PERGAMON

Phytochemistry 50 (1999) 377–382

PHYTOCHEMISTRY

Senescence-related serine protease in parsley

Wei Bo Jiang [1], Amnon Lers *, Ella Lomaniec, Nehemia Aharoni

Department of Postharvest Science of Fresh Produce, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

Revised 1 July 1998

Abstract

During leaf senescence protein degradation is enhanced. In order to obtain information on the enzymes involved in this process, a study was initiated to identify and characterize proteases whose activity is elevated in artificially senescing parsley leaves. A 70-kDa serine protease (EC 3.4.21) was identified by an activity gel assay. This protease activity, which is low in young leaves, was found to increase considerably in parallel to the advance of senescence and the reduction in the protein content of the leaves. A high correlation between the progress of senescence and the increase in the activity of the 70 kDa serine protease was demonstrated. Treatments with CO_2 or gibberellic acid, which retard senescence, reduced the protease's activity, whereas acceleration of senescence with ethylene enhanced it.

Keywords: *Petroselinum crispum*; Umbelliferae; Parsley; Senescence; Proteolysis; Serine protease

1. Introduction

Net loss of protein is a dominant feature of leaf senescence. Much of the protein is probably catabolized by specific proteases, which have been induced and the derived amino acids are transported to areas of growth or storage (for review, see Peoples & Dalling, 1988; Huffaker, 1990; Feller & Fischer, 1994). The substrate for the proteolytic enzymes in senescing leaves includes mainly enzymes and membrane proteins which function in photosynthesis. Large-scale hydrolysis of proteins occurs early in leaf senescence and is likely to depend upon the integrated action of groups of enzymes. In earlier studies changes in total proteolytic activity in crude extracts prepared from senescing leaves was followed and correlated with the loss of total protein (Peoples & Dalling, 1988; Huffaker, 1990; Feller & Fischer, 1994). Such correlations were found, but it is necessary to identify and characterize specific senescence-related proteases in order to understand the proteolytic mechanism which is responsible for senescence-induced protein degradation. Recently a few cDNAs representing senescence-related proteases, mainly cysteine proteases, have been isolated (Buchanan-Wollaston, 1997).

In our study of artificial senescence occurring during postharvest storage of leafy vegetables, we use parsley (*Petroselinum crispum* Mill.) as our model system. Our main interest, in this system, is to gain knowledge about artificial senescence per se as part of our long-term objective to improve the quality of stored fresh produce. Study of artificial senescence may be relevant for natural senescence too, however, care must be taken before general conclusions are drawn.

The soluble protein content in parsley is steadily reduced during postharvest senescence, whereas amino acids, which can not be exported from the detached leaves, accumulate. We have shown previously that treatments which are known to retard senescence, including gibberellin (GA3) (Aharoni, Dvir, Chalupowicz, & Aharon, 1993) and CO_2 (Aharoni, Reuveni, & Dvir, 1989), slow down protein loss and amino acid accumulation during postharvest senescence of parsley (Lers, Jiang, Lomaniec, & Aharoni, 1998). On the other hand, ethylene, which is well known to accelerate senescence (Mattoo & Aharoni, 1988), markedly enhanced protein loss and amino acids accumulation during parsley postharvest

* Corresponding author.

[1] Present address: Department of Food Science, China Agricultural University, 100094 Beijing, People's Republic of China.

0031-9422/98/$ - see front matter
PII: S0031-9422(98)00546-9

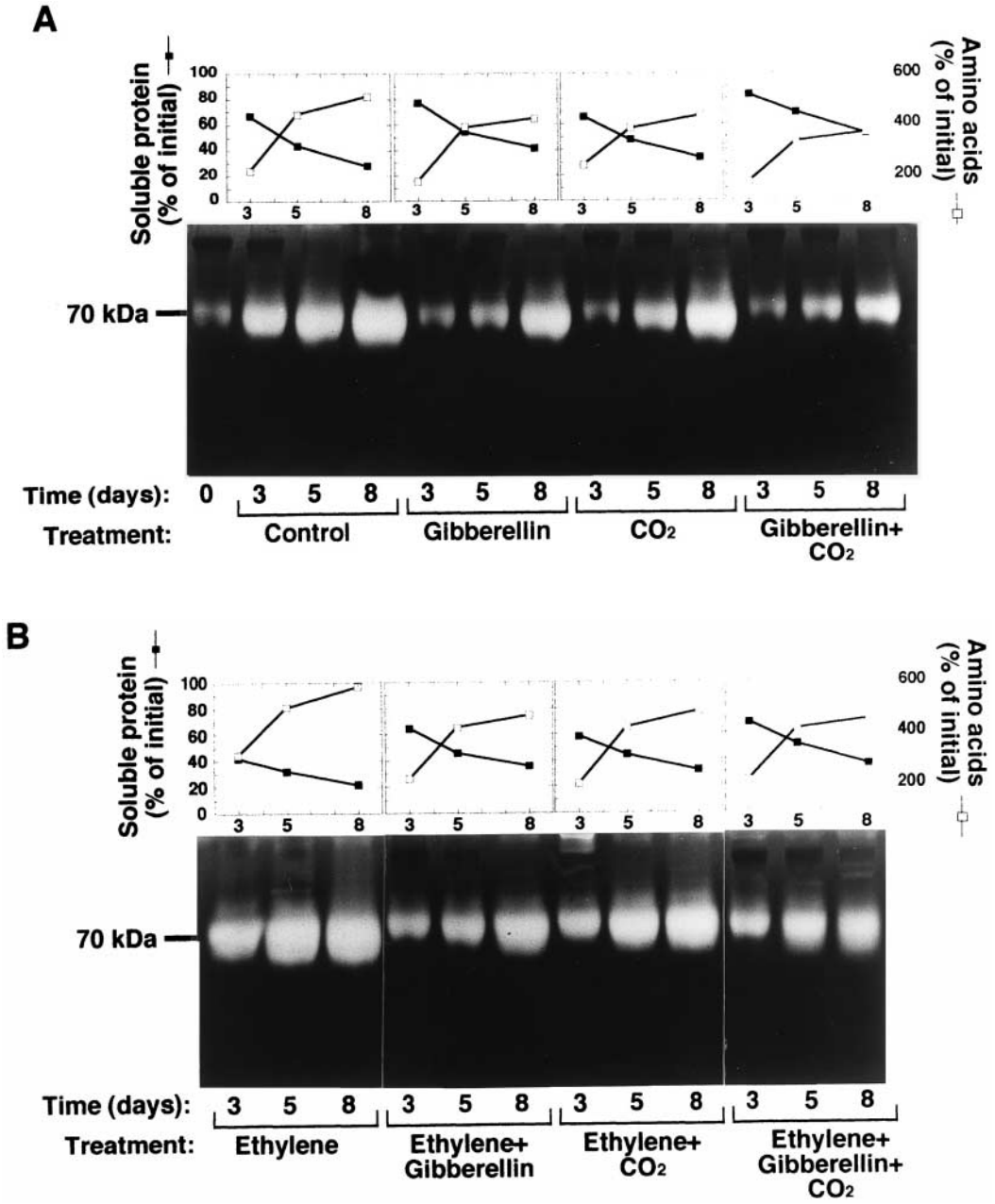

Fig. 1. Activity gel analysis of changes in protease activity during senescence of detached parsley leaves and the parallel changes in soluble and amino acid content. Parsley bunches were incubated in darkness under a flow-through system of air, 10% CO_2 in air, or 10 μl l^{-1} ethylene in air. Gibberellin (GA_3) treatment was given as a single spray of 50 μM, 1–2 h before harvest of the leaves. (A) Protease activity in non-treated leaves (control) and in leaves in which senescence was retarded with gibberellin or CO_2. (B) The effect of ethylene and a combination of ethylene with the senescence-retardants gibberellin and CO_2. Protein samples (20 μg) extracted on different days of the experiment were resolved on 10% gelatin-impregnated SDS-gel. Following renaturation, incubation and staining, protease activities are observed as clear bands. Parallel changes in soluble protein (filled symbols) and amino acid (open symbols) content are presented by the graphs located above the activity gel analysis (values are given as percentage of initial content).

senescence (Lers et al., 1998). In order to identify specific proteolytic activities, which may play a role in the senescence-induced protein degradation, we employed a gel activity assay. A serine-type protease activity was identified, the level of which was increased in parallel to the advance of parsley leaf senescence.

2. Results and discussion

2.1. Analysis of protease activity in senescing parsley leaves

Under our experimental conditions full yellowing of the parsley leaves was reached 8–10 days following

harvest and initiation of dark incubation. As reported earlier, degradation of both chlorophyll and protein was observed following the first day of the experiment (Lers et al., 1998). In order to identify proteolytic activities enhanced during senescence, protein samples, extracted at different stages of senescence, were resolved on SDS-PAGE containing gelatin substrate. Following renaturation and protein staining, the proteolytic activity was visualized as clear bands in which the substrate was hydrolyzed. In this assay we observed a single proteolytic activity with an estimated molecular weight of 70 kDa (Fig. 1). This activity, which was of a low level in young green leaves, was enhanced in parallel to the progress of senescence (Fig. 1A). The figure of 70 kDa MW is a rough estimate, since the gelatin substrate in the gel may interfere with the electrophoretic migration and a reducing agent was not present. A remarkable increase in the activity level was observed after only 3 days of incubation of the control leaves. The protease activity level was further enhanced, to reach a maximum on the 8th and last day of the experiment. At that time most leaves were completely yellow and the protein content had declined to ~30% of the initial level (Fig. 1A). The same protease activity was observed when we used either BSA, casein or parsley leaf protein instead of gelatin in the activity gel assay (data not shown). The observation of only one type of proteolytic activity may result from our inability to detect in this experimental system enzymes which can not retain activity following SDS-PAGE, need specific cofactors, or are substrate-specific. To test the correlation between the increase of the protease activity and progress of senescence, we examined the effect of senescence-retarding treatments. Leaves were treated with either GA_3, given as a single pre-harvest application, or with a continuous flow of 10% CO_2 during incubation. As shown in Fig. 1(A), both GA_3 and CO_2 treatments retarded protein loss and a combined treatment resulted in an additive effect. These two senescence-retarding treatments were able also to slow down the senescence-related increase in the 70-kDa protease activity with similar efficiency (Fig. 1A). When both GA_3 and CO_2 treatments were applied, an even more pronounced inhibition of the 70 kDa protease activity compared with the control was observed (Fig. 1A). This additive effect of GA_3 and CO_2 was similar to their combined inhibitory effect on protein and chlorophyll degradation during senescence (Lers et al., 1998).

Ethylene, shown before to accelerate senescence of detached parsley leaves, had a positive effect on the induction of the 70 kDa protease activity (Fig. 1B). Concomitantly, ethylene accelerated the decline in protein content and the accumulation of free amino acids (Fig. 1B). Both GA_3 and CO_2 overcame the enhancing effect of ethylene and reduced the 70 kDa activity to levels even lower than those observed in the control (Fig. 1B). However, protease activity in leaves treated with ethylene together with either GA_3 or CO_2 was higher than that observed when only GA_3 or CO_2 was present (Fig. 1). Treatment with both GA_3, and CO_2 could reduce even more the enhancing effect of ethylene on the protease activity (Fig. 1B).

The demonstrated correlation between the level of the 70 kDa protease activity and the progress of senescence raises the possibility that it plays some role during this process. The low-level protease activity observed in green leaves suggests that it has a role also in protein metabolism of non-senescing leaves. It was suggested before that in daylily (Guerrero, De la Calle, Reid, & Valpuesta, 1998) and tomato (Drake et al., 1996), senescence-induced cysteine proteases are involved in regular protein turnover. This suggestion was based on the observation that the expression of their encoding genes could be detected also in non-senescing tissue.

2.2. Temperature and pH optimum of the 70 kDa protease

The optimal temperature for the activity of the 70 kDa protease was determined. Multiple protein samples of senescing leaves were resolved on gelatin-gel. Following electrophoresis and protein renaturation different gel slices were incubated at different temperature and pH conditions. The optimal temperature was found to be 37°C (Fig. 2), which was used subsequently in all our experiments. The significant decrease in activity observed at temperatures above 37°C indicates that this enzyme is sensitive to high temperatures. The determined optimal temperature reflects probable temperature requirements for both the proteolytic reaction and diffusion in the gel of the enzyme, substrate or degradation products.

The results for the optimal pH demonstrate activity in a wide range of pH levels (Fig. 3). The maximal activity was observed at pH 7–8, with a gradual decrease when the pH was higher or lower than this level. Interestingly, even at the low pH of 4 some activity could be observed.

2.3. The 70 kDa protease is a serine protease

The mechanistic class of the 70-kDa protease was determined by treatment with different inhibitors diagnostic for the different classes of proteolytic enzymes. The protease activity was significantly inhibited by phenylmethylsulphonyl fluoride (PMSF) and the related substance 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (AEBSF) (Fig. 4), demonstrating that the proteolytic activity belongs to the serine-type proteases. Other tested inhibitors, including E-64 and

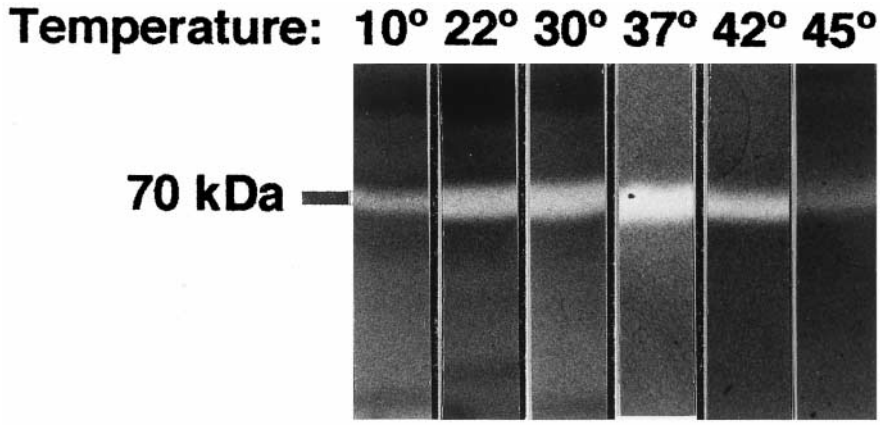

Fig. 2. Effect of temperature on the protease activity in the gel assay. Gelatin gel slices containing resolved protein (20 μg) extracted from senescing parsley leaves (day 8) was incubated in 50 mM Tris–HCl, pH 7.5 buffer, at the different temperatures for 6 h.

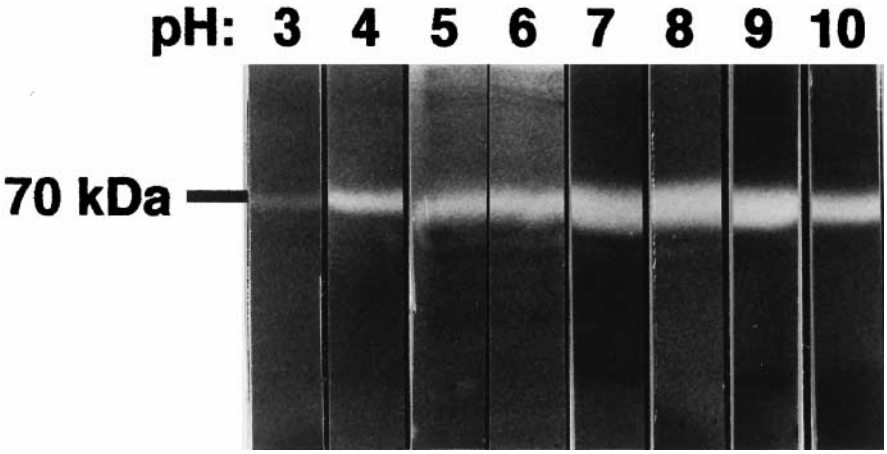

Fig. 3. Effect of the incubation buffer pH on the protease activity. Gelatin gel slices containing resolved protein (20 μg) extracted from senescing parsley leaves (day 8) were incubated at 37°C for 4 h in the appropriate 50 mM Tris–HCl buffer.

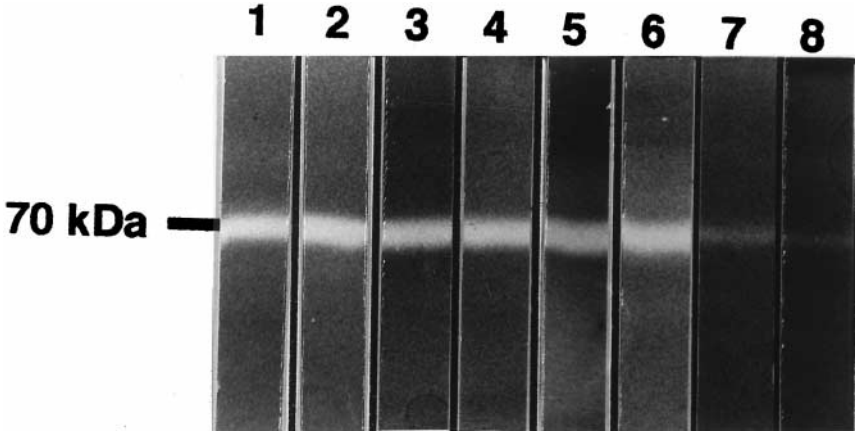

Fig. 4. Effect of different protease inhibitors. Protein samples (20 μg) and gel slices were treated with the different inhibitors as described in Section 3. 1, Control; 2, E-64 (4 μM); 3, EDTA (4 mM); 4, pepstatin (2 μM); 5, 1,10-phenanthroline (10 mM); 6, iodoacetic acid (100 μM); 7, AEBSF (4 mM); 8, PMSF (2 mM).

iodoacetic acid, cysteine protease inhibitors; pepstatin, an aspartic protease inhibitor; and EDTA and 1,10-phenanthroline, metal protease inhibitors, did not reduce the level of the 70 kDa protease activity. Likewise, dithiothreitol and β-mercaptoethanol reducing agents did not affect the protease activity (data not shown). Thus, the 70 kDa activity is a serine-type protease. Serine proteases seem to be the most abun-

dant protease group, and are extremely widespread and diverse. Characteristics of serine proteases include maximal activity at slightly alkaline pH, no absolute activator requirements and diverse substrate specificity (Barrett, 1986).

Not enough knowledge exists today to enable us to draw a clear conclusion about the relative importance of the different protease classes in the process of protein degradation during senescence. Most of the senescence-related protease genes identified in the last few years encode for cysteine proteases (Buchanan-Wollaston, 1997), suggesting that this group of enzymes plays an important role in the bulk degradation of cellular proteins during senescence. The involvement of serine protease activity in maize leaf senescence was suggested on the basis of protease inhibitor studies (Mau & Hageman, 1986). A senescence-induced 38 kDa protein detected in thylakoids of spinach was suggested to be a serine protease based on its covalent binding to diisopropylfluorophosphate and inhibition of this binding by the protease inhibitor PMSF (Kawasaki & Takeuchi, 1989). Serine proteases were detected also in two other developmental stages, during which an elevated protein degradation process takes place. A 55 kDa serine-type carboxypeptidase was shown to be induced during germination in cotyledons of bean seeds (Yamaoka, Ohba, Takeuchi, & Morohashi, 1993). Serine protease activities were found also to be induced during tracheary element differentiation in *Zinnia* (Ye & Varner, 1996; Beers & Freeman, 1997). Recently the cDNAs of the serine proteases P69A and P69B were cloned from tomato (Tornero, Conejero, & Vera, 1996, 1997). These proteases, which have an M_r similar to that of the parsley senescence-related protease, were identified as pathogenesis-related subtilisin-like proteases. It is interesting to note that pathogenesis-related proteins were recently found in parsley to be induced during leaf senescence (Lers, Jiang, Lomaniec, & Aharoni, 1998).

To the best of our knowledge, the results presented in this study are the first direct demonstration of a serine protease activity, which is increased during senescence. High correlation between the changes in the level of this protease activity and the advance of senescence, including protein degradation, was shown using treatments which accelerate or inhibit leaf senescence. These results support the possibility that this protease has a role in senescence-related protein degradation.

3. Experimental

3.1. Plant material and treatments

Experiments were performed with green, fully-expanded mature leaves of parsley (*Petroselinum crispum* Mill.), which were freshly harvested from the field. Bunches of parsley stems (~20 cm long, 100 g) were enclosed in 2-l jars fitted with inlet and outlet ports and stored for 8 days in the dark at 25° in order to promote senescence. The jars were sealed and connected to a flow-through system of air, 10% CO_2 in air, 10 μl l^{-1} ethylene in air, or 10% CO_2 plus 10 μl l^{-1} ethylene in air; the flow rate was maintained at 50 ml min^{-1}. The gases were bubbled through sterile water to maintain humidity in the jars. Where indicated, gibberellic acid (GA_3, Sigma, St. Louis, MO) treatment was applied by spraying the parsley plants in the field, 1–2 h before harvest, with a 50 μM hormone soln containing 0.05% BB-5 (Plaaskem, Gauteng, South Africa) as a surfactant.

3.2. Extraction and measurement of amino acids and proteins

Soluble protein and amino acids were extracted and levels were determined essentially as described by Lers et al. (1998).

3.3. Protease activity gel assay

To follow protease activities we used the SDS-gelatin-PAGE assay (Hellmich & Schauz, 1987) with some modifications. Regular SDS-PAGE (Laemmli, 1970) was performed in a mini-PROTEAN II® apparatus (Bio-Rad), except that the 10% resolving gel contained 0.15% gelatin. Protein samples were mixed with an equal volume of a non-reducing sample buffer (0.1 M Tris–HCl, pH 6.8; 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% bromophenol blue) and incubated at 42°C for 20 min before loading on the gel. Following electrophoresis at room temperature, the gel was washed in renaturing buffer (2% Triton X-100, 50 mM Tris–HCl, pH 7.5) for 20 (0.75-mm-thick gels) or 40 (1.5-mm-thick gels) min. Protease activities were investigated by incubating the gel in 50 mM Tris–HCl, pH 7.5, for 3 h at 37°C or for 12–16 h at room temp (24–26°C). After staining the gel with amido black (0.1% amido black, 10% acetic acid, 30% MeOH) and destaining with a soln of 5% acetic acid and 15% MeOH, white bands representing the site of protease activity were visualized. For determination of optimal pH, multiple protein samples of senescing leaves were fractionated on gelatin-gels as described above. Following electrophoresis and renaturation single-lane gel slices were incubated at 37° for 4 h in different Tris–HCl buffers ranging in their pH from 3 to 10. Staining was carried out as described above. Temperature effect on the protease activity was examined similarly by incubation of the gel slices in 50 mM Tris–HCl, pH 7.5, for 6 h at the different temperatures, followed by staining.

3.4. Protease inhibitor treatment

Different protease inhibitors were selected in order to determine the mechanistic class of the protease (Beynon & Salvesen, 1993). The inhibitors were added to the senescing leaves protein samples prior to electrophoresis. Following renaturation, gel slices were incubated in 50 mM Tris–HCl, pH 7.5 buffer, with the appropriate inhibitor at 37°C for 4 h. Inhibitors included pepstatin (2 μM, Sigma), 1,10-phenanthroline (10 mM, Sigma), EDTA (4 mM), PMSF (2 mM, Sigma), AEBSF (4 mM, Boehringer Mannheim), E-64 (4 μM, Sigma), and iodoacetic acid (100 μM, Sigma).

Acknowledgements

This research was supported by grant No. 408-0018-96 from the Chief Scientist of the Israel Ministry of Agriculture. We thank Mr. Zion Aharon for excellent technical assistance. Contribution from the Institute for Technology and Storage of Agricultural Products, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 405/98.

References

Aharoni, N., Dvir, O., Chalupowicz, D., & Aharon, Z. (1993). *Acta Horticulturae, 344,* 69.

Aharoni, N., Reuveni, A., & Dvir, O. (1989). *Acta Horticulturae, 258,* 255.

Barrett, A. J. (1986). In: *Plant proteolytic enzymes vol. 1*, (p. 1). Boca Raton, FL, USA: CRC Press.

Beers, E. P., & Freeman, T. (1997). *Plant Physiology, 113,* 873.

Beynon, R. J. & Salvesen, G. (1993). In: R. J. Beynon, & J. S. Bond, *Proteolytic enzymes: a practical approach* (p. 241). Oxford, UK: IRL Press.

Buchanan-Wollaston, V. (1997). *Journal of Experimental Botany, 307,* 181.

Drake, R., John, I., Farrell, A., Cooper, W., Schuch, W., & Grierson, D. (1996). *Plant Molecular Biology, 30,* 755.

Feller, U., & Fischer, A. (1994). *Critical Reviews in Plant Sciences, 13,* 241.

Guerrero, C., de la Calle, M., Reid, M. S., & Valpuesta, V. (1998). *Plant Molecular Biology, 36,* 565.

Hellmich, S., & Schauz, K. (1987). *Experimental Mycology, 12,* 223.

Huffaker, R. C. (1990). *New Phytologist, 116,* 199.

Kawasaki, S., & Takeuchi, J. (1989). *Plant Physiology, 90,* 338.

Laemmli, U. K. (1970). *Nature, 227,* 680.

Lers, A., Jiang, W., Lomaniec, E., & Aharoni, N. (1998). *Journal of Food Science, 63,* 66.

Lers, A., Jiang, W., Lomaniec, E., & Aharoni, N. (1998). *Physiologia Plantarum, 103*, 497.

Mattoo, K. M. & Aharoni, N. (1988). In L. D. Noodén, & A. C. Leopold (Eds.), *Senescence and aging in plants* (p. 241). San Diego, CA, USA: Academic Press.

Mau, T., & Hageman, R. H. (1986). *Soil Science and Plant Nutrition, 32,* 285.

Peoples, M. B. & Dalling M. J. (1988). In: L. D. Noodén, & A. C. Leopold, *Senescence and aging in plants* (p. 181). San Diego, CA, USA: Academic Press.

Tornero, P., Conejero, V., & Vera, P. (1996). *Proceedings of National Academy of Science USA, 93,* 6332.

Tornero, P., Conejero, V., & Vera, P. (1997). *The Journal of Biological Chemistry, 272,* 14412.

Yamaoka, Y., Ohba, Y., Takeuchi, M., & Morohashi, Y. (1993). *Plant Science, 95,* 1.

Ye, Z.-H., & Varner, J. E. (1996). *Plant Molecular Biology, 30,* 1233.