for their ability to affect the hydrolysis of [methyl-$^{14}$C] α-casein by the garlic proteasome (**Table 2**). As compared to reactions not containing any protease inhibitor, six of those tested reduced the proteasome's activity by more than 50%; the greatest inhibition was 79.57%, by TLCK.

## DISCUSSION

We had observed proteolytic activity in extracts of garlic using [methyl-$^{14}$C] α-casein as the substrate (unpublished results) and sought to pinpoint the existence of the 26S proteasome. The procedure to purify the garlic proteasome, described in this paper, yielded a homogeneous enzyme as indicated by the appearance of a single band by PAGE under nondenaturing conditions (data not shown).

On SDS−PAGE, the multiple subunits resembled those obtained from other sources including human (*10*), rat (*13*), and spinach (*16*, *17*), exhibiting polypeptides having molecular masses in the range of 21−35 (components of the 20S catalytic core) and 55−100 kDa (components of the 19S regulatory units). After 2D PAGE, mass fingerprint analysis on one protein spot identified it as an α-5 type proteasome subunit.

A garlic cDNA library was constructed and screened for the existence of proteasome subunits. An α-3 type proteasome subunit was identified by sequencing the cDNA obtained by means of PCR.

More than just a mechanism for removing proteins, which are faulty or damaged by oxidation (*4*−*6*), we now know that the proteasome serves multiple functions in species within the animal and plant kingdoms. These include control of the cell cycle, apoptosis, hormone signaling (auxin response), embryogenesis, circadian rhythms, photomorphogenesis, and pathogen defense (*27*−*32*).

Proteasomal dysfunction has been linked to cell cycle arrest and apoptosis (*29*, *30*) and many diseases in humans including Alzheimer's disease, Huntington's disease, Parkinson's disease, and cystic fibrosis (*33*).

```
Spinach     ... ..G ... ..T ... ... ... ..G ... ..G ... ..T ..G ... ..C ... ..G ..T ... ..A  60
Petunia     ... ... ... ..T ..G ... ... ..T ... ..A ... ... ..C ... ..T ... ... ... TC. T.T  60
Potato      ... ... ... ... ... ... ... ..T ... ..A ... ... ..T ... ..T ... ... ... ... T.C  60
Rice        ... ..G ... ... ..G ... ... ..G ..C ..G ..C ... ..G TCG ..C C.G ... G.. C.. ..G  60
Garlic      ... ... ... ... ... ... ... ..A ... ..T ..C ... ..C TCT ..A ... ... ..T C.T ..C  60

Consensus   CAG GTT GAG TAC GCA ATG GAG GCD ATT GGD AAT GCA GGB AGT GCH ATA GGC ATC TTG GCN
Translation Q   V   E   Y   A   M   E   A   I   G   N   A   G   S   A   I   G   I   L   A

Spinach     ..A ... ..T ..T ..T T.. A.T ..T ..G ..A ..G ..G ..T ... ... ..T ... ... ... ..C 120
Petunia     ..G ... ..G ... ..A ... ..A ..C ... ... ... ... ..A ... ... ..G ... ... ... ... 120
Potato      ..G ... ..G ... ..G T.. ..A ..T ... ... ... ... ..A ... ... ..G ... ... ... ... 120
Rice        GCC ..C ..C ... ..C ..C ..C ..C ..G ... ..G ..C ..C ..C ... ..C ..C ... ... ..G 120
Garlic      ..A ... ..T ..A ..T ..T ..T ..A ... ... ... ... ..T ..A ... ..T ..C ..A ..A ... 120

Consensus   AAV GAT GGB GTG GTN CTG GTH GGH GAA AAG AAA GTA ACH TCT AAG CTB CTT CAG ACC TCA
Translation K   D   G   V   V   L   V   G   E   K   K   V   T   S   K   L   L   Q   T   S

Spinach     ..C ... ..C ..A ... ... ... ... ... ..C ... ... ..A ..T ... ..T ..G ... ..T ..A 180
Petunia     ..A ... .GT ... ... ... ... ... ... ... ... ... ..G ..A ... ..C ..A ... ... ..T 180
Potato      ..T ..T .GT ... ... ... ... ... ..T ... ... ... ..G ..G ..C ..T ..A ... ... ..T 180
Rice        CGC ..C G.C ... ... ... ... ... ... ..C TCC ..C C.C ..C ..C ..C ..C ..C ..G ..C 180
Garlic      .AA ... ..G ... ..A ... ... ..A ... ... ..C ... C.T ..T ... ..G ..T ... ... ..C 180

Consensus   ACH TCA ACB GAG AAG ATG TAC AAG ATC GAT GAT CAT GTN GCN TGT GCB GTN GCT GGA ATH
Translation T   S   -   E   K   M   Y   K   I   D   D   H   V   A   C   A   V   A   G   I

Spinach     ... ... ... ..A ... ..T ..T ... ..T ..A ..T ..T ... ..A ... ... ..T ..C ..A ... 240
Petunia     ... ... ... ... ... ..T ..A ... ... ..T ..C A.G ... ..G ... ... ... ... ..A ..C 240
Potato      ... ... ... ... ... ..A ..G ... ... ..G ..C A.G ..T ..G ... ... ... ... ..G ... 240
Rice        ... ..G ..C ... ... ..C ..C C.. ... ..G ..G ..C C.C ..C ..C ... ... ..C G.C C.C 240
Garlic      ... ... ... ... ... ..C ..C ... ... ..T ..A ..T ..T ..A ..A ..A A.G ... G.G ... 240

Consensus   ATG TCT GAT GCC AAC ATH CTN ATC AAC ACD GCN CGB GTC CAV GCT CAG CGC TAT ACV TTT
Translation M   S   D   A   N   I   L   I   N   T   A   R   V   Q   A   Q   R   Y   T   F

Spinach     T.T ..C ... ... ..G ... ..T ..T ... ..G ... ... ... ..T ..T ... ..T ... ... ..G 300
Petunia     T.G ... ... ... ... ... ..A ..C ..A ... ... ... ... ... ... ... ... ... ... ... 300
Potato      G.T ... ... ... ... ... ..A ..T ... ... ... ... ... ... ... ... ... ... ... ... 300

Rice        T.C ..C ..G ..G ... ..C ..C ..C ... ..G ..C ..C ..A ..C ..C ..C ... ... ... ..G 300
Garlic      T.A ... ... ... ..T ... ..T ..A ... ... ..A ..A ..A ... ... ... ... ..T ... ... 300

Consensus   TCN TAT CAA GAA CCC ATG CCH GTH GAG CAA CTT GTT CAG TCA CTA TGT GAC ACC AAG CAA
Translation S   Y   Q   E   P   M   P   V   E   Q   L   V   Q   S   L   C   D   T   K   Q

Spinach     ... ... ..T ... ... ..T ... ..A ..A ..A ... ... ... ... ... ..G ... ..T ..G ... 360
Petunia     ... ... ..A ... .A. ..T ..A ..C .C. ... ... ... ... ... ... ... ... ..A ... ... 360
Potato      ... ... ..A ... ... ..C ... ..T ... ... ... ... ... ... ... ... ... ..A ... ... 360
Rice        ..G ... ..C ... ..C ..C ..C ..C ... ..C ..C ..C ..C ..C ..C ... ..C ..C ..C ... 360
Garlic      ..G ... ..T ..A ... ..A ... ..A ... ... ... ..G ..A ..G ... ..T ... ..G ... ... 360

Consensus   GGT TAC ACH CAG TTT GGH GGG CTH CGC CCT TTT GGT GTT TCA TTT CTC TTT GCN GGT TGG
Translation G   Y   T   Q   F   G   G   L   R   P   F   G   V   S   F   L   F   A   G   W

Spinach     ... ..G ... ... ..G ... ..A ... ... ... ... ... ... ... ..G ... ... ... ..G ... 420
Petunia     ... ... ..T TT. ... ... ... ... .T. ... ..C ... ... ... ..A ... ... .C. ..T ... 420
Potato      ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ..A ... ..C ... ..T ... 420
Rice        ..C ..G C.. CA. ... ... ... ..C ... ... ..C ... ..C TCC ..C ..C ..C A.C ..C ... 420
Garlic      ..C ... ..T ... ..A ..T ... ... ..T ... ... ... ... ... -.C ... ... ... ..A ... 419

Consensus   GAT AAA AAC TAT GGC TTC CAG CTT TAC ATG AGT GAC CCA AGT GGV AAT TAT GGT GGN TGG
Translation D   K   N   Y   G   F   Q   L   Y   M   S   D   P   S   G   N   Y   G   G   W

Spinach     ... ..T A.T ..T ..C 435
Petunia     ... ..G ..A ..A ... 435
Potato      ... ... ..A ..A ... 435
Rice        ..G ... ..C ..C G.C 435
Garlic      ... ... A.C ..C GA. 434

Consensus   AAA GCC GCH GCH ATT
Translation K   A   A   A   I
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**Figure 6.** Alignment of cDNAs for a portion of proteasome subunit type α-3. The nucleotide sequence of the amplified cDNA from garlic (*Allium sativum*) was aligned with sequences from spinach (*Spinacia oleracea*), petunia (*Petunia x hybrida*), potato (*Solanum tuberosum*), and rice (*Oryza sativa* L.). Only nucleotides in disagreement with the consensus sequence are shown. Standard IUPAC ambiguity codes are used where there is no consensus. Codons that result in amino acids that differ from the consensus translation are shaded in black. Nucleotide substitutions that are silent are shaded in gray. Of the 145 codons shown, 120 (82.8%) are identical (16) and conserved (104). A single nucleotide gap was inserted in the garlic sequence to optimize the alignment (position 403). Only one of the codons was not conserved within this group of plant species. All entries for this codon (consensus ACB) are shaded in black.

There is genetic evidence that the genes encoding proteasomes have been conserved during evolution (*34*). There are so many similar, yet varied, subunits that have been identified in an abundance of species; therefore, we can observe that the 26S proteasome is a ubiquitous complex of nonidentical components whose functions are vital to cellular proliferation, regulation, and survival.

## ABBREVIATIONS USED

Bq, becquerel; ChT-L, chymotrypsin-like; DMSO, dimethyl sulfoxide; E-64, *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane; MES, 2(N-morpholino) ethanesulfonic acid; MPC, multicatalytic protease complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PGPH, pep-

**Table 2.** Effect of Protease Inhibitors on the Garlic (*A. sativum*) 26S Proteasome[a]

| inhibitor | final concentration | % inhibition |
|---|---|---|
| phenylmethylsulfonyl fluoride[a] | 0.1 mM | 75.36 |
| *p*-chloromercuribenzoic acid | 0.1 mM | 6.40 |
| N-ethylmaleimide | 1.0 mM | 31.91 |
| iodoacetic acid | 1.0 mM | 54.04 |
| leupeptin | 13.3 $\mu$g/mL | 53.19 |
| pepstatin[b] | 13.3 $\mu$g/mL | 76.09 |
| antipain | 13.3 $\mu$g/mL | 74.04 |
| aprotinin | 13.3 $\mu$g/mL | 53.62 |
| cystatin | 13.3 $\mu$g/mL | 46.38 |
| E-64 | 13.3 $\mu$g/mL | 71.15 |
| TLCK | 133 $\mu$g/mL | 79.57 |

[a] The hydrolysis of [methyl-$^{14}$C] $\alpha$-casein by the garlic 26S proteasome was carried out in the absence and presence of protease inhibitors. See Materials and Methods. Stock solutions of the inhibitors were prepared in an aqueous buffer (phosphate-buffered saline or Tris-HCl, pH 8.0) or [b]ethanol.

tidylglutamyl peptide bond hydrolyzing; PVP, poly(vinylpyrrolidone); SDS, sodium dodecyl sulfate; Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide; T-L, trypsin-like; TLCK, N$\alpha$-*p*-Tosyl-L-lysyl-chloromethyl ketone.

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