Regulation of the development of the first leaf of oats (*Avena sativa* L.)

Characterization and subcellular localization of proteases

Regulatie van de ontwikkeling van het eerste blad van haver (*Avena sativa* L.)

Karakterisering en subcellulaire lokalisatie van proteasen

Promotor: **Dr. J. Bruinsma**
hoogleraar in de fysiologie der planten
Co-promotor: **Dr. ir. L. C. van Loon**
universitair hoofddocent

Henry C. P. M. van der Valk

Regulation of the development of the first leaf of oats (*Avena sativa* L.)

Characterization and subcellular localization of proteases

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
Dr. C. C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 11 september 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1. Een toename van de protease-activiteit is geen noodzakelijke voorwaarde voor de daling van het eiwitgehalte in verouderende haverbladeren.

   Dit proefschrift

2. Eiwitafbraak tijdens de bladontwikkeling vindt niet plaats door redistributie van de zure protease van de vacuole naar het cytoplasma.

   Dit proefschrift

3. Voor onderzoek naar compartimentalisatie is de gebruikelijke aanname dat, na lysering, per protoplast één vacuole vrijkomt, te voorbarig.

4. De hypothese, dat polyaminen een rol spelen bij de bladveroudering door remming van protease-activiteit, wordt onvoldoende ondersteund door experimentele gegevens.

   Kaur-Sawhney, R. et al. (1982). FEBS Lett., 145: 345-349.

5. Het is hoogst onwaarschijnlijk dat DNA sequenties van slechts 4-12 nucleotiden zelfstandig weefselspecifieke gen-expressie reguleren.

   Botanical Congress, Berlin (1987).

6. Ten onrechte suggereert Griesbach met de term genetische transformatie dat overdracht van chromosomen via microinjectie moleculair is bewezen.

   Griesbach, R.J. (1987). Plant Science 50: 69-77.

7. Belangrijk wetenschappelijk werk, als gedaan door Balz, wordt internationaal niet op zijn waarde geschat door gebrek aan belangstelling voor niet-Engelstalige publicaties.

   Balz, H.P. (1966). Planta 70: 207-236.

8. Samenstellers van een woordenboek behoeven meer kennis van de biologische wetenschappen, daar de omschrijving van de vacuole als "een blaasvormige holte in het protoplasma, met celvocht gevuld", de capaciteit van de plantecel tot compartimentalisatie onderbelicht.

   Van Dale, Groot Woordenboek der Nederlandse Taal, 11e druk (1984).

9. De "N-end rule" is een voorbeeld van hoe door juiste interpretatie van resultaten van een goed experiment, gedaan om de verkeerde reden, nieuwe wetenschappelijke hypothesen kunnen ontstaan.

   Bachmair, A. et al. (1986). Science 234: 179-185.

10. De motivatie om de volgorde te bepalen van de DNA-basen van het complete menselijk genoom staat in schril contrast tot de onverschilligheid tegenover de afbraak van het leefmilieu.

11. Ontwapeningsonderhandelingen worden geloofwaardiger als diplomaten openlijk worden vergezeld door vertegenwoordigers van belanghebbende instanties en industrieën.

12. Het werkwoord vogelen heeft ten onrechte slechts kwalijke en dubbelzinnige betekenissen.

13. De opdracht in het proefschrift "dankzij de partner" is minder verèrend voor zowel de partner als de promovendus dan "ondanks de partner".

Stellingen behorende bij het proefschrift: "Regulation of the development of the first leaf of oats (Avena sativa L.). Characterization and subcellular localization of proteases." door Henry C.P.M. van der Valk.

Wageningen, 11 september 1987.

The death of plants or plant parts may be of positive ecological or physiological value.

A.C. Leopold

VOORWOORD

Het onderzoek naar de eiwitafbraak tijdens de bladveroudering is eigenlijk al gestart in 1976 toen de eerste student zich vastbeet in een doctoraalonderzoek over dit onderwerp. Er volgden er nog enkelen, waaronder ikzelf. Doordat Professor dr. J. Bruinsma mij de gelegenheid bood tijdens mijn vervangende dienstplicht de technieken verder te optimaliseren, kon een voorstel geschreven worden voor een onderzoeksproject. Het onderzoek dat na honorering van het projectvoorstel is uitgevoerd wordt beschreven in dit proefschrift.

Ik ben Professor Bruinsma zeer dankbaar voor zijn initiatief het onderzoek op deze manier mogelijk te maken. Zijn functie als promotor heeft zijn schaduw vooruitgeworpen en zou uitgebreid kunnen worden tot initiator. Als regulator heeft hij mij gesteund met aanmoedigingen en waardevolle opmerkingen.

Mijn co-promotor, Dr. ir. L.C. van Loon, geestelijk vader van het project en dagelijks begeleider wist mij steeds opnieuw te motiveren als er weer een sterk concurrerend artikel gepubliceerd werd. Ik heb hopelijk veel geleerd van zijn wonderbaarlijk geheugen, scherp analytisch vermogen en acurate wijze van formuleren. Daarnaast is hij het wellicht geweest die mijn sluimerende oenologische belangstelling heeft gewekt.

De studenten Miriam Budde, Leonne Plegt, Hans Kapteyn, Ans Nahuis, Ad Jespers en Maringa van Bentum hebben zeer veel vacuolen en enzymen geïsoleerd. Hun betrokkenheid en enthousiasme hebben mij erg geholpen. Ook Yvonne Gerritsen heeft mij bij enkele experimenten bereidwillig bijgestaan.

Met Hans Klerk, mijn kamergenoot en collega in een parallelproject, hebben de studenten en ik prettig samengewerkt. Hij had altijd gelegenheid het electroforeren uit te leggen en de apparatuur uit te lenen. Gezamenlijk konden we riskante hypothesen bedenken en verwerpen.

Jan Verburg, Gerrit van Geerenstein, Aart van Ommeren en Wilbert Alkemade zorgden ervoor dat er altijd planten waren om mee te experimenteren. Technische problemen werden door Ruth van der Laan en Ben van der Swaluw soepel en snel opgelost. De tekeningen, posters en foto's waren steeds op tijd klaar dankzij Allex Haasdijk, Paul van Snippenburg en Sybout Massalt.

In het bijzonder wil ik Folkert Hoekstra bedanken voor zijn attente geste de boeken van Dalling voor mij aan te schaffen. Dankzij de financiele steun van de Stichting "Fonds Landbouw Export Bureau 1916/1918" was ik in de gelegenheid deel te nemen aan de "Workshop on Plant Vacuoles" in Sophia-Antipolis in Frankrijk en de meeste vacuologen persoonlijk te leren kennen.

Vooral dankzij Frank Dumoulin, die met zijn enthousiaste hulp, ook in het weekend, assisteerde bij het maken van de lay-out heb ik zeer goede herinneringen aan de tijd die ik op de vakgroep Plantenfysiologie heb doorgebracht.

Mijn ouders hebben mij gestimuleerd en de mogelijkheden geboden te gaan studeren. Ik ben ze daar zeer dankbaar voor.

CONTENTS

CHAPTER I

General introduction

Using new high-yielding varieties, in many agricultural crops substantial increases in yield have been achieved during the last few decades. Plant breeding and genetic manipulation in order to further increase crop yield constitute active fields of investigation, but much attention is also focussed on the improvement of crop quality. Protein content is an important parameter for the nutritional value of many crop plants for humans and farm animals [1]. In general, high protein contents in the harvested parts of the plant are pursued. In addition, studies are directed towards improvement of protein quality e.g. a better balance of essential amino acids [2]. Toxic proteins, e.g. protease inhibitors [3], cause serious problems in food crops, and should be eliminated. On the other hand, they can be useful for farmaceutical and baking purposes, as well as in the beer industry [4].

In cereals, the grains are the main product and studies concerning the protein content of the seeds are numerous [1]. Protein quality in the grains is important in terms of viscoelasticity and hardness for making bread and pasta products. In contrast, in the breeding of barley for malting, minimal protein content of the grains is pursued. Therefore, breeding programs with the aim to improve protein content and quality depend on the crop and for the use it is intended.

During the vegetative growth phase of plants, continuous protein turnover takes place. The degradation products formed in senescing organs are mobilized to young, growing parts, e.g. apical leaves and roots. After the transition to the generative phase, flowers, fruits and seeds become major sinks for assimilates and nitrogenous compounds. Cereals display monocarpic senescence: when the grains are filled and start ripening, the whole plant senesces and dies off. In such a late stage of plant development, when all remaining leaves turn yellow, the protein in the leaves is rapidly hydrolysed. A substantial part of the degradation products formed (e.g. amino acids) is translocated up to the ears [5], where they constitute the major source for protein synthesis in the developing grains, particularly under

Table I. Proteases in leeves of cereal crop plants. BSA; bovine serum albumin, CBZ; N-carbobenzoxy, Hb; hemoglobin, Rubisco; ribulose-1,5-bisphosphate carboxylase.

	number	pH-optimum	substrate	type	localization	reference
Endopeptidases						
oats	1	4.2	Hb	serine-		[10]
	1(2)	4.2(6.8)	Hb, BSA,	sulphydryl-	etioplast	[11]
wheat	6	4.2-5.5	azocasein, Hb, Rubisco	sulphydryl-	vacuole	[12,13]
	1	7.5	azocas.			[14]
barley	1	5.5-5.7	Hb, azocas.,	sulphydryl-	vacuole	[15]
	1	5.5-5.7	Rubisco		vacuole	[15]
rice	1(2)	4.5(5.5)	Hb			[16]
	1	7.0	Hb	sulphydryl		[16]
Exopeptidases						
aminopeptidases						
oats	1	6.6	Hb	sulphydryl		[10]
	5	7.6-8.3		napthylamidases		[17]
wheat	3	7.4-7.6		naphtylamidases	cytosol	[12,18,19]
barley	1(4)				chloroplast	[15]
rice	1	8.0		sulphydryl		[16]
carboxypeptidases						
wheat	1(5)	5.3	CBZ-phenyl-alanine-l-alanine	serine	vacuole	[20]
barley	1				vacuole	[15]
rice	1	5.5		sulph/serine-		[16]

dry conditions [6]. This degradation of protein in senescing leaves precedes the loss of chlorophyll but already involves the reduction of photosynthetic capacity, as the major protein present in leaves, ribulose-bisphosphate carboxylase necessary for CO_2 fixation, is most rapidly degraded [7]. The rate at which valuable metabolic products are mobilized, is one of the factors determining seed yield. On the one hand, a prolonged phase of active photosynthesis leads to a higher yield of starch in the developing seeds. On the other hand, a rapid mobilization of nitrogen during the later phase of seed filling is important to obtain a high content of protein in the seeds. Wheat lines with rapidly yellowing leaves possess a relatively high content of protein in the seeds [8]. Conversely, spraying the leaves with hormones that retard senescence increases the period of active photosynthesis but impedes mobilization and, at least in soybean, decreases seed yield both quantitatively and qualitatively [9]. To establish physiological criteria for the improvement of agricultural crops, there is a need to understand senescence as well as photosynthesis and translocation. When one aims to improve the protein content of the grains by breeding and genetic manipulation, it is crucial to obtain information about the physiology of protein synthesis and degradation in the leaves.

Proteases in green and senescing leaves (Table I) are attracting increasing attention as possible regulatory enzymes in protein turnover and breakdown during senescence [21]. These enzymes degrade proteins by hydrolyzing peptide bonds. Proteases are divided into endopeptidases, or proteinases, that cleave internal peptide bonds, and exopeptidases with specificity for protein N- or C-termini. The endopeptidases can be further classified according to their active site: serine-, cysteine-, aspartic- or metallopeptidases. The exopeptidases are classified according to substrate specificity. Those specific for the N-terminus are aminopeptidases and remove single amino acids. Similarly, dipeptidylpeptidases remove dipeptides from peptides and proteins. Carboxypeptidases release C-terminal residues from peptides and proteins, whereas peptidyldipeptidases cleave off a C-terminal dipeptide [22]. In this thesis the terms "acidic" and "neutral" proteases will be used to designate relatively poorly characterized proteases with well-established pH optima.

Changes in protease activities may control the rate of protein breakdown during senescence. Much attention has been paid to changes in enzyme acti-

vities in extracts from cereal leaves and to correlations of these changes with protein loss. Marked increases in endopeptidase activities were observed in both naturally senescing leaves attached to the plant [23], and in detached leaves during artificial ageing in the dark [24-26]. However, in dark-incubated detached leaves of corn [27], oats [28] and wheat [29], protein breakdown has been observed to occur in the absence of an increase in total endopeptidase activity. Also in senescing legume leaves, in general no good correlation was found between protease activities and protein degradation [30-33]. In tobacco, protease activity has also been shown to decline during leaf senescence [34,35].

Alternatively, compartmentalization of enzymes and substrates has been suggested as a mechanism for controlling protein breakdown through changes in the distribution or spatial separation of enzymes and substrates. Many hydrolytic enzymes with acidic pH optima are localized inside the vacuole [36]. Consequently, the vacuole has been suggested to function as a lysosomal organelle in plant cells [37]. In many plant species, including wheat [38] and barley [39], endopeptidases and carboxypeptidases were found to be present mainly in vacuoles (Table I). As to how far these vacuolar proteases can function in cytoplasmic and organellar protein degradation is largely unknown. Minor endopeptidases seem to be present in the cytoplasm [29], which raises the question about the function of these different types of proteases. Aminopeptidases in particular seem to be located in the cytoplasm (Table 1). High activities of these latter enzymes in young and full-grown leaves support the hypothesis that these enzymes are involved in protein turnover or processing rather than in senescence. However, their contribution to protein metabolism has not been sufficiently elucidated.

The first leaf of the seedling of oats demonstrates the typical pattern of cereal leaf senescence [40], and has therefore been chosen as a model system to study the regulatory function of proteases in protein degradation in cereal leaves. Varga and Bruinsma [41] previously established that artificial, but not natural cytokinins retard senescence in this system, casting doubt on the role of cytokinins in the regulation of senescence under natural conditions. Martin and Thimann [26] suggested that the cytokinins act by suppressing the formation of proteases responsible for the

breakdown of protein during senescence and, thus, an increase in protease activity would be required for senescence to be expressed.

In this thesis it is demonstrated that the loss of protein during senescence of oat leaves does not depend on changes in protease activity. Rather, young leaf cells already turn out to possess sufficient protease activity to degrade all proteins present within two hours in vitro (Chapter 2.).

A study to determine the distribution and subcellular localization of proteases in developing leaves demands careful measurements of protease activities in subcellular fractions and compartments. In Chapter 3 a method is described to reliably determine protease activities in isolated protoplasts, a first step to further elucidate the subcellular localization of the enzymes.

To be able to follow the localization of the proteases in the course of leaf development, vacuoles were isolated from leaves up to an advanced stage of senescence (Chapter 4). The distribution of the protease activities and the relative amounts present in purified vacuoles were determined to study the possible role of vacuolar enzymes in the process of protein breakdown (Chapter 5).

To study whether additional proteases can be distinguished that might play a role in leaf senescence, extracts were fractionated and the major proteases were partly purified and characterized (Chapter 6). A different method for identifying proteases, activity staining on polyacrylamide gels after electrophoretic separation, was finally employed to more fully characterize the spectrum of proteases present in oat leaves (Chapter 7).

Research on the properties of leaf proteases may help to provide parameters for further study, control and improvement of agricultural crops in order to increase the level and quality of plant protein in harvested organs.

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CHAPTER 2

Changes in Protease Activity in Leaves during Natural Development and Accelerated Ageing upon Detachment

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Summary

Oat (*Avena sativa* L. cv. Victory) leaves contain two major proteases with pH optima at 4.5 («acidic» protease) and 7.5 («neutral» protease). In naturally developing primary oat leaves both proteases decreased in activity during senescence, whether activities were measured in the presence of endogenous proteins as substrates or of a saturating amount of haemoglobin. In both oat and tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants, younger leaves, once fully-expanded, possessed higher protease activities than older leaves.

When detached oat leaves were aged in darkness, loss of protein and chlorophyll was similar whether leaf segments were placed horizontally in Petri dishes on wet filter paper, or held vertically in plastic holders with their bases in water. In horizontally placed leaves, the activities of the two proteases tended to increase but in leaves kept vertically protease activities decreased as in attached leaves. In light, both chlorophyll and protein loss occurred at about half the rate observed in darkness. However, in vertically held leaves the acidic protease started to increase by day 2, whereas the neutral protease first declined but increased later. These increases were not mimicked by incubation on sucrose in darkness and neither could they be attributed to a greater accumulation of free amino acids in the light.

Kinetin and, to a lesser extent zeatin, maintained chlorophyll and protein levels and counteracted both the increases and decreases in protease activities observed during ageing in light or darkness, respectively. Since accelerated loss of protein in darkness was not accompanied by increases in protease activities, these observations are at variance with those of Martin and Thimann (Plant Physiol. 49: 64, 1972) and suggest that additional synthesis of the major proteases is not a prerequisite for protein breakdown during senescence.

Key words: Avena sativa, Nicotiana tabacum, protease activity, leaf senescence, attached/detached leaves, light, cytokinin.

Introduction

The protein content of leaves passes through a maximum during rapid leaf growth and gradually declines during senescence. Cytokinins delay senescence and the accompanying loss of protein primarily by inhibiting proteolysis (Thimann, 1980). Martin and Thimann (1972) showed that both kinetin and cycloheximide suppress an increase in protease activity during ageing of detached oat leaves placed horizontally on slides over moist filter paper in darkness. These authors therefore suggested that cytokinins might act by suppressing protease formation.

Abbreviations: EDTA = ethylene diamino tetraacetic acid; MCE = 2-mercaptoethanol; SDS = sodium dodecyl sulphate; TCA = trichloroacetic acid.

Although inhibitors of nucleic acid synthesis or of protein synthesis on cytoplasmic ribosomes retard senescence in leaf segments or disks of various plant species (Thomas and Stoddart, 1980; Yu and Kao, 1981), actinomycin D and cycloheximide accelerated the senescence of tobacco leaf discs and interfered with the anti-senescence action of benzyladenine (BA) (Takegami, 1975). Since cycloheximide suppressed ^{14}C-leucine incorporation into protein equally in the presence and absence of BA, the action of BA in retarding senescence must also involve protein synthesis. Hence, suppression of protease synthesis by cytokinins, as proposed by Martin and Thimann (1972) may not be a general effect of the hormone.

The importance of such a mechanism in the regulation of leaf senescence may be further questioned on the following grounds: 1. Since severing the leaf from the stem cuts off the supply of nutrients and hormones from other plant parts, the course of the accelerated artificial ageing of detached leaves is not necessarily identical to that of the natural senescence of attached leaves. Besides, wounding due to cutting increases the activity of several hydrolytic enzymes, e.g. RNase (Udvardy et al., 1967) and protease (Miller and Huffaker, 1985). 2. Varga and Bruinsma (1973) showed that in detached oat leaves chlorophyll is retained by the artificial cytokinins kinetin and BA to a far larger extent than by the natural ones, zeatin and isopentenyladenine. It may be questionable, therefore, whether the slower ageing of attached as compared to detached leaves can be attributed to efficient suppression of protease synthesis under natural conditions. 3. Martin and Thimann (1972) determined protease activity on an exogenous substrate, haemoglobin, and did not measure the activity on the endogenous leaf proteins that are degraded during senescence. This may be relevant because the affinity of proteases for different substrates may vary greatly, as experienced also by Martin and Thimann (1972) for different exogenous substrates.

Martin and Thimann (1972) initially found two peaks of protease activity in oat leaves, at pH 3 («acid» protease) and pH 7.5 («neutral» protease) with a minimum at pH 5. Later, however, purification by use of haemoglobin-affinity chromatography revealed the presence of two proteases with pH optima of 4.2 and 6.6, and indicated the occurrence of a third enzyme, active at pH 3.5 (Drivdahl and Thimann, 1977). In view of the substantial shift between the earlier and the later values and the possibility of multiple forms of both the acid and the neutral protease, the pH-activity profile of oat leaf protease was reexamined, both in the absence and in the presence of exogenous substrate and after establishing optimal extracting conditions for the two proteases. To further elucidate the relationship between senescence, protein content and protease activity, the course of protease activity and protein breakdown in attached leaves was compared to that in detached leaves. The effects of the two types of cytokinins were further investigated in detached leaves.

Materials and Methods

Plant material

Oats (*Avena sativa* L. cv. Victory), were grown in soil in a glasshouse under natural lighting conditions, supplemented with illumination from high pressure mercury halide Philips HPI/T

lamps to ensure a minimum light intensity of 18 W m^{-2} for 16 h day^{-1}. Minimum temperature was 20 °C during the day and 19 °C at night.

First leaves were used unless stated otherwise. To follow the fate of attached leaves, these were harvested at regular intervals and the top 4.5 cm segment was used for the determinations. For incubation of detached leaves, 5 cm apical portions of leaves sampled on day 8 were used. Ten leaf segments were placed vertically in plastic holders with their bases in water or cytokinin solution. For comparison, in some experiments the segments were held horizontally in Petri dishes on wet filter paper. This last set-up was similar to the one used by Martin and Thimann (1972) and Varga and Bruinsma (1973) except that glass slides were omitted. Incubation was carried out at 25 °C either in the dark or under two cool white fluorescent tubes yielding 5.0 and 3.7 mW cm^{-2} in the blue and the red, respectively, at leaf height; relative humidity exceeded 70 %.

Chlorophyll was retained to a far larger extent near the base than in the rest of the leaf segments. To avoid this interference due to wounding (Farkas et al., 1964; Miller and Huffaker, 1985), the basal 0.5 cm of the segments were cut off and discarded before extraction.

Tobacco (*Nicotiana tabacum* L. cv. Samsun NN) leaves from plants grown for 9 weeks as described above, were used for comparison in a few experiments.

Extraction of protease

Four g of 4.5 cm leaf segments were homogenized with a mortar and pestle at 2 °C in 22 ml extraction medium containing 50 mM Tris-HCl, 0.2 % (w/v) ascorbic acid, pH 8.0. This medium proved optimal for extraction of the acidic protease, but was routinely supplemented with 3.5 % (w/v) NaCl, 0.1 % (v/v) 2-mercaptoethanol (MCE) (Martin and Thimann, 1972) and 2 mM EDTA (Frith et al., 1975) to ensure adequate extraction of the neutral protease. The homogenate was strained through four layers of hydrophilic gauze and centrifuged successively for 15 min at 15,000 g and 30 min at 30,000 g. The resulting supernatant was dialysed overnight against 1 l of 10 mM Tris-HCl, 3.5 % NaCl, 0.1 % MCE, pH 7.0. After centrifugation at 30,000 g for 30 min, the dialysed solution was used for protease determination. The whole procedure was carried out at 0–4 °C.

Protease determination

Protease activity on endogenous substrates (e.g. the soluble proteins present in the dialysed enzyme solution) was determined at pH 4.5 and 7.5 by mixing 4 ml 0.2 M phosphate-citrate buffer, pH 4.5 or 0.2 M Tris-HCl, pH 7.5 with 1 ml water and 2 ml enzyme solution at 40 °C. After 0, 60 and 120 min incubation in a rotatory shaking water bath, 1.75 ml samples were withdrawn and added to 0.25 ml of ice-cold 40 % (w/v) trichloroacetic acid (TCA). After standing for at least 4 h at 2 °C, the mixture was centrifuged at 5000 g for 15 min. Aliquots of the supernatant solutions were analyzed for free amino acids by the ninhydrin method, as modified by Rosen (1957). Release of amino acids was checked for linearity and protease activity was expressed as nmoles of amino acid released per h per g of leaf fresh weight, using glycine as a standard.

To measure protease activity in the presence of exogenous substrate, the 1 ml water in the reaction mixture was replaced by 1 ml 4 % (w/v) haemoglobin solution (Martin and Thimann, 1972). Casein and bovine serum albumin were similarly tested as substrates. In the presence of 4 % haemoglobin, substrate is present in large excess and protease activity is not limited by substrate availability. Under these conditions, the relative potentials for protein breakdown by protease can be compared.

Determinations of protein, free amino acids and chlorophyll

Soluble protein and free amino acid content were determined in the 30,000 g supernatant before dialysis. Aliquots were mixed with an equal volume of ice-cold 10 % (w/v) TCA and free amino acids in the supernatant after centrifugation determined as described above, correcting

for buffer blanks. The precipitate was dissolved in 0.1 N NaOH and the protein content measured according to Lowry et al. (1951).

For total protein and chlorophyll determinations, leaf samples were homogenized at 2 °C in 80 % (v/v) acetone, containing 1 % (w/v) ascorbic acid. After centrifugation at 5000 g for 10 min, chlorophyll was estimated in the supernatant solution after Bruinsma (1963). The pellet was resuspended in 0.1 N NaOH. After standing overnight, the residue was removed by centrifugation and the solubilized protein was determined similarly to the procedure used for the soluble proteins.

Gel electrophoresis

Protein samples after incubation at different pH values were precipitated with equal volumes of 10 % TCA. After centrifugation, the precipitates were dissolved in sample buffer [63 mM Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) MCE, 5 % (v/v) glycerol, 0.001 % (w/v) bromophenolblue, pH 6.8], boiled for 5 min at 100 °C and subjected to electrophoresis in 12.5 % (w/v) polyacrylamide gel slabs containing SDS according to Laemmli (1970). Gels were stained with 0.25 % (w/v) Coomassie Brilliant Blue in methanol : acetic acid : water (5 : 1 : 4, v/v) and destained in methanol : acetic acid : water (5 : 8 : 88, v/v).

All determinations were made in duplicate and each experiment was repeated at least once. Quantitative differences occurred between experiments but the observed effects were reproducible and qualitatively similar. In all cases, data from representative experiments are presented.

Results

Protease activity in developing oat leaves

In the first leaf of 8-day-old oat seedlings, protease activity showed maxima around pH 4.5 and 7.5 with added haemoglobin as a substrate (Fig. 1 A). Shoulders of activity were present at pH 3.5 and 5.5, suggesting that the protease activity at low pH values may consist of several forms differing in pH optimum. A similar pH-activity profile was revealed in young tobacco leaves (Fig. 1 B), indicating that proteases with similar pH optima are present in different plant species. Both in oat and in tobacco leaves, substantial hydrolysis of haemoglobin occurred at all pH values measured, i.e. between 3 and 9. Degradation of the endogenous proteins present in the enzyme solution likewise occurred at all pH values. However, less prominent peaks of activity were noticeable and in oat leaves the activity declined below pH 5.5, perhaps due to the lower solubility of the leaf proteins at these lower pH values.

Since the proteases active at pH 4 to 5.5 («acidic» protease) and 7 to 8 («neutral» protease) apparently constitute the major proteases present in leaves, these were examined in further experiments. Although the presence of 0.1 % MCE reduced acidic protease activity by 12 ± 6 %, its inclusion in the extraction medium increased neutral protease activity up to 60 %, possibly through stabilization of the enzyme (Drivdahl and Thimann, 1977). 2 mM EDTA had no effect on the acidic protease but increased neutral protease activity a further 20 %. No increase in activity of either protease occurred when 0.1 % Triton X-100 was included in the extraction medium.

When added as exogenous substrate in the protein determination mixture, haemoglobin stimulated the release of ninhydrin-positive material more than two-fold at pH 4.5 but by no more than about 25 % at pH 7.5. In contrast, in the presence of

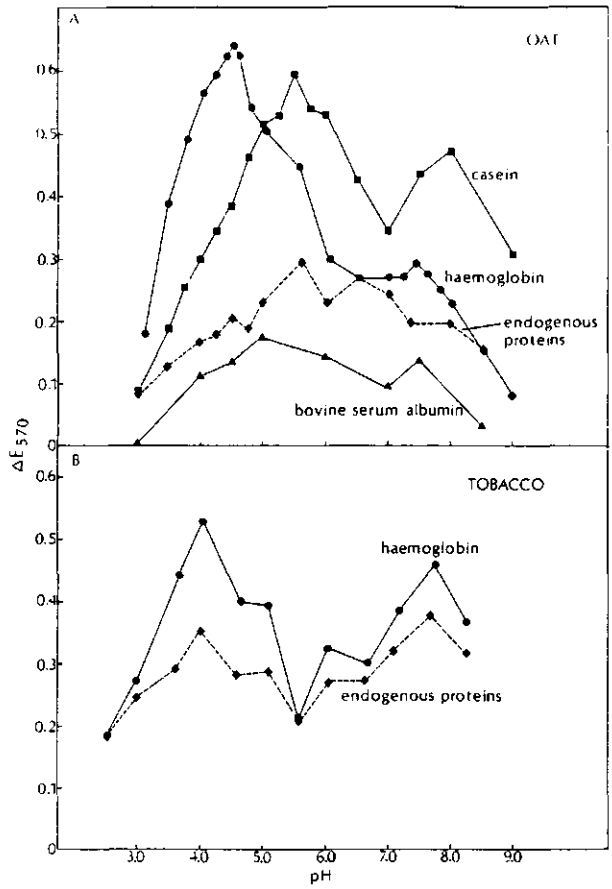

Fig. 1: Effect of pH on protease activity in extracts from A) 8-day-old primary oat leaves and B) young leaves from 9-week-old tobacco plants, using either (●) haemoglobin, (■) casein, (▲) bovine serum albumin or (◆) endogenous proteins present in the extracts as substrates.

added casein, protein breakdown was stimulated about twofold over the entire pH range, with maxima at pH 5.5 and 8.0. Whereas haemoglobin was preferentially degraded at pH 4.5, the neutral protease more readily used casein as a substrate. Bovine serum albumin was inhibitory to protein breakdown at all pH values and slightly more so at neutral than at low pH. The acidic and the neutral proteases further differed in inactivation temperature and stability *in vitro* at different pH's (Table 1), both proteases being most stable at their pH optima.

Gel electrophoresis of the oat leaf proteins after incubation at different pH values showed that all detectable proteins were readily degraded at pH 5.5 and at a lower rate at lower pH values (Fig. 2), indicating that this activity was endoproteolytic in nature. Above pH 6, protein breakdown appeared to be restricted to a small number

Table 1: Properties of acidic and neutral proteases from developing oat leaves.

	Acidic protease	Neutral protease
pH-optimum (substrate: haemoglobin)	4–5	7–8
Inactivation temperature (10 min)	75 °C	65 °C
($T_{½}$, 10 min)	58 °C	52 °C
Stability *in vitro*		
($t_{½}$, 0 °C, pH 4.5)	14 days	2 days
($t_{½}$, 20 °C, pH 4.5)	4 days	1 day
($t_{½}$, 0 °C, pH 7.5)	5 days	4 days
($t_{½}$, 20 °C, pH 7.5)	1 day	2 days

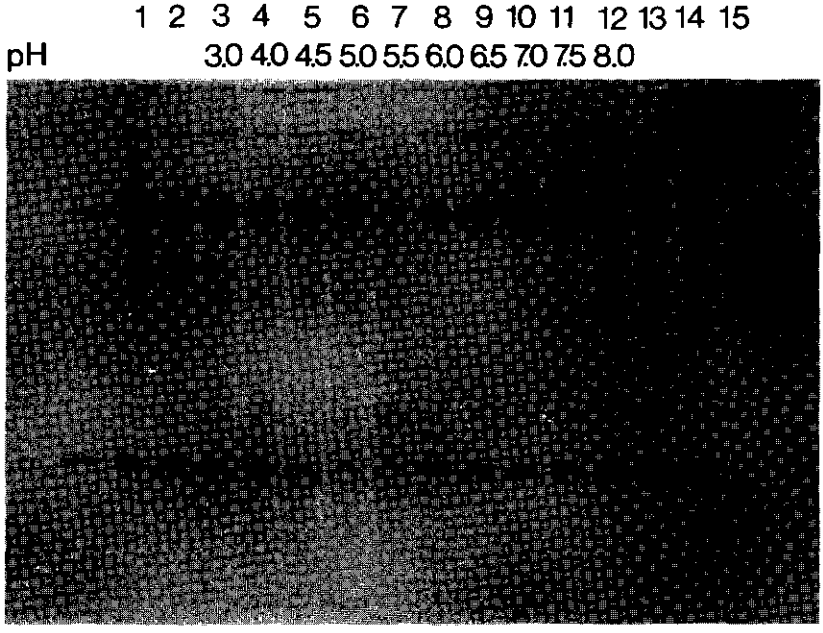

Fig. 2: Gel electrophoretic profiles of oat leaf proteins after incubation of extracts for 6 h at 40 °C at (lane 3) pH 3.0, (lane 4) pH 4.0, (lane 5) pH 4.5, (lane 6) pH 5.0, (lane 7) pH 5.5, (lane 8) pH 6.0, (lane 9) pH 6.5, (lane 10) pH 7.0, (lane 11) pH 7.5, (lane 12) pH 8.0. Lanes 1, 2, 13, and 14 contain 0 h controls (no incubation) at pH's 4.5, 5.5, 7.0, and 8.0, respectively. Phosphate-citrate buffer was used at pH values from 3.0 to 6.5 and Tris-HCl buffer from pH 7.0–8.0. Lane 15 contains the mol. wt markers phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400). The positions of the large and small subunit of ribulosebisphosphate carboxylase are indicated by arrows.

of protein bands only. Notably, the subunits of ribulosebisphosphate carboxylase were stable under these conditions. Although the presence of exoproteolytic activity

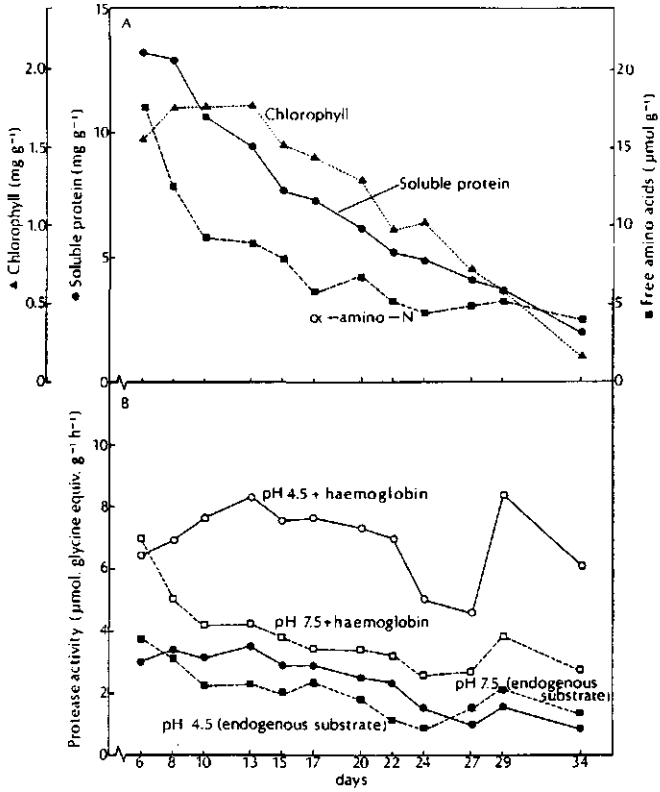

Fig. 3: Changes in (A) (●) soluble-protein, (▲) chlorophyll and (■) free-amino acid content and (B) activities of (○, ●) acidic and (□, ■) neutral protease using either (○, □) haemoglobin or (●, ■) soluble leaf proteins present in the extracts as substrates, during natural development of the primary leaf on intact oat plants.

cannot be ruled out, the complete disappearance of specific bands from the profile suggests that also at neutral pH the main protease is of the endo-type.

Changes in protease activities during leaf development

To follow the course of the protease activities during leaf development, oat plants were grown and their first leaves detached at regular intervals for protease determinations. The activity of the acidic protease on endogenous substrates remained virtually constant from 6 to 13 days after sowing, when the leaf expanded to its final size, then gradually declined with a passing upsurge around day 29, when the leaf withered and died (Fig. 3 B). With haemoglobin the activity increased about 30% up to day 13, then decreased in parallel with the activity on endogenous substrates, before showing a pronounced increase on day 29. A final increase was also manifested by the neutral

protease, both in the absence and in the presence of haemoglobin. However, this enzyme showed maximal activity already on day 6 at leaf emergence, and only declined afterwards up to day 24 (Fig. 3 B).

Whereas chlorophyll content remained maximal up to day 13, soluble protein content started to decline already upon leaf emergence, decreasing at an almost linear rate to about 20% of the initial value by day 34. Hence, at least in the two weeks between days 13 and 27, the continuous breakdown of protein was not accompanied by an increase in protease activity. Only at the final stage of leaf death, when 70 to 80% of the protein had been degraded, was the capacity of both enzymes for protein degradation increased. The decrease in protein content with leaf age was accompanied by a decrease in free amino acids, apparently resulting from the transport of the liberated amino acids to other plant parts (Fig. 3 A).

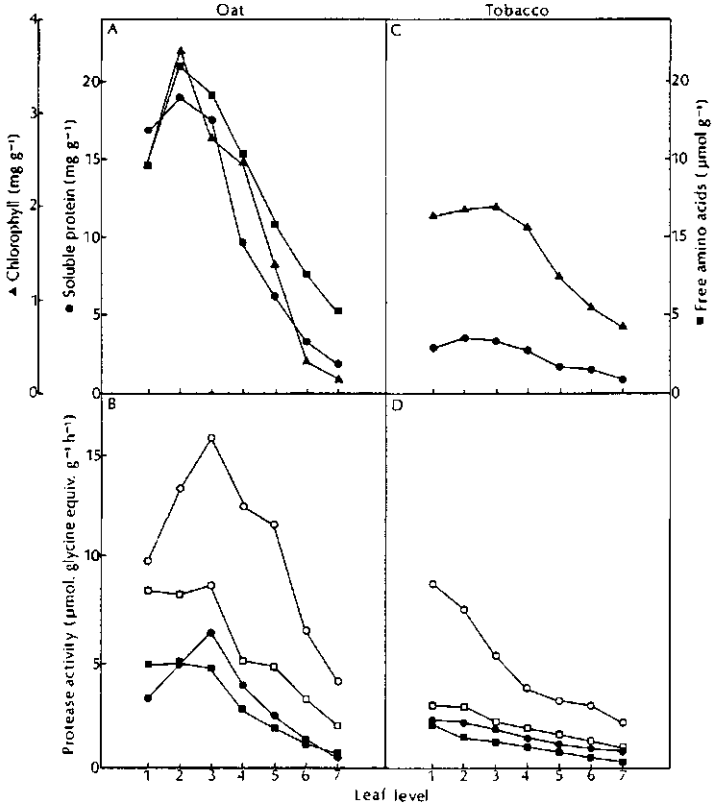

Fig. 4: Content (A, C) of (▲) chlorophyll, (●) soluble protein and (■) free amino acids and (B, D) activities of (○, ●) acidic and (□, ■) neutral protease using either (○, □) haemoglobin or (●, ■) soluble leaf proteins present in the extracts as substrates in (A, B) consecutive leaves of 34-day-old oat plants and (C, D) consecutive groups of leaves of 9-week-old tobacco plants. Leaf levels are numbered from top (youngest leaves) to bottom (oldest leaves).

Since protease activities were found to decrease with leaf age, it would be expected that at any one time, younger leaves on a plant would possess higher protease activities than older ones. Fig. 4 shows that this is indeed the case, both for oat and for tobacco plants. In oats, acidic protease activity increased from leaf 1 to leaf 3, associated with leaf expansion, then decreased in parallel with protein content in consecutively older leaves. Neutral protease likewise declined. Similar results were obtained when selected leaves at different insertion levels were analyzed at different stages of their development (data not shown). In tobacco leaves, both protein content and protease activities were much lower than in oat (Fig. 4 C, D). However, also in tobacco, both acidic and neutral protease were highest in the young leaves and declined gradually throughout maturity and senescence.

Changes in protease activities during accelerated ageing of detached oat leaves

The apical 5 cm of 8-day-old primary leaves were cut off and placed vertically in holders with their bases in water and kept either in darkness or in light. In darkness, accelerated protein breakdown was evident by day 2 and maintained up to day 4, when 70% of the protein had been degraded. During this period, free amino acid content increased over 11-fold (Fig. 5 A). As shown in Fig. 5 B, protease activities were not increased when the accelerated protein breakdown was initiated, and up to day 2 followed a course similar to those in attached leaves. Only on day 3 to 4 did the acidic protease show a small but significant increase with haemoglobin as a substrate, reminiscent of the increase on day 29 in attached leaves. Apart from a slight increase in the degradation of endogenous proteins at pH 7.5 on day 3, the activities of both proteases on endogenous substrates declined, associated with substrate depletion.

In the light, both chlorophyll and protein loss occurred at about half the rate observed in darkness. Free amino acids accumulated less than seven-fold (Fig. 5 C). Using haemoglobin as a substrate, the acidic protease activity increased almost two-fold between days 2 and 6 and started to decline slowly only by day 10 (Fig. 5 D). Neutral protease activity declined as in attached leaves up to day 8, after which a more than two-fold increase occurred over the following four days before a large drop between 12 and 14 days. Without added substrate only a minor increase in acidic protease activity was evident on day 4, and none in neutral protease activity. Apparently, protease activity on endogenous substrates was limited by substrate availability. Thus, protein was lost more slowly than in the dark, in spite of the large increases in protease activities.

To see whether the increase in the protease activities in the light might result from sustained energy metabolism due to photosynthesis, leaf segments were incubated in the dark on 20 mM sucrose (Goldthwaite, 1974). As shown in Table 2 for a typical experiment, neither the acidic nor the neutral protease activity increased under these conditions.

Effect of experimental set-up on changes in protease activity

Martin and Thimann (1972) described a large, transitory increase in acidic protease activity and a smaller, continuous increase in neutral protease activity during se-

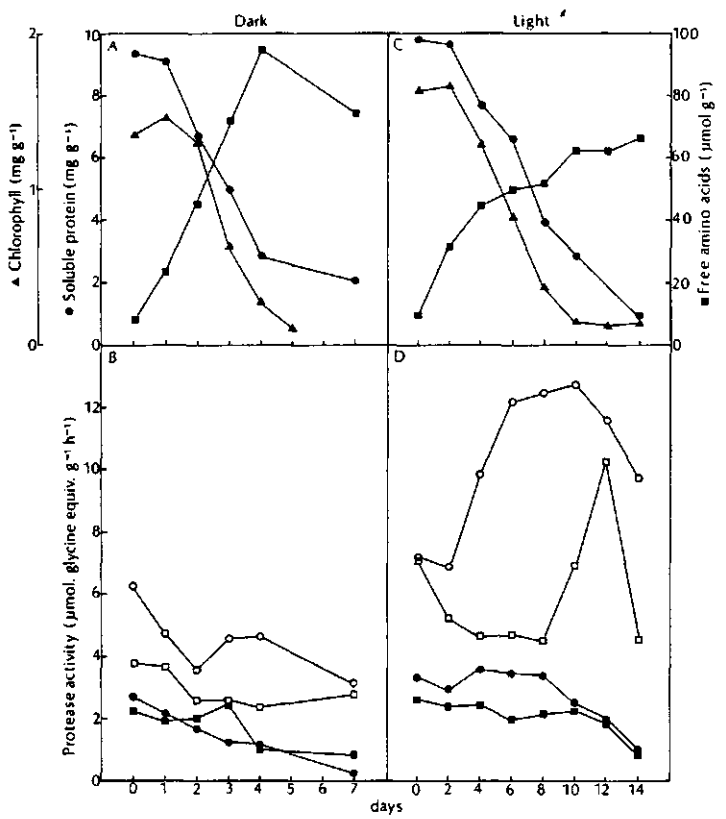

Fig. 5: Changes in (A, C) (▲) chlorophyll, (●) soluble protein and (■) free amino acids and (B, D) activities of (○, ●) acidic and (□, ■) neutral protease using either (○, □) haemoglobin or (●, ■) soluble leaf proteins present in the extracts as substrates during incubation of detached oat leaves held vertically in plastic holders with their bases in water in (A, B) darkness or (C, D) light.

nescence of detached oat leaves in darkness. Under our conditions such increases were evident neither in leaf segments in darkness, nor in attached leaves on plants growing under natural conditions in a greenhouse, but were rather similar to those produced by detached leaves kept in continuous light (Fig. 5 D). Since Martin and Thimann (1972) kept their leaf segments horizontally on wet filter paper, whereas we placed them vertically with their bases in water, we investigated whether this positional difference might affect the change in the protease activities. As shown in Table 3, in leaves held vertically both protease activities declined, as shown previously in Fig. 5 B. However, in the leaf segments placed horizontally, the acidic protease activity consistently increased, whereas the neutral protease activity was more variable. Losses of chlorophyll, total and soluble protein were similar whether the

Table 2: Effect on protease activities of incubation of oat leaf segments in darkness on sucrose.

Incubating conditions	Acidic protease activity (μmol glycine equiv. $g^{-1} \cdot h^{-1}$)	Neutral protease activity (μmol glycine equiv. $g^{-1} \cdot h^{-1}$)
Control (day 0)	4.96	4.43
4 days on water	5.43	3.56
4 days on 20 mM sucrose	4.92	3.10

Table 3: Relative values of parameters in leaf segments upon incubation for 4 days in darkness when held vertically in plastic holders with their bases in water or kept horizontally in Petri dishes on wet filter paper.

Parameter	Vertically	Horizontally
Acidic protease activity	87± 7%	138± 24%
Neutral protease activity	68± 7%	89± 26%
Chlorophyll content	11± 6%	15± 6%
Total-protein content	45±11%	47± 9%
Soluble-protein content	26± 7%	36± 4%
Free-amino acid content	602±27%	814±129%
Free amino acids liberated into solution	98±31%	41± 13%

Values are means ± standard deviation; values in fresh leaf segments (day 0) were taken as 100%. Protease activities were assayed in the presence of haemoglobin.

leaves were placed horizontally or vertically, indicating that ageing progressed similarly under both conditions. Free amino acids accumulated to a greater extent in horizontally than in vertically kept leaves, associated with a smaller loss into solution. It thus appears that the increases reported by Martin and Thimann (1972) were caused by the leaf segments being placed in a horizontal rather than a vertical position. It is unlikely that these increases are due to a higher content of free amino acids in the leaf segments, as leaves held vertically in the light contained less free amino acids than similar leaves kept in darkness, yet showed increases rather than decreases in the protease activities (cf. Fig. 5). Also, transferring the leaves daily to fresh solutions in order to avoid uptake of amino acids liberated into the water, had no effect on the increase in protease activity in the light.

Effects of cytokinins on the changes in protease activity

Leaf segments were incubated on a concentration range of kinetin or zeatin and protease activities, protein and free amino acid contents determined after 4 days in darkness or 11 days in the light (Fig. 6). Kinetin was at least 10 times more active than zeatin in reducing the loss of protein and accumulation of free amino acids in the dark, and at least 100 times more effective in the light (Fig. 6 A, C), confirming previous results (Varga and Bruinsma, 1973; Biddington and Thomas, 1978) that the

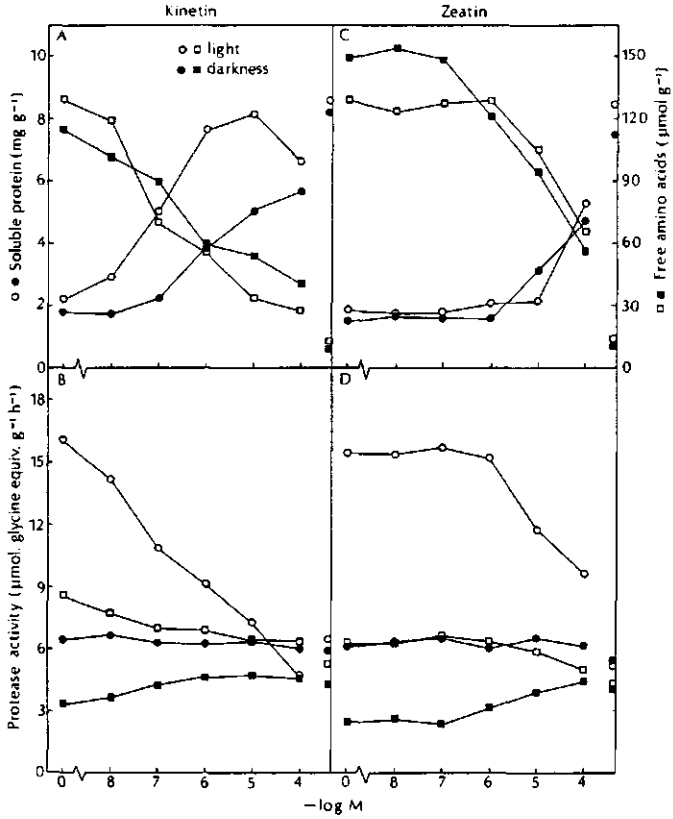

Fig. 6: Effects of a concentration range of (A, B) kinetin and (C, D) zeatin on (A, C) content of (○, ●) soluble protein and (□, ■) free amino acids, and (B, D) (○, ●) acidic and (□, ■) neutral protease activity using haemoglobin as a substrate in detached oat leaves (closed symbols) 4 days after incubation in darkness or (open symbols) 11 days after incubation in light. Leaf segments were incubated vertically in plastic holders with their bases in water or cytokinin solution. Symbols on the right vertical axes refer to values at the time of leaf detachment (day 0 control).

natural cytokinins are far less active in retarding senescence in detached oat leaves than artificial ones such as kinetin.

In darkness, neither kinetin nor zeatin had any significant effect on acidic protease activity. However, both counteracted the decrease in neutral protease activity, 10^{-7} M kinetin or 10^{-5} M zeatin maintaining protease activity at the day 0 level (Fig. 6B, D). In contrast, in the light, the cytokinins effectively reduced the increases in both acidic and neutral protease activity at concentrations of 10^{-8} M and higher for kinetin and exceeding 10^{-6} M for zeatin.

Discussion

Oat leaves contain two major proteases, an «acidic» and a «neutral» one, which degrade haemoglobin optimally at pH 4.5 and 7.5, respectively, and further differ in various properties. Both proteases may actually constitute groups of proteolytic enzymes with similar pH optima since they differed in some of their reactions to substrates and inhibitors from the purified proteases characterized by Drivdahl and Thimann (1977).

Using the soluble proteins extracted together with the proteases as substrates, protein degradation was less clearly pH-dependent, possibly due to the low solubility of the leaf proteins below pH 5.5. Ribulosebisphosphate carboxylase subunits were degraded below pH 6, indicating the endoproteinase nature of the enzyme(s). All proteins appeared to be degraded under these conditions, in contrast to extracts incubated at neutral pH where some protein bands were selectively lost. Addition of haemoglobin increased the rate of appearance of ninhydrin-positive material between 1 and 2 times at pH 7.5 and between 2 and 3 times at pH 4.5. These ratios did not change appreciably during the course of senescence in either attached or detached leaves, except at the last stage when most of the endogenous protein had already been degraded and thus, substrate must have become limiting. Although casein was a good substrate for both proteases, its solubility does not extend to the pH optimum of the acidic protease. As haemoglobin is soluble at pH 4.5, addition of excess haemoglobin was found to ensure reliable determination of enzyme activities. Proteolytic enzymes present in wheat or soybean leaves were likewise found to degrade haemoglobin well (Wittenbach, 1978; Peoples et al., 1979; Ragster and Chrispeels, 1981).

Both acidic and neutral protease activities were already high in emerging leaves. Acidic protease activity increased about 25 % during leaf expansion, when protein content was already declining, but, like neutral protease activity, decreased progressively during the period in which further loss of protein and chlorophyll marked the onset of senescence. The same pattern was evident in consecutive leaves, older leaves possessing lower protease activities than younger ones. Since tobacco plants showed the same course, a decrease in protease activities with leaf age appears to be a more general phenomenon. A similar trend has been reported in rice leaves senescing on the plant (Cheng and Kao, 1984). Also, darkening of leaves of *N. rustica* senescing on the plant accelerated protein loss without an increase in endopeptidase activity (Weckenmann and Martin, 1984). Hence, senescence of leaves does not seem to require additional proteolytic activity. Only in the last stage of senescence of oat leaves, when most of the protein had already been broken down, an increase in both protease activities was evident, but clearly these increases occur too late to influence the course of senescence.

That synthesis of additional protease is apparently not required for the onset of protein breakdown can be concluded from the results obtained during the accelerated ageing of detached leaves in darkness. Protein breakdown began about one day after detachment when protease activities were declining. No major increases in protease activity occurred during the subsequent days when most of the protein was degraded.

This effect was obtained when leaf segments were incubated vertically with their bases in water rather than horizontally on wet filter paper. Protease activities did increase under the latter condition, as was also the case in leaf segments incubated vertically in the light. However, in spite of the higher protease activities attained, protein degradation was not accelerated. The large increases in protease activity in the light could not be mimicked by adding sucrose in darkness, nor be ascribed to a greater accumulation of free amino acids. Apparently, the increases in detached leaves incubated either horizontally in darkness or vertically in light, are caused by some kind of stress, which can be alleviated by cytokinins (cf. Martin and Thimann, 1972). In non-stressed leaves, cytokinins tended to increase rather than decrease protease activities, in line with the higher protease activities found in physiologically younger leaves.

In detached leaves the natural cytokinin, zeatin, was less effective than the artificial cytokinin, kinetin, in preventing protein loss. This differential effect may be due to differences in the rate of metabolism, which may also play a role during leaf senescence in whole plants. Nevertheless, it is clear that the cytokinins do not act by suppressing the formation of protease. Young leaf cells must already possess the full capacity to degrade their proteins, in spite of numerous reports linking the onset of senescence with an increase in protease activity (Frith and Dalling, 1980). Rather, compartmentation of protease, as initially suggested by Balz (1966) may be gradually lost during senescence. There is ample evidence that at least the acidic protease may be compartmentalized in the vacuole (Boller and Kende, 1979; Heck et al., 1981; Waters et al., 1982). The action of cytokinins might then be sought in the stabilization of the protease-containing cell compartment, thereby protecting the proteins from degradation.

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Erratum: The values for the protease activity in Fig. 1, Table II, and Fig. 2 of Chapter 3 have to be multiplied by 75.

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DETERMINATION OF PROTEASES IN ISOLATED WASHED PROTOPLASTS: INACTIVATION OF PROTEASES IN CELL WALL-DEGRADING ENZYME MIXTURES USED IN PROTOPLAST ISOLATION

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Protease activity was determined in oat leaf protoplasts after isolation and washing. Protoplasts were isolated with a cellulase R-10, macerozyme R-10 mixture, which contains high proteolytic activity. Proteases in this cell wall-degrading enzyme mixture were inactivated by heating the enzyme preparation at 50°C for 10 min at pH 6.5. This treatment did not impair the cell wall-degrading activity. Protease activity in protoplasts isolated with heated enzyme was similar after washing to that in protoplasts isolated with untreated enzymes. This provided proof that contaminating proteases were effectively removed during protoplast washing and that the protease activity measured in isolated protoplasts was derived from the protoplasts themselves.

Key words: Avena sativa; protoplasts; protease; heat inactivation; cellulases; pectinases

Introduction

Isolated protoplasts are important tools to gain insight into the subcellular localization of specific enzymes. Reliable determinations of protease activities are difficult to achieve, however, because during protoplast isolation cell wall-degrading enzymes must be used which may be heavily contaminated with proteolytic enzymes. These contaminating proteases may adsorb to the protoplasts, making it difficult to remove them even by repeated washing. The proof of the effectiveness of the washing procedure can only be given by eliminating the protease activity in the cell wall-degrading enzymes and by comparing the protease activity in protoplasts isolated with treated and untreated cell wall-degrading enzymes.

The proteolytic activity in Cellulysin™ was substantially reduced with phenylmethylsulfonylfluoride (PMSF) [1]. However, the effectiveness of PMSF largely depends on the sensitivity of the proteolytic enzymes to this serine-protease inhibitor. Moreover, the PMSF must be quantitatively removed to avoid its contact with the proteases to be measured in the isolated protoplasts. In all other reports to date, the agents used to inhibit protease activity in cell wall-degrading enzyme mixtures proved only partially effective. Lin and Wittenbach [2] took the essentially similar specific activity of proteases in protoplasts and leaves of wheat and corn as an indication that there was no contamination with proteases from the cell wall-degrading enzymes. As part of the protease activity is present extracellular [3], the specific activity of protease in protoplasts is expected to be lower than in intact leaves.

The isolation enzymes are usually removed from the protoplast preparation by repeated washing or by density centrifugation in order

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid disodium salt; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride.

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to separate the protoplasts from contaminating debris. To assess the efficiency of these procedures, isolation-enzyme mixtures labelled with ^{125}I have been used [4]. In these studies low levels of the isolation enzymes were retained in protoplast preparations. β-Glucosidase has been used as a marker for contaminating enzymes [5] because this enzyme was not detected in washed mesophyll protoplasts. However, adsorptive binding of proteases to the protoplasts could not be excluded.

In this report I present a simple and rapid method to inactivate proteolytic enzymes in commercial cellulase and pectinase preparations, which does not impair their cell wall-degrading activities.

Materials and methods

Plant material

Oat plants (*Avena sativa* L. cv. Victory) were grown in pot soil, in a growth cabinet in 70% relative humidity with a photoperiod of 18 h at 25°C and 17°C during the dark period. Eight days after sowing the distal parts of the first leaves 4.5 cm were cut and used for protoplast isolation.

Protoplast isolation

After removal of the lower epidermis, 20 leaf segments were floated in a Petri dish on 10 ml of a preplasmolyticum consisting of 0.7 M mannitol, 0.5 mM dithiothreitol (DTT) and 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/KOH (pH 5.7). The solution was replaced after 20 min by 10 ml of a cell wall-degrading enzyme mixture (0.8% cellulase Onozuka R-10, 0.4% macerozyme Onozuka R-10, Yakult, Nishinomiya, Japan) in the same medium. Incubation was continued for 3 h at 28°C with slight, continuous shaking. The protoplasts liberated were collected by suction and washed 3 times with 0.6 M mannitol, 0.5 mM DTT and 25 mM MES/KOH (pH 5.7) upon centrifugation for 10 min at 65 × *g*. The washed protoplasts were resuspended in 0.6 M sucrose, 0.5 mM DTT, and 25 mM MES/KOH (pH 5.7) and centrifuged for 15 min at 180 × *g*. The intact, floating protoplasts were collected with a Pasteur pipette. The protoplasts were counted in a haemocytometer. Recovery from leaf material was calculated on the basis of chlorophyll content, determined according to Ref. 6.

Determination of protease activity

The protoplasts were resuspended in a buffer consisting of 50 mM TRIS/HCl, 0.2% ascorbic acid, 3.5% NaCl, 2 mM ethylenediamine tetraacetic disodium salt (EDTA), and 0.5 mM DTT (pH 8.0), containing 0.1% Triton X-100 to lyse the protoplasts. The lysate was dialysed overnight against 10 mM TRIS/HCl (pH 7.0), 3.5% NaCl, and 0.5 mM DTT, and centrifuged for 30 min at 30 000 × *g*. The resulting supernatant was used for protease determination. To determine protease activity of whole leaves, leaf segments were homogenized in the former buffer. The homogenate was centrifuged for 15 min at 15 000 × *g* and the resulting supernatant for 30 min at 30 000 × *g* before dialysis as above. Protease activity was determined from the amount of trichloroacetic acid-soluble, ninhydrin-positive material [7], liberated from 4% hemoglobin, which was added as a substrate during incubation at 40°C for 2 h in phosphate—citrate buffer, at pH 4.5. Protease activity is expressed as nmol glycine equivalents liberated per h. As 1 g leaf material contains 1.67 mg chlorophyll, the amount of protoplasts containing 1.67 mg chlorophyll was taken to represent 1 g leaf material.

Heat treatment of cell wall-degrading enzymes

Solutions of 0.8% macerozyme R-10 or 1.6% cellulase R-10 in 25 mM MES/KOH, 0.7 M mannitol and 0.5 mM DTT, were heated for 10 min at temperatures ranging from 45° to 60°C and pH-values from 5.0 to 7.0, and then rapidly cooled. As a control, untreated enzyme solutions were stored at 4°C. The macerozyme and cellulase solutions were then mixed in a 1 : 1 ratio. The pH was adjusted to 5.7 with HCl or KOH and the mixtures were used for protoplast isolation. To determine

protease activity, part of the same solution was diluted with lysis buffer and dialysed overnight as described above.

Results and discussion

Both macerozyme R-10 and cellulase R-10 contained high proteolytic activity (Fig. 1). Activities were highest around pH 4, but even at pH-values above 6 measurable protease activity remained. The optimum around pH 4 closely resembles the pH optimum of the acidic protease present in oat leaves [8]. Thus, when using these cell wall-degrading enzymes to isolate protoplasts from oat leaves, it is essential to be able to discriminate between protease activity present in the protoplast and activity from protease in the cell wall-degrading enzyme mixture.

Attempts to inhibit the proteolytic enzymes in the cell wall-degrading enzyme mixtures with specific inhibitors were unsuccessful. For instance, PMSF inhibited the proteolytic activity in the cellulase R-10 macerozyme R-10 mixture by only 20% (data not shown).

Conversely a distinction might be made by specifically inhibiting the oat leaf proteases upon protoplast lysis. Kaur-Sawhney et al. [9] claimed that oat leaf proteases are inhibited in vitro by polyamines. However, the highly basic spermine was found to affect the buffering pH, bringing it to the value of 5.2, where the oat leaf proteases show only 70% of the activity present at pH 4.5. Using their procedure with Azocoll as the substrate 5 mM and 10 mM spermine inhibited the Azocollase activity by only 2% in both cases.

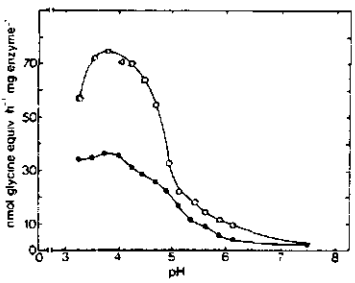

Fig. 1. The effect of pH on protease activity. 0.1% Macerozyme Onozuka R-10 (o——o) and 0.1% cellulase Onozuka R-10 (•——•) were adjusted with phosphate-citrate to below pH 7.0 or with 0.2 M TRIS/HCl to pH 7.0 and 7.5.

Heating of the cellulase R-10 and macerozyme R-10 effectively decreased proteolytic activity in the cell wall-degrading enzyme solutions (Table I). protease activity was measured at pH 4.5, but equal extents of inactivation were observed upon protease determinations of pH-values between 3.5 and 5.5. Heat inactivation was strongly pH-dependent: when heating was carried out at pH 5.0 proteolytic activity in the cellulase R-10 solution, measured subsequently at pH 4.5, decreased by 23%, at pH 6.5 by 96%; in the macerozyme R-10 solution the proteolytic activity decreased by 10% at pH 5.0 and 92% at pH 6.5 (Fig. 2).

Table I. The effect of heating cell wall-degrading enzymes at pH 6.5 on their protease activities. Protease activity in untreated cellulase R-10 28.6 nmol · h^{-1} · mg $enzyme^{-1}$, in macerozyme R-10 63.8 nmol · h^{-1} · mg $enzyme^{-1}$. Protoplast yield with untreated enzymes 7 x 10^6 per gram leaf.

Treatment	Relative protease activity (% of untreated enzyme)		Relative protoplast yield
	Cellulase R-10	Macerozyme R-10	
10 min at 45°C	10	15	100
10 min at 50°C	4	8	100
10 min at 55°C	—	5	71
10 min at 60°C	—	0	25

Table II. Effect of heating cell wall-degrading enzymes at 50°C for 10 min on protoplast yield and subsequent protease activity in isolated protoplasts (± S.D.; $n = 3$).

	Protoplasts isolated with untreated enzymes	Protoplasts isolated with heated enzymes	Leaf
Yield · g leaf^{-1} ($\times 10^{-6}$)	7.5 ± 1.2	7.1 ± 1.6	
Protease activity in washed protoplasts (nmol glycine · h^{-1} · g leaf^{-1})	54.9 ± 6.7	61.6 ± 16.1	
Protease activity in leaf (nmol glycine · h^{-1} · g leaf^{-1})			95.9 ± 22.1

Heating the cell wall-degrading enzymes for 10 min at 50°C at pH 6.5 did not influence the protoplast yield (Tables I and II). During protoplast isolation about 30—40% of the protease activity was lost, as compared to the activity present in the whole leaf extract (Table II). This was irrespective of whether the cell wall-degrading enzymes were heated or not. This difference is due to the extracellular localization of a part of the protease activity at pH 4.5 (Van der Valk and Van Loon, in preparation).

The protease activity in protoplasts isolated with heated cell wall-degrading enzymes was about the same as the activity in protoplasts isolated with untreated enzymes, indicating that the washing procedure effectively removed the proteolytic enzymes present in the cell wall-degrading enzyme mixture. These results demonstrate that the protease activity in cell wall-degrading enzymes can be distinguished from protease activity in protoplasts by inactivating the proteolytic activity of the former.

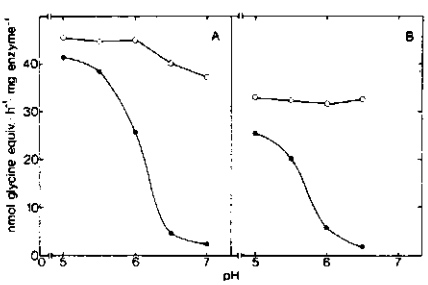

Fig. 2. The effect of pH at heating on subsequent protease activity at pH 4.5. Protease activity in 0.1% macerozyme Onozuka R-10 (A) and 0.1% cellulase Onozuka R-10 (B) was measured at pH 4.5, after heating the enzymes at 50°C for 10 min at different pH in 25 mM MES/KOH, 0.7 M mannitol, and 0.5 mM DTT. o——o, enzyme which underwent pH adjustment but no heating; •——•, 10 min at 50°C.

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CHAPTER 4

ISOLATION OF VACUOLES FROM DEVELOPING OAT LEAVES

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The text of this chapter has been accepted for publication in Plant Science.

Abstract

Procedures for the isolation of vacuoles from the first leaf of oats at different stages of development were compared as to yield, size and purity of the vacuoles isolated. Mechanical disruption or polybase-induced lysis of isolated protoplasts did not lead to clean vacuole preparations. In contrast, relatively pure vacuoles were obtained by phosphate-dependent osmotic lysis. Liberation of intact vacuoles required a minimum concentration of K_2HPO_4 in the lysis medium, lower concentrations leading to vacuole fragmentation. The maximum concentration by which complete lysis occurred decreased progressively when leaves were fully-grown and started to senesce. Only vacuoplasts (vacuoles with adhering cytoplasm) could be obtained from leaves older than 17 days. The implications for the control of senescence in oat leaves are discussed.

Key words: Avena sativa, leaf development, senescence, vacuole isolation, vacuoplasts.

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid disodium salt; MES, 2-(N-morpholino)ethanesulfonic acid; PNP, para-nitrophenol.

Introduction

Isolation of vacuoles from plant cells [1] has enabled their characterization as a lysosomal organelle [2]. Many hydrolytic enzymes are compartmentalized in the central vacuole [3]. These enzmes have been implicated in defense reactions after wounding or pathogenic attack, as well as in the control of developmental processes such as senescence. So far, it is unknown how the loss of protein during leaf senescence is controlled, and in how far vacuolar proteases participate in this process [4-7]. To be able to address this question, it was attempted to isolate vacuoles from leaves at different stages of development.

It has been reported that the tonoplast is a physically stable membrane until late in senescence [4]. From detached wheat leaves senescing in the dark, vacuoles were isolated when 80% of the protein had been lost [8]. However, reports of vacuole isolation procedures resulting in clean vacuole preparations, representative of leaves at successive stages of development, are lacking. Vacuoles are commonly isolated from protoplasts by either osmotic shock, polybase-induced lysis, or mechanical disruption of the plasma membrane. The method of choice differs for different plant species and organs, and numerous adaptations required under different conditions have been described [9]. However, yields are usually low and the purity of such vacuolar preparations can often be questioned. Cytoplasmic contaminants tend to adhere to the tonoplast upon protoplast rupturing. Furthermore, vacuole preparations often are seriously contaminated with unlysed protoplasts, that are insufficiently separated from the liberated vacuoles during further purification. Also "vacuoplasts", spheres consisting of vacuoles with adhering cytoplasm within a resealed plasma membrane, contribute to cytoplasmic contamination [10]. Therefore, conditions for lysis should be optimized to obtain vacuoles without vacuoplasts and unlysed protoplasts. In this report we describe conditions required to isolate pure, representative vacuoles from the first leaf of oats at different stages of its development.

Material and methods

Plant material and protoplast preparation

Oat plants (Avena sativa L. cv. Victory) were grown in pot soil. Plants grown in a glasshouse under conditions as described in [7] were not suitable for protoplast preparation and subsequent vacuole isolation throughout the year. To guarantee a reproducible course of leaf development and representative yields of protoplasts and vacuoles in various experiments throughout the year, plants were grown in a growth cabinet. Temperature was maintained at 25°C during a photoperiod of 18 h, and 17°C during the dark period. Light intensity was 12000 Lux from fluorescent tubes (Osram 20R/Cool White and Philips 33RS) supplemented with weak incandescent light (Philips Superlux). Relative humidity was 70%. The distal 4.5 cm of the first leaves was harvested at regular intervals for protoplast preparation. Protoplasts were isolated from stripped leaf segments by treatment with 0.8% cellulase and 0.4% macerozyme Onozuka R-10 (Kinki Yakult, Nishinimiya, Japan), as described earlier [11]. After purification, protoplasts were resuspended in isolation medium containing 600 mM mannitol, 25 mM MES/KOH and 1 mM DTT at pH 5.8.

Isolation of vacuoles

Three methods of vacuole isolation were compared. Firstly, vacuoles were liberated from protoplasts by mechanical disruption of the plasma membrane by means of the shearing forces of ultracentrifugation as described by Guy et al. [12]. Secondly, vacuoles were obtained by polybase-induced lysis, using a modification of the method developed for isolating vacuoles from wall-less yeast cells [13]. Protoplasts were lysed by adding isolation medium containing DEAE-dextran until a concentration of 7.5 µg DEAE-dextran/10^6 protoplasts was reached. Upon gentle shaking for 2-7 min at room temperature the protoplasts lysed, and threads of broken protoplasts and clumped chloroplasts became apparent. Subsequently, dextran sulphate was added to a final concentration of 5 µg/10^6 protoplasts. The lysate was then carefully layered over a cushion of isolation medium containing 5% Ficoll in a centrifuge tube. After centrifugation for 10 min at 3,000 g,

vacuoles were collected from the interfase.

Thirdly, vacuoles were isolated by a modification of the osmotic-lysis method described in [14]. Protoplasts suspended in isolation medium were mixed with 10 vol of lysis medium, containing 80-160 mM K_2HPO_4, 1 mM DTT and 5 mM EDTA adjusted to pH 8.0 with HCl. During gentle shaking for 2-5 min at 30°C protoplasts lysed and intact vacuoles were released. After addition of 0.33 vol of a solution of 600 mM mannitol, 5 mM EDTA, 20% (w/v) Ficoll-400 and 1% (w/v) neutral red, chloroplasts and residual protoplasmic membranes (broken protoplasts) clumped together and were removed by filtration through a single layer of hydrophylic gauze. The filtrate was centrifuged for 10 min at 3,000 g. Under these conditions, vacuoles floated to the surface. The vacuoles were collected by suction, resuspended in a solution containing 150 mM mannitol, 5mM EDTA, 250 µM DTT, 5% (w/v) Ficoll and phosphate at the corresponding concentration, and centrifuged for 10 min at 3,000 g. The purified vacuoles were collected from the surface and counted in a haemocytometer.

The diameters of individual protoplasts and vacuoles were measured with a calibrated ocular micrometer.

Assays

Glucose-6-phosphate dehydrogenase, NADH cytochrome-c-reductase and α-mannosidase activities as markers for cytoplasm, endoplasmic reticulum and vacuoles, respectively, were measured using assays described in [14]. Chlorophyll content of the leaves was determined according to Bruinsma [15]. Protein concentrations were estimated by the method of Bradford [16], using bovine serum albumin as a standard.

Results and discussion

Protoplast isolation

Protoplasts were prepared from the apical parts of first leaves 8-18 days after sowing, during the period of rapid leaf expansion and subsequent incipient senescence. The lower epidermis was easily peeled from leaf apices until 15 days old, and the cell wall-degrading enzymes penetrated the entire leaf segment. In contrast, the epidermis of older leaves proved difficult to be removed from the distal 1 cm of the leaf segment, and the cell wall-degrading enzymes had no entry to this part of the leaf. Consequently, protoplasts from 15- to 18-days-old leaves represented cells from the proximal 3.5 cm of the 4.5 cm leaf segments. The average yield of intact protoplasts from 8-days-old leaves was 5.10^6 per g leaf material. Yields passed through a maximum of 8.10^6 per g when leaves were 10 days old and decreased after leaves were fully-grown and started to senesce (Table I).

Table I. Effect of leaf age on the yield of protoplasts per g of 4.5 cm leaf apices and on the yield of isolated vacuoles as a percentage of the protoplasts isolated, using optimal concentrations of phosphate. Data are means ± SD for n independent experiments.

Leaf age (days)	n	Protoplast yield$\times 10^{-6}.g^{-1}$ fresh weight of leaf	n	Vacuole yield (% of protoplasts)	K_2HPO_4 (mM)
8	11	4.9±1.1	17	20.4±7.2	130
10	6	7.9±1.7	9	10.7±7.9	120
14	4	6.3±0.8	8	8.7±5.1	110
17	3	2.1±0.1	3	4.2±2.2	90

Table II. Effect of leaf age on chlorophyll and protein content in leaves and in protoplasts prepared from 4.5 cm leaf apices. Data are means ±SD for n independent experiments.

Leaf age (d)	Chlorophyll content				Protein content			
	n	leaf ($mg.g^{-1}$ fresh weight)	n	protoplasts ($mg.10^{-7}$)	n	leaf ($mg.g^{-1}$ fresh weight)	n	protoplast ($mg.10^{-7}$)
8	5	1.64±0.07	5	1.15±0.06	3	13.5±0.8	1	13.0
10	3	1.85±0.08	1	1.04	3	10.3±1.3	1	10.4
17	4	1.35±0.12	1	0.55	3	6.3±2.3	1	7.1

Protein contents in protoplasts isolated from 8, 10, and 17-days-old leaves were fully representative of the protein contents of the leaves, as depicted in Table II. However, protoplasts contained only 70-40% of the amount of chlorophyll present in the leaves from wich they were isolated. The same phenomenon has been observed in protoplasts from young leaves of Avena ativa L. cv. Gelbhafen-Flämingskrone [17]. There are no reports indicating that substantial amounts of proteins adhere to, or are taken up by protoplasts during the isolation and purification procedures. In wheat [18] and barley [19] leaf protoplasts the number of chloroplasts has been found to be constant up to a late stage of leaf senescence, making it highly unlikely that cells with a small number of chloroplasts were selected for. Thus, the reason for the reproducibly observed loss of chlorophyll in the present study is not clear.

Senescing leaves of 17 days old contained about 30% less chlorophyll than the fully-grown leaves of 10 days old (Table II). Protoplasts from 17-days-old leaves showed a similar additional loss of chlorophyll when compared to protoplasts from 10- and 8-days-old leaves. Thus, the protoplasts from 17-days-old leaves seem fairly representative of the leaves from which they were isolated. There are no indications that protoplasts with a relatively high content of chlorophyll and protein were selected during the protoplast isolation procedure.

Vacuole isolation

1. Mechanical disruption

Mechanical disruption of the protoplasts by the shearing forces of ultracentrifugation through step-density Ficoll-gradients did not result in pure vacuole preparations, an observation also reported by Haas *et al.* [17]. The yield was low and contamination with unlysed protoplasts and vacuoplasts was high.

2. Polybase-induced lysis

Intact vacuoles were released from protoplasts prepared from leaves at different developmental stages by disrupting the plasma membrane with DEAE-dextran. The amount of DEAE-dextran proved critical, however. Amounts too low or too high resulted in serious contamination with unlysed protoplasts and fragmented vacuoles, respectively. It was possible to titrate the protoplast suspension with the DEAE-dextran until threads of broken protoplasts and clumped chloroplasts became visible. Adding dextran-sulphate at this stage stabilized the vacuoles and prevented sticking of the chloroplasts to the vacuoles. However, the yield of uncontaminated vacuoles was highly variable and usually below 1%. Boudet *et al.* [20] improved the method by adding the DEAE-dextran and dextran-sulphate to gradients with increasing concentrations of Ficoll. Under our conditions, centrifugation of oat-leaf protoplasts through polybase-containing gradients with increasing concentrations of Ficoll did not result in 100% lysis. Neither were pure vacuole preparations obtained when protoplasts were suspended in 15% Ficoll and vacuoles were released by floatation through step gradients of layers of 10 and 5% Ficoll containing DEAE-dextran and dextran-sulphate, respectively.

Thus, isolation of satisfactorily pure vacuoles from oat protoplasts was not possible by using the methods of mechanical disruption or polybase-induced lysis.

3. Phosphate-dependent osmotic lysis

Vacuoles were liberated from protoplasts by osmotic lysis in a solution containing K_2HPO_4 and EDTA at pH 8.0. Under these conditions lysis of the

protoplasts proved critically dependent on the phosphate concentration. As shown in Fig. 1, clean vacuole preparations from 14-days-old leaves were obtained only if the medium contained less than 130 mM K_2HPO_4. At higher phosphate concentrations vacuole preparations remained contaminated with unlysed protoplasts that could not be removed during further purification. Vacuole preparations with less then 5% unlysed protoplasts were obtained at any concentration between 80 and 120 mM K_2HPO_4.

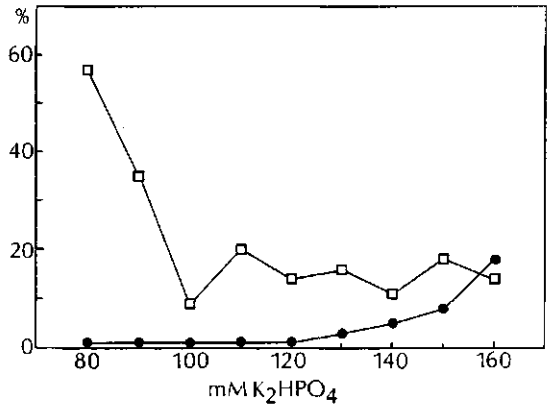

Fig. 1. Effect of the concentration of K_2HPO_4 in the lysis medium on the yield of vacuoles (▫ - ▫) and unlysed protoplasts (● - ●) expressed as percentage of the protoplasts, isolated from the 4.5 cm apices of 14-days-old leaves.

At concentrations of K_2HPO_4 above 100 mM the yield of vacuoles remained constant, but the percentage of unlysed protoplasts increased progressively. It is conceivable that a population of stable vacuoles was selected at these different K_2HPO_4 concentrations and that those protoplasts that did not lyse at the higher K_2HPO_4 concentrations would never have contributed to the vacuole population. This would mean that stable vacuoles were obtained only from easily-lysing protoplasts.

Protoplasts isolated from 9-days-old-leaves had diameters ranging from 23 to 58 µm (Fig. 2A). The average size of the vacuoles was smaller than the size of the protoplasts (Fig. 2C, 3C). Vacuoles showed a less uniform

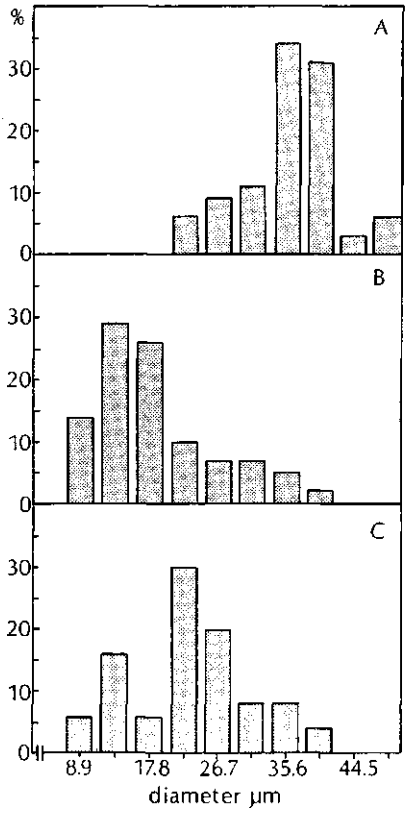

Fig. 2. Effect of the concentration of K_2HPO_4 in the lysis medium on the diameter of released vacuoles, isolated from 9-days-old leaves; A: isolated protoplasts, B: vacuoles released in 90 mM K_2HPO_4, C: vacuoles released in 120 mM K_2HPO_4.

distribution, but concentrations of K_2HPO_4 below 100 mM in the lysis medium resulted in the dominance of many small vacuoles (fig. 2B, 3B), the more so as the concentration was lowered below 80 mM. The small vacuoles contained neutral red and apparently resulted from fragmentation of the large central vacuole, giving rise to many vesicles as suggested by [21]. Within yeast cells large vacuoles easily split into small vesicles, which can fuse again during the cell cycle [22]. In cultured plant cells also fragmentation and fusion of vacuoles during the growth cycle has been observed [23, 24]. It is also possible that cells contain not only one large vacuole but also small vacuolar vesicles that pinch off from the central compartment through

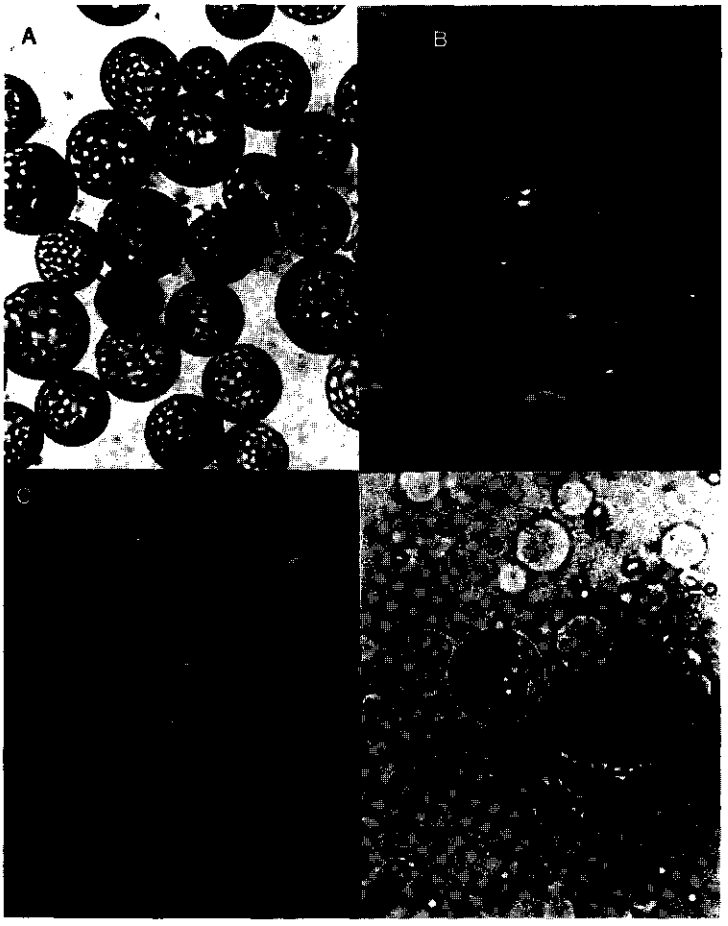

Fig. 3. Photomicrograph of isolated protoplasts (A) vacuoplasts (D) and vacuoles from 9 days-old leaves, isolated with 90 mM (B) and 120 mM K_2HPO_4 (C) in the lysis medium.

the hypotonic conditions or mechanical forces during the isolation procedures. Populations of relatively small vacuoles have already been observed when protoplasts were osmotically ruptured [25, 26].

Cytoplasmic contamination of purified vacuole preparations, as measured by glucose-6-phosphate dehydrogenase and NADH cytochrome-c-reductase

activities, usually varied between 1 and 3% and never exceeded 10% of the activities measured in protoplast preparations. The protein content of the isolated vacuoles was between 5 and 10%. Isolated protoplasts and vacuoles showed α-mannosidase activities of 109±25 (n=5) and 97±38 (n=7) nmol PNP/10^6/hour, respectively. As the α-mannosidase turns out to be associated with the vacuoles, this enzyme is a good marker enzyme for vacuoles isolated from oat leaves, similar to what has been shown for other plant species [14].

Influence of leaf age

During the course of leaf development the highest yields of isolated vacuoles were reproducibly obtained from protoplasts prepared from 8-days-old leaves: 20.4±7.2%, with 3±1% unlysed protoplasts (n=12). Yields of vacuoles from older leaves were lower and more variable, but commonly in the order of 10% (Table I). In particular the K_2HPO_4 concentration in the lysis medium at which more than 5% of the protoplasts remained unlysed, was correlated with leaf age. As shown in Fig. 4, a concentration of K_2HPO_4 below 90 mM always gave rise to preparations with many small vacuoles (region I). In contrast, high concentrations of K_2HPO_4 in the lysis medium resulted in vacuole preparations with more than 5% unlysed protoplasts (region III). Whereas 150 mM K_2HPO_4 was still suitable to obtain clean vacuole preparations from 8- to 9-days-old leaves, protoplasts prepared from older leaves required lower K_2HPO_4 concentrations in the lysis medium in order to obtain uncontaminated vacuole preparations.

Region II in Fig. 4 depicts the conditions in which clean vacuoles of relatively uniform size (Fig. 3C) were obtained from developing leaves. This region is bounded by a lower phophate concentration below small vacuoles dominated the vacuole preparation (region I), and a higher concentration above which more than 5% of the protoplasts remained unlysed (region III). These limits approached each other with increasing leaf age. Thus, it proved no longer possible to isolate pure vacuoles from leaves more than 17 days old by this method. By that time 53% of the soluble protein in the leaves had already been degraded (Table II). Obviously, the tonoplast remained physically intact up to this stage of senescence.

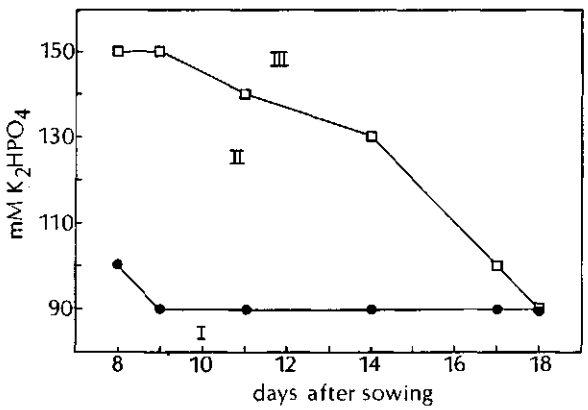

Fig. 4. Dependence of the isolation of vacuoles from leaves of different ages on phosphate concentration. Region I: preparations with small, apparently fragmented vacuoles, region II: clean, large vacuoles, and region III: vacuoles contaminated by more than 5% unlysed protoplasts.

As most of the protoplasts from 17-days-old leaves started to lyse already during their preparation, the tonoplast turned out to be more stable than the plasma membrane. Under these conditions the protoplasts took the shape of vacuoplasts: vacuoles surrounded by plasma membrane and cytoplasmic particles located as a cap on one side of the vacuole (Fig.3D).

The use of 90 mM K_2HPO_4 in the lysis medium for protoplasts from 18-days-old leaves resulted in small vacuoles only. At higher concentrations, mainly vacuoplasts were obtained. At this stage of development, neither the tonoplast, nor the plasma membrane could stand the manipulations necessary for the isolation of clean protoplasts and vacuoles. These observations are suggestive of alterations in membrane properties, which may be related to senescence. Particularly, more easy disruption of the tonoplast might be linked to facilitated leakage of vacuolar contents into the cytoplasm, before, at a very late stage of senescence, compartmentation within the tissue is fully lost.

Using the phophate-dependent osmotic lysis method it was possible to liberate uncontaminated vacuoles from mesophyll protoplasts. The conditions became more stringent when leaves were fully-grown and started to senesce.

Liberation of intact vacuoles required a minimum concentration of K_2HPO_4 of 90 mM in the lysis medium, lower concentrations leading to vacuole fragmentation. As the origin of such small vacuoles is unknown, the use of such vacuole preparations for compartmentation studies appears questionable. The upper limit decreased from 150 mM to 100 mM from day 8 to day 17 of leaf development. At higher concentrations, vacuole preparations were contaminated with more than 5% unlysed protoplasts. Only vacuoplasts could be obtained from leaves older than 17 days. By choosing the phosphate concentrations as depicted in region II (Fig. 4) one can reproducibly obtain representative, uncontaminated vacuoles from oat leaves at different stages of development.

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CHAPTER 5

SUBCELLULAR LOCALIZATION OF PROTEASES IN DEVELOPING LEAVES OF OATS (*AVENA SATIVA* L.)

H.C.P.M. VAN DER VALK and L.C. VAN LOON

Abstract

The distribution and subcellular localization of the two major proteases present in oat leaves was investigated. Both the acidic protease, active at pH 4.5, and the neutral protease, active at pH 7.5, are soluble enzymes, a few percent of which are ionically bound or loosely associated with organellar structures sedimenting at 1,000 *g*. On an average 16% of the acidic protease could be washed out of the intercellular space of the leaf. Since isolated protoplasts contained correspondingly lower activities as compared to crude leaf extracts, part of the acidic activity is associated with cell walls. No neutral protease activity was recovered in intercellular washing fluid. Of the activities present in protoplasts, the acidic protease was localized in the vacuole, whereas the neutral protease was not. The localization of the acidic protease in vacuoles did not change during leaf development up to an advanced stage of senescence, when more than 50% of the leaf protein had been degraded. These observations indicate that protein degradation during leaf senescence is not due to a redistribution of acidic protease activity from the vacuole to the cytoplasm.

Key words: *Avena sativa* L.; cell wall; intercellular fluid; protoplasts; leaf senescence; vacuoles.

Abbreviations: DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid (disodium salt); IWF, intercellular washing fluid; MES, 2-(N-morpholino)ethane sulfonic acid; PNP, p-nitrophenol; Tris, Tris (hydroxymethyl) amino methane.

Introduction

The loss of chlorophyll during the senescence of cereal leaves is preceded by a gradual decline in protein content. It has been suggested that proteases responsible for the degradation of the proteins are instrumental in regulating senescence progress [1,2]. Thus, in oats Martin and Thimann [3] observed increases in the activity of the two major proteases, with pH optima at 4.5 ("acidic" protease) and 7.5 ("neutral" protease), in detached leaves senescing in the dark. However, depending on the conditions of incubation of the leaves, protein breakdown can occur at a similar rate without increases in the protease activities and although protease activity may increase during leaf senescence, young leaf cells appear to already possess sufficient protease activity to degrade all proteins present [4]. Thus, it has been proposed that in those cells the proteases are spatially separated from their protein substrates. During senescence, compartmentalization might be lost, leading to the accelerated loss of protein. In this hypothesis, spatial contact between substrates and proteases rather than protease amount or activity would be the mechanism regulating the rate of protein degredation and, hence, senescence.

Protease activities have been detected in isolated vacuoles of many plant species [5], including wheat [6-8] and barley [9,10]. The presence of endopeptidases and carboxypeptidases inside the vacuole suggests that the vacuolar sap is involved in the digestion of cytoplasmic proteins [11,12]. The proteins to be degraded are located in the cytoplasm and in organelles such as chloroplasts and mitochondria, and are thus spatially separated from the proteolytic enzymes. So far, it is unknown how protein degradation during leaf senescence is controlled, and as to how far vacuolar proteases participate in this process. In principle, cytoplasmic constituents might be engulfed by the vacuole in a process resembling endocytosis, to be degraded by the enzymes present [11]. Degradation of endogenous proteins inside isolated vacuoles of Acer pseudoplatanus has been reported by Canut et al. [13]. These authors suggested that the specificity of the degradation is due to a selective transfer of proteins from the cytoplasm into the vacuole rather than selective proteolysis inside the vacuole [14]. However, the mechanism of transport of proteins into the vacuole is not understood.

Alternatively, proteases synthesized and compartmentalized during early

leaf development might be gradually liberated into the cytoplasm, as initially suggested by Balz [15]. Thus, senescence might be controlled by passage of proteases out of the vacuole. Under conditions where protease activity does not increase during senescence, the amount of protease present in vacuoles is then expected to decrease during leaf development. Up to now there is no evidence that vacuolar proteases are released into the cytosol, except when in a final stage of senescence lysis of the whole vacuole occurs [16]. Recently, we obtained representative vacuoles from oat leaves at various stages of development [17], and observed that the vacuolar membrane remained physically intact up to an advanced stage of senescence, when more than 50% of the protein had already been degraded. These data offer the possibility to probe the subcellular localization of the two major proteases of oat leaves up to this stage and to address the question as to how far vacuolar proteases can be involved in cytoplasmic protein degradation.

Materials and Methods

Plant material

Oat plants (Avena sativa L. cv. Victory) were grown in pot soil, in a growth cabinet as described previously [17]. For comparison, plants grown in a greenhouse [4] were also used. To establish the subcellular distribution of the proteases throughout leaf development, first leaves were harvested at regular intervals. The distal 4.5 cm of the leaves were homogenized in a mortar with pestle in extraction medium containing 50 mM Tris, 0.2% ascorbic acid (w/v), 0.1% MCE (v/v), 2 mM EDTA and 3.5% NaCl. The pH was set at 8.0 with HCl. The tissue/buffer ratio was 4 g/15 ml (final volume).

Fractionation

Subcellular fractionation was carried out by successive centrifugations of the extract at 1,000, 15,000 and 30,000 g. Each pellet was washed once by gentle resuspension in extraction medium, after which the centrifugation step was repeated. The supernatant thus obtained was combined with the

previous one, whereas the pellet was resuspended in extraction buffer containing also 0.1% Triton X-100. The fractions were dialysed overnight against extraction buffer without ascorbic acid at pH 7.0, prior to protease determination.

Isolation of intercellular washing fluid

Intercellular washing fluid (IWF) was obtained essentially as described in [18]. Leaf apices were vacuum-infiltrated with the extraction medium containing a trace of Tween 20 (infiltration medium). The infiltration procedure was repeated up to two times, until the entire leaf segments had turned dark green. The leaf surfaces were dried by blotting with filter paper. The leaves were then placed in a tube with a perforated bottom within a centrifuge tube and centrifuged at 1,000 g for 20 min. IWF was collected at the bottom of the centrifuge tube.

Isolation of protoplasts and vacuoles

Protoplasts were isolated by incubating stripped leaf apices in a cell wall-degrading enzyme mixture consisting of 0.8% cellulase and 0.4% macerozyme Onozuka R-10 (Kinki Yakult, Nishinimiya, Japan), 0.7 M mannitol, 0.5 mM DTT and 25 mM MES/KOH pH 5.8, as described earlier [19]. Vacuoles were liberated from protoplasts by lysis in appropriate concentrations of phosphate buffer, and purified on Ficoll gradients [17]. After purification protoplasts and vacuoles were resuspended and lysed in the extraction buffer described above, supplemented with 0.1 % Triton X-100.

Enzyme, protein and chlorophyll determinations

After centrifugation and dialysis of the extracts, the protease activities were determined by measuring the increase in ninhydrin-positive material after incubation at 40°C for 2 h with hemoglobin as the substrate [4]. Protease activity was expressed either as the amount of glycine equivalents liberated.g^{-1} of leaf fresh weight or as µ mol glycine equivalents.$vol^{-1}.h^{-1}$ related to the activity of α-mannosidase expressed as mol p-nitrophenol formed.$vol^{-1}.h^{-1}$ (relative units). α-Mannosidase, glucose-6-

phosphate dehydrogenase and NADH-cytochrome-c-reductase activities (as markers for the vacuoles, cytosol and endoplasmic reticulum, respectively) were measured using standard assays [20]. Protein concentrations were estimated by the method of Bradford [21], using bovine serum albumin as a standard. Chlorophyll content of the leaf apices was determined from 1 g of leaf material homogenized in 80% (v/v) acetone, containing 1% (w/v) ascorbic acid. After centrifugation, chlorophyll was estimated in the supernatant after [22].

Table I. Protease activities at pH 4.5 and 7.5 in subcellular fractions (percent of total) and total activity in extracts (µmol glycine equiv.$g^{-1}.h^{-1}$) from 4.5 cm apices of the first leaf from 9-days-old oat plants.

Pellet obtained by centrifugation at	Extraction medium containing					
	no addition		0.5 M NaCl		0.5 M sucrose	
	pH 4.5	7.5	4.5	7.5	4.5	7.5
10 min 1,000 g	7	6	4	3	5	4
15 min 15,000 g	4	0	0	0	4	0
30 min 30,000 g	0	0	0	0	0	0
supernatant	89	94	96	97	91	96
total activity (%)	100	100	100	100	100	100
total activity (µmol glycine.$g^{-1}.h^{-1}$)	10.4	7.4	11.6	8.9	12.4	7.9

Table II. Distribution of total protease activity at pH 4.5 and 7.5 in apices, isolated protoplasts and stripped epidermis from first leaves of 8-days-old oat seedlings. Protease activities were calculated on the basis of the amount of protein recovered in each fraction (i.e. specific activities multiplied by the total amount of protein present).

	protease activity			
	pH 4.5		pH 7.5	
	µmol.$g^{-1}.h^{-1}$	%	µmol.$g^{-1}.h^{-1}$	%
leaves	4.18	100	2.88	100
lysed protoplasts	2.84	68	2.36	83
epidermis	0.40	9	0.03	1
missing		23		16

Results

Subcellular fractionation

In previous investigations, media containing 0.5 M NaCl were used to extract both the acidic and the neutral protease from oat leaves. Under these conditions, more than 95% of both protease activities were soluble, a few percent only remaining associated with the 1,000 g pellet (Table I). Omission of the salt or its replacement by sucrose in order to retain ionically-bound activity and stabilize subcellular organelles, respectively, affected the distribution of the acidic protease in that a larger percentage was retained in the 1,000 g pellet and some activity remained associated with the 15,000 g pellet. The distribution of the neutral activity was not significantly altered. In no case was any activity found in the 30,000 g pellet, nor was activity in this fraction generated upon the addition of various detergents. These observations indicate that both the acidic and the neutral proteases are soluble enzymes but suggest that particularly part of the acidic protease activity is associated with easily sedimentable structures.

Table II shows results from a representative experiment comparing protease activities in leaf apices and their protoplasts. When the amount of protease activity in isolated protoplasts was compared with the total activity present in leaves, parts of both the acidic and the neutral activity were missing. Stripped epidermis contained relatively substantial amounts of the acidic but negligible neutral protease activity. Still, 23% of the acidic and 16% of the neutral activity were not recovered indicating that part of both protease activities had been lost during protoplast preparation. Either the isolation procedure itself or an association of protease with the removed cell walls might be responsible for these losses.

To investigate whether any proteases are present extracellularly, ionically bound to cell walls, the intercellular space of the leaves was infiltrated with extraction buffer containing 0.5 M NaCl, and IWF collected by centrifugation.

Glucose-6-phosphate dehydrogenase activity in the IWF was below the level of detection and NADH-cytochrome-c-reductase activity was only 0.25% when

calculated on the basis of leaf weight. The amount of ninhydrin-positive material was negligible. Apparent protein content in IWF was 0.1 % of that present in the leaves. These data demonstrate that significant leakage of large molecules into the IWF could be excluded.

Taking the protease activities in untreated leaf apices as 100%, 16% of the acidic protease activity was recovered from the IWF whereas in the same IWF protease activity at pH 7.5 was below the limit of detection (Table III). The remaining leaf tissue retained 65% of the protease active at pH 4.5 and 60% of the protease active at pH 7.5. Also under these conditions, parts of both the acidic and the neutral protease activities appeared to be lost.

Table III. Distribution of acidic and neutral protease activities in leaves from 8-days-old plants. Data are means ±SD from n independent experiments.

	protease activity					
	pH 4.5			pH 7.5		
	n	$\mu mol.g^{-1}.h^{-1}$	%	n	$\mu mol.g^{-1}.h^{-1}$	%
leaves	8	8.3 ± 0.7	100	7	6.3 ± 0.4	100
IWF[a]	4	1.4 ± 0.2	16	3	b.l.d.[b]	0
remaining leaf tissue	5	5.4 ± 0.4	65	3	3.8 ± 0.1	60

[a] intercellular washing fluid

[b] below level of detection

When NaCl was omitted from the infiltration medium the yield of IWF was not significantly different from that containing NaCl. Under these conditions, acidic protease activity in the IWF was only 60% of that recovered in the presence of NaCl. Again, protease activity at pH 7.5 was not detectable. The amount of protease activity recovered could be increased up to the level obtained with infiltration medium containing NaCl by successive infiltrations with increasing concentrations of NaCl (data not shown).

These observations indicate that part of the acidic protease activity is ionically bound to the cell walls.

Vacuolar localization

To elucidate the subcellular localization of the soluble proteases, the relative amounts present in vacuoles were determined. To check that these enzymes retained their activities during the preparation of the vacuoles, protease activities were determined in the Ficoll solutions in which the protoplasts were lysed (solution I) and in which the vacuoles were subsequently washed (solution II), and expressed in relative units on the basis of the vacuolar marker enzyme α-mannosidase (Table IV).

In Ficoll solution I protease activities relative to α-mannosidase were similar to those in leaf homogenates, indicating that the Ficoll did not affect the protease activities or their measurement. In the washing Ficoll solution II relatively low protease activities were found. In contrast, the vacuoles floating in solution II contained high protease activity at pH 4.5, whereas protease activity at pH 7.5 was only just above the level of detection.

Based on the total α-mannosidase activity associated with the vacuoles [17] a mean of 59% of the acidic protease activity present in the protoplasts was calculated to be located in the vacuoles (Table V). When the activities were calculated on the basis of the numbers of protoplasts and vacuoles, counted with a haemocytometer, vacuoles were found to possess a mean of 82% of the acidic protease present in protoplasts. No neutral protease activity was found in purified vacuoles. Based on α-mannosidase activity, in the vacuolar preparations glucose-6-phosphate dehydrogenase and NADH-cytochrome-c-reductase usually showed activities between 1 and 5% of those measured in the leaf extracts. Thus, cytoplasmic contamination of the isolated vacuoles was low.

Table IV. α-Mannosidase and protease activities in crude leaf extract, protoplasts lysed in *Ficoll solution I*, *Ficoll solution II after washing of liberated vacuoles*, and purified vacuoles from leaves of 8-days-old plants (representative experiment).[a]

	α-mannosidase $\mu mol.h^{-1}.vol^{-1}$	protease activity			
		pH 4.5		pH 7.5	
		$\mu mol.h^{-1}.vol^{-1}$	rel. units[c]	$\mu mol.h^{-1}.vol^{-1}$	rel. units
crude leaf extract	1.79	4.20	2.30	2.60	1.45
Ficoll solution I[b] with lysed protoplasts	0.72	1.26	1.70	0.93	1.29
Ficoll solution II[b] (washing solution)	0.14	0.18	1.20	0.28	2.00
vacuoles in Ficoll solution II	1.06	2.05	1.93	0.04	0.04

[a] Activities are presented as measured in the respective solutions without corrections for differences in volume or yield.

[b] Composition of the Ficol solutions: I: 150 mM mannitol, 5mM EDTA, 250 μM DTT, 5% (w/v) Ficoll and 0.12 M K_2HPO_4; II: identical to I, except that the phosphate was omitted.

[c] μmol glycine equiv. liberated.mol^{-1} PNP formed.

Table V. Protease activities in isolated protoplasts and vacuoles from leaves of 8-days-old plants. Data are means of 5 independent experiments with SD.

	protease activity			
	pH 4.5		pH7.5	
	rel. units	%	rel.units	%
protoplasts	2.45 ± 0.43	100	1.71 ± 0.40	100
vacuoles	1.44 ± 0.61	59	b.l.d.	0

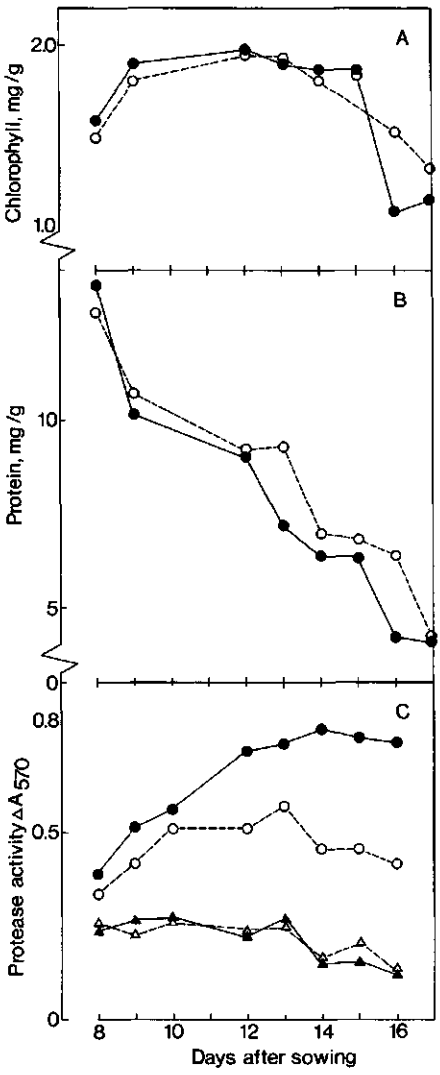

Fig. 1 Changes in the contents of (A) chlorophyll and (B) soluble protein, and (C) in the activities of the acidic protease (o) and neutral protease (▵) during natural development of the primary leaf of intact oat plants grown either in a greenhouse (open symbols), or in a growth cabinet (closed symbols).

During leaf development, chlorophyll and protein content decreased similarly, whether plants were grown in a growth cabinet or in a greenhouse (Fig. 1A, B). However, whereas protease activities tended to decrease under the latter conditions [4], in the growth cabinet the acidic protease activity increased substantially up to the stage when chlorophyll content started to decline (Fig. 1C). Since the isolation of clean vacuoles requires plants grown under controlled conditions [17], the localization of the proteases during leaf development was investigated when, for the most part, net protease activities were still increasing.

Protoplasts and vacuoles were prepared at 8, 10, 14 and 17 days of development. This last stage was the latest one at which representative vacuoles could be prepared, and marked an advanced stage of senescence when more than 50% of the protein had been lost. Yet, at all four stages essentially all of the acidic protease activity present in protoplasts was found in the vacuoles (Table VI). Activities were based on the -mannosidase activity present in each fraction. The later activity was highly constant throughout leaf development, corroborating its usefulness as a marker. These results thus establish that even when gradual protein breakdown proceeds, all of the acidic protease activity present in protoplasts is retained within the vacuoles. In contrast, at no stage was any neutral activity present in this compartment (data not shown).

Table VI. Protease activity at pH 4.5 in relative units in leaves at different stages of development and in protoplasts and vacuoles isolated from these leaves. α-Mannosidase activity in $\mu mol.g^{-1}.h^{-1}$ in leaves at the same stages of leaf development.

leaf age (days)	α-mannosidase in leaf $\mu mol.g^{-1}.h^{-1}$	exp no.	protease activity (relative units)		
			leaf	protoplast	vacuole
8	3.86	1	1.18	1.25	1.44
		2	1.98	1.77	2.20
		3	2.28	- [a]	1.82
		4	3.12	3.25	-
		average	2.15	2.09	1.82
10	3.59	1	1.90	2.39	2.15
		2	1.86	-	2.24
		3	1.48	-	-
		4	2.72	-	2.20
		average	2.00	2.39	2.20
14	3.69	1	4.94	4.24	4.90
		2	4.14	-	3.72
		average	4.54	4.54	4.31
17	3.86	1	4.83	4.37	4.66

[a] not determined.

Discussion

Both fractionation and protoplast isolation established that about one sixth of the acidic protease activity present in oat leaves is localized extracellularly associated with the cell wall. Intercellular space has previously been shown to contain peroxidase, ribonuclease, glycosidases and phosphatases [23] with acidic pH optima. In this latter respect these enzymes resemble vacuolar enzymes . Matile [16] suggested that the cell wall is part of the lytic compartment of the plant cell. Thus, it does not seem surprising that proteases are also present extracellularly. To our knowledge only an Azocoll-digesting proteolytic enzyme present in bean leaves has so far been reported to be associated with the cell walls [24]. However, this enzyme has a pH optimum at 9.0 and was not active at pH values below 6.0. Such proteases may function in degrading enzymes secreted by micro organisms present in the intercellular space. Whether such proteases can also degrade cell wall proteins remains to be demonstrated.

The majority of the protease activity at pH 4.5 was soluble and located within the vacuole. In initial experiments designed to probe the subcellular localization of both proteases, on an average 59% of the activity present in protoplasts was recovered in the vacuoles. However, in later experiments, values up to 100% were detected. The reasons for these variations are not clear. It is noteworthy, however, that upon protoplast preparation and vacuum infiltration of the leaves losses of up to 40% of both the acidic and the neutral protease were common. Since both these procedures entailed plasmolysis of the tissue, it is possible that this condition may lead to inactivation of proteases. Loss from the tissue appears highly improbable: no leakage of macromolecules was detected upon vacuum infiltration; loss of neutral protease activity was usually greater than that of the acidic one, yet its activity in IWF was negligible.

The enzyme active at pH 7.5 was absent from isolated vacuoles. By measuring low protease activities at pH 7.5 in the washing Ficoll layer II, we proved that the enzyme could be detected, if present, under the conditions of the vacuole isolation and purification procedures. The enzyme active at pH 7.5 must then be located within the cytoplasmic compartment. The recovery of a few percent of the neutral protease in the 1,000 g pellet fraction suggests that it may be loosely associated with readily sedi-

mentable structures.

Growing plants under different conditions greatly affected the level of protease activity, but had no significant effect on the rate of protein loss. The acidic protease remained compartmentalized inside the vacuole throughout the period in which the protein content decreased by more than 50%. Thus, protein degradation during leaf development is not due to a redistribution of acidic protease activity from the vacuole to the cytoplasm. The only way in which the vacuolar proteases could have a function in the degradation of cytoplasmic protein, is when proteins to be degraded are transferred into the vacuole [13,14,25]. In that case, the regulation of senescence must depend on more or less specific transport of proteins over the tonoplast.

Alternatively, proteases outside the vacuole, i.e. the neutral protease and perhaps exopeptidases might be responsible for protein breakdown during senescence. Evidence is available that chloroplasts [8,26] and mitochondria [27] contain minor proteases that might function in the organellar protein degradation (e.g., of ribulose-1,5-bisphosphate carboxylase), but the origin and relative importances of these enzymes are largely unknown. Cytoplasmic protein degradation might be influenced by additional factors, as ATP and pyridine nucleotides [28] and fatty acids [29] have been suggested to play a role in the regulation of protein degradation.

The acidic protease activity located in the vacuole might only function in the late stages of senescence when leakage of the tonoplast occurs. However, during the life of the cell it may serve a protection function in that it ensures rapid hydrolysis of proteins upon wounding or pathogenic attack. The activity present in the intercellular space appears to be eminently suited to serve the latter functions.

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CHAPTER 6

PROTEOLYTIC ENZYMES IN DEVELOPING LEAVES OF OATS (*AVENA SATIVA* L.)
I. PARTIAL PURIFICATION AND CHARACTERIZATION OF THE MAJOR ENDOPEPTIDASES

H.C.P.M. VAN DER VALK and L.C. VAN LOON

Abstract

By using extraction at pH 4.5, followed by gel filtration and anion-exchange chromatography, the acidic and the neutral proteases present in oat leaves were purified 87 and up to 22 times, respectively. The enzymes were only marginally separated, however, indicating that they have similar molecular weights and are rather similarly charged between pH 6 and 9. Both enzymes appeared to be endopeptidases. The neutral protease was inhibited by inhibitors of metallopeptidases, whereas the acidic protease was not. However, both were inhibited to varying extents by sulphydryl- or serine-type protease inhibitors.

Inhibition of enzyme activity by polyamines could not be ascertained. Contrary to a previous report, serine did not activate the enzymes. These discrepancies may be due to anomalies encountered in the ninhydrin staining used for determination of the enzyme activity.

key words: *Avena sativa* L.; endopeptidase; leaf senescence; protease.

Abbreviations: DMFA, dimethylformamide; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid (disodium salt); IAc, iodoacetamide; MCE, 2-mercaptoethanol; NEM, N-ethylmaleimide; PCMPS, *p*-chloromercuriphenyl-sulfonate; PMA, phenylmercuricacetate; PMSF, phenylmethylsulphonylfluoride; Tris, Tris (hydroxymethyl) aminomethane.

Introduction

Oat leaves contain two major proteolytic enzyme activities with pH optima at 4.5 ("acidic protease") and 7.5 ("neutral protease"), respectively. Both proteases are endopeptidases, but they differ in substrate preference, inactivation temperature and stability in vitro [1]. Furthermore, the acidic protease is localized predominantly in the vacuole and to a smaller extent in the intercellular space associated with cell walls, whereas the neutral protease is absent from these compartments and is localized presumably in the cytoplasm, perhaps loosely associated with membranous structures [2].

Martin and Thimann [3] found increases in both acidic and neutral protease activities during dark-induced senescence of detached leaves, suggesting that these increases were instrumental in the rapid loss of protein occurring under these conditions. However, loss of protein during senescence of either detached or attached leaves can occur without concomitant increases in protease activity [1], suggesting that additional synthesis of the major proteases is not a prerequisite for protein breakdown during senescence. Possibly other, minor proteases might function in the breakdown of proteins in cytoplasm and organelles. The vacuolar, acidic protease could be involved, however, if proteins to be degraded were transported into the vacuole [2,4,5]. The cytoplasmic, neutral protease might function in protein turnover and hydrolyse proteins marked for degradation [6]. Since little is known about the regulatory mechanisms of protein degradation in senescing leaves and the proteases involved, it was desirable to further characterize the proteases in oat leaves.

Although the acidic protease shows maximal activity at pH 4.5, shoulders of activity at pH 3.5 and 5.5 suggested that the protease activity at low pH values may consist of several forms differing in pH optimum [1]. By using gel filtration and ion-exchange chromatography, Frith and Dalling were able to distinguish six hemoglobin-degrading proteolytic enzymes from wheat leaves [7]. Miller and Huffaker [8] separated two proteases with pH optima of 5.5 and 5.7 from barley leaves. From oat leaves, Drivdahl and Thimann [9,10] separated and partially purified two proteases with pH optima of 4.2 and 6.6, respectively. Particularly the acidic protease was activated in vitro by the presence of L-serine in the incubation medium

[11], and was classified as a serine peptidase [9]. The neutral protease was activated and stabilized by mercaptoethanol (MCE) and, thus, considered to be a sulphydryl peptidase. However, the yield of both enzymes was low and the pH optima did not correspond to those in crude extracts, suggesting that the purified enzymes may be minor proteases selected during the purification procedure. Therefore, it was attempted to separate and purify the different proteases that might be present in oat leaves.

Materials and methods

Plant material

Oat plants (Avena sativa L. cv. Victory) were grown in a greenhouse under natural light conditions, supplemented with illumination from high-pressure mercury-halide Philips HPI/T lamps to ensure a minimum light intensity of 18 $W.m^{-2}$ for 16 $h.day^{-1}$. Minimum temperature was 20°C during the day and 17°C at night. First leaves were harvested when the seedlings were 8 days old, as neutral protease activity is highest at this stage [1].

Preparation of extracts

Apical 4.5 cm leaf segments were homogenized in 2 vol. (v/w) of extraction buffer in a Waring blendor at 2°C. The extraction buffer consisted of 50 mM Tris, 3.5% NaCl, 0.2% (w/v) ascorbic acid, 0.1% (v/v) MCE, 2 mM EDTA, set at pH 8.0 with HCl. Alternatively, extraction was carried out at pH 4.5, using a buffer containing 100 mM phosphate/50 mM citrate buffer, 3.5% NaCl, 0.2% ascorbic acid and 0.1% (v/v) MCE. The homogenate was passed through three layers of gauze and centrifuged for 15 min at 15,000 g and 30 min at 30,000 g, successively. Solid ammonium sulphate was then added until 70% saturation was reached and the protein precipitated for a minimum of 2 h at 2°C. The mixture was centrifuged for 20 min at 20,000 g and the protein pellet was resuspended in 10-15 ml 50 mM Tris-HCl, pH 7.5, 0.1% MCE.

Gel-filtration chromatography

The protein solution was loaded onto a column (50 x 2.5 cm Ø) of Sephadex G-50 as described by [7]. Alternatively, columns of Sephadex G-100 (92 x 4.3 cm), Ultrogel AcA 44 (50 x 2.5 cm), or Sephacryl S-300 (50 x 2.5 cm) were used. Elution was performed with 50 mM Tris-HCl pH 7.5, 0.1 % MCE, at a flow rate of 0.5 $ml.min^{-1}$ for the Sephadex columns or 1-3 $ml.min^{-1}$ on the other two columns. Fractions of 2.5-5 ml were collected and tested for protease activities. Fractions containing proteolytic activity were pooled and further fractionated.

Anion-exchange chromatography

A DEAE-Trisacryl column (30 x 2.5 or 17 x 1.5 cm) was washed overnight with 50 mM Tris-HCl pH 7.5, 0.1% MCE, unless stated otherwise. Upon loading of protease-containing fractions, the column was washed with the same buffer until the eluate was free of absorbance at 280 nm and of protease activity. Subsequently, proteins were eluted with a linear gradient from 0-0.3 M NaCl in the same buffer. The column was then washed with 2 M NaCl in buffer to remove all protein, and regenerated by washing with buffer only.

Chromatofocusing

Active fractions from the ion-exchange column were dialysed overnight against 25 mM imidazol-HCl pH 7.4 or desalted over Sephadex G-25, equilibrated with the imidazol buffer. A column (23 x 0.8 cm) of Polybuffer exchanger 94 (Pharmacia) was equilibrated with 250 ml 25 mM imidazol-HCl pH 7.4. The protease-containing solution was loaded and the column eluted with 280 ml of Polybuffer 74 brought to pH 4 with HCl. Fractions of 2.5 ml were collected and tested for protease activity.

Affinity-chromatography

Active fractions from the ion-exchange column were dialysed overnight against either 100 mM Tris-HCl pH 7.5, 0.1% MCE, or 100 mM

phosphate/50 mM citrate pH 4.5, 0.1% MCE. The solutions were then loaded onto a benzamidine-Sepharose column (6,5 x 1.6 cm) equilibrated with the buffer at pH 7.5 or 4.5. The column was washed with the buffer and subsequently eluted with a 0-0.3 M NaCl gradient in the same buffer, or by changing the buffer composition from pH 4.5 to 8.0, or from 7.5 to 4.0. A hemoglobin-Sepharose column was similarly used.

Determination of proteolytic enzyme activities

Proteolytic activity was determined at pH 4.5 and 7.5 with hemoglobin as the substrate, as described previously [1]. Centrifuged extracts were dialysed against 50 mM Tris-HCl pH 7.0 buffer containing 3.5% NaCl and 0.1% (v/v) MCE. To determine the effect of enzyme inhibitors, various compounds were dissolved in the incubation buffer and mixed with the enzyme solution 30 min prior to the addition of substrate. The inhibitors NEM and o-phenanthroline were dissolved in DMFA, PMSF in DMSO or ethanol (5% final concentration in the incubation mixture).

Electrophoretic separation and activity staining of aminoacyl-2-naphthylamidases was performed as described in the accompanying paper [10].

Results

Purification

To determine the activity of the acidic and neutral proteases present in oat leaves, extracts were routinely prepared using a buffer at pH 8.0 to ensure optimal solubility of the proteins and minimize interference by phenolic compounds. However, when the pH of the extraction medium was changed to 4.5, more than 90% of the protein was not solubilized, but protease activities at pH 4.5 and 7.5 were not significantly different from those obtained at pH 8.0. Thus, extraction at pH 4.5 resulted in a purification of 14 times for both types of enzymes (Table I). Upon addition of ammonium sulphate to the extracts after centrifugation, both proteases precipitated at similar concentrations and were fully precipitated at 70% saturation. In the subsequent purification steps, the acidic protease

Table I. Purification of acidic and neutral proteases from 4.5 cm apices of first leaves of 8-days-old plants after extraction at pH 8.0 and 4.5.

	protein $mg.g^{-1}$	protease activity units[a].g^{-1} leaf fresh weight					
		pH 4.5			pH 7.5		
		units	yield (%)	purification factor	units	yield (%)	purification factor
homogenate pH 8.0	14	8.1	100	1	4.4	100	1
homogenate pH 4.5	1	8.1	100	14	4.4	100	14
gel-filtration extract pH 8.0	2.5	5.8	74	4	3.2	70	4
gel-filtration extract pH 4.5	0.5	6.3	78	22	3.4	77	22
anion-exchange chromatography extract pH 4.5	0.12 0.35	6.0	74	87	1.8	41	16

[a] One unit of protease activity is defined as liberating 1 µmol glycine equival.h^{-1}.

activity proved quite stable but the neutral protease activity was gradually lost, in spite of the presence of the stabilizing MCE. For this reason, the recovery of the neutral protease activity highly depended on the speed with which purification was carried out. Due to their relatively slow flow rate, Sephadex columns proved disadvantageous. Moreover, gel filtration on Sephadex G-50 did not result in a substantial purification of the proteases, in contrast to the observations reported by Drivdahl and Thimann [7]. With Sephacryl S-300 and Ultrogel AcA 44 relatively high flow rates were achieved. However, only on Sephacryl S-300 were the proteases eluted in one distinct peak well separated from the bulk of the protein eluted at the void volume (Fig.1). Independent of the type of gel-filtration column, the acidic and neutral protease activities always eluted in the same fractions, indicating that they must have rather similar molecular weights.

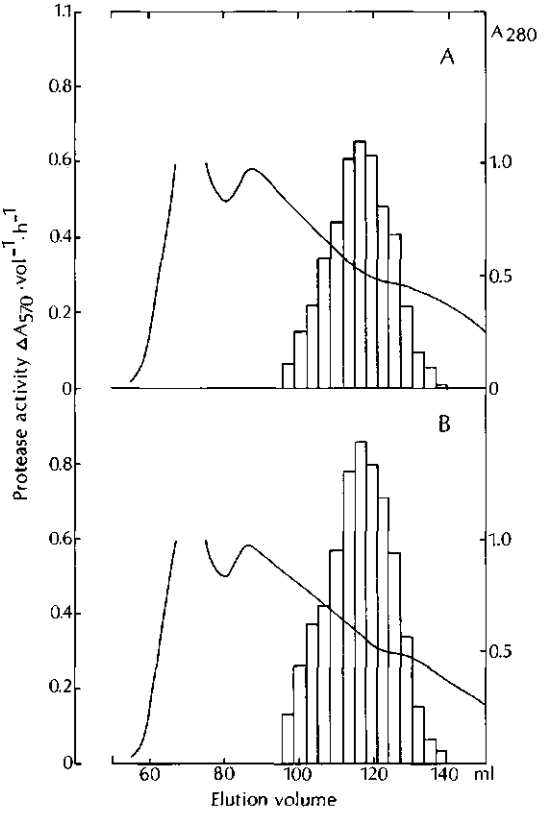

Fig. 1 Gel filtration chromatography of acidic (A) and neutral (B) proteases in an extract of 9-days-old oat leaf apices on Sephacryl S-300.

When pooled protease-containing fractions were further subjected to anion-exchange chromatography, on an average 10% of both the acidic and the neutral activity was not bound to the column. However, the amount of activity flowing through was quite variable. Furthermore, in their properties these activities resembled those of the main protease peak, which was eluted at a salt concentration around 0.15 M. If extraction had been carried out at pH 8.0, no separation of the acidic and the neutral proteases was observed. In contrast, when extraction had previously been at pH 4.5, the peak of the neutral protease activity was eluted reproducibly 2-3 fractions after the peak of the acidic activity (Fig. 2). However, further separation could not be achieved, neither by varying the pH of the loading and elution buffer between 6.0 and 9.0, nor by reducing the elution velocity, using shallower salt gradients or using longer columns. Neither protease was bound to

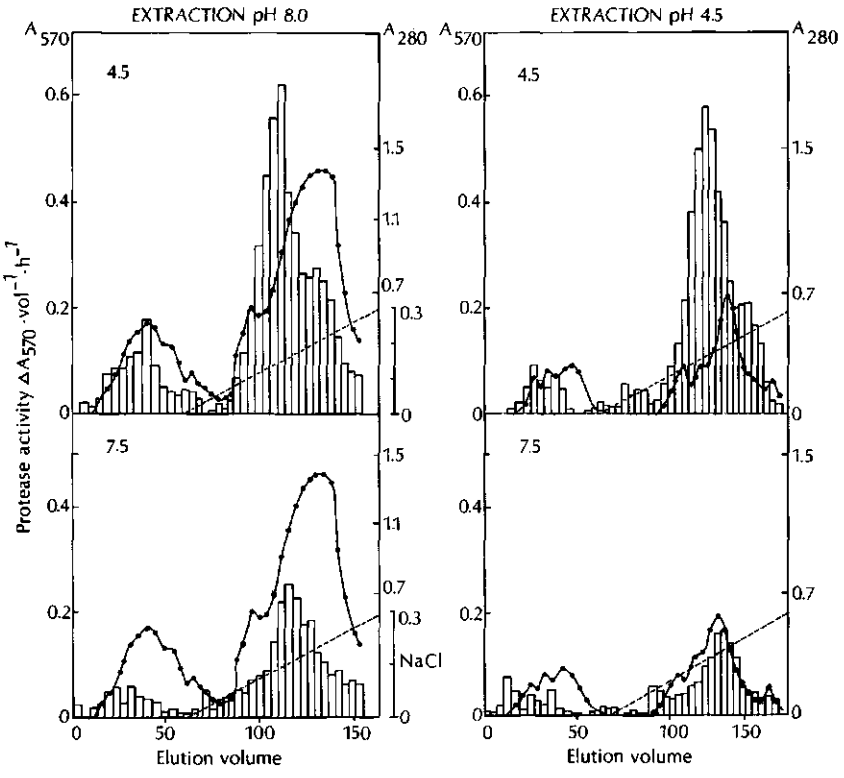

Fig. 2 Anion-exchange chromatography of proteases active at pH 4.5 and 7.5 from 9-days-old oat leaf apices after extraction at pH 8.0 and 4.5.

cation-exchangers over the range from pH 4.5 to 6.0. Thus, both proteases also appear to have rather similar charges.

Further attempts to separate the protease activities involved chromatofocusing and affinity-chromatography on benzamidine-Sepharose (specific for trypsin-like proteases) and hemoglobin-Sepharose (since hemoglobin is a good substrate for both proteases). However, no further separation or purification could be achieved. The polybuffer constituents used for generating a pH gradient in chromatofocusing interfered with the ninhydrin determination of protease activity and could not be sufficiently removed by gel filtration. Neither protease bound to the benzamidine-Sepharose column. On hemoglobin-Sepharose, part of both activities was retained. However, the binding was highly irreproducible and did not allow separation of the proteases.

Characterization

Activity staining for aminoacyl-2-naphthylamidases of proteins separated on polyacrylamide gels revealed that pH 4.5 extracts contained the methionine-specific aminopeptidase, as well as part of the benzoyl-arginine-2-naphthylamide-hydrolysing endopeptidase [10]. Both activities were bound to the DEAE-Trisacryl column, but neither was eluted exactly coinciding with the peak of protease activity. The aminopeptidase activity was present only in the last fractions of the protease peak, whereas the endopeptidase was eluted slightly earlier, starting when the protease activity peaked. These observations support our earlier conclusion [1] that the acidic and neutral proteases must be endoproteolytic enzymes. Since the aminopeptidase and the arginine-specific endopeptidase were still eluted when protease activity had returned to low values, the former enzymes do not appear to contribute significantly to the protease activity measured in these experiments.

In extracts made at pH 4.5 the metal chelators EDTA and o-phenanthroline did not affect the acidic-protease activity (Table II). In contrast, the neutral-protease activity was inhibited 25% by 5 mM EDTA and almost completely (93%) by 10 mM o-phenanthroline. Thus, the neutral protease appears to be a metallopeptidase. The acidic activity was partly inhibited by 1 mM leupeptin or 5 mM PCMPS, whereas the neutral activity was completely blocked by PCMPS and also inhibited by leupeptin. Iodoacetamide partially inhibited (31%) the neutral activity but NEM had no significant effect. From these results it seems that both types of enzymes have free sulphydryl groups participating in catalysis. Of the serine-type protease inhibitors PMSF was somewhat more inhibitory to the acidic protease than to the neutral one, whereas PMA inhibited neutral but not acidic protease activity. Solvents used for administering the inhibitors did not significantly influence the protease activities.

Table II. Effects of inhibitors and solvents on the activities of the acidic and neutral proteases in leaves of 8-days-old plants.

compound	concentration mM	residual activity (% of control)	
		pH 4.5	pH 7.5
EDTA	5	100	75
o-phenanthroline	10	100	7
leupeptin	1	48	66
PCMPS	5	51	0
IAc	10	[a]	69
NEM	1	100	93
PMSF	10	42	56
PMA	1	93	47
DMFA	5%	100	85
DMSO	5%	100	100
ethanol	5%	100	100

[a] Not determined due to interference with the ninhydrin staining.

Using ion-exchange chromatography fractions relatively enriched in acidic protease activity (ascending slope of the protease peak in Fig. 2) or neutral protease activity (descending slope) the effect of inhibitors on enzyme activity at pH 4.5 and 7.5 was also determined. With the exception of PMSF, all inhibitors tested showed similar inhibition of the protease activities in the partly enriched fractions. However, PMSF was less inhibitory, 62 and 89 percent remaining of the acidic and neutral activity, respectively.

The effect of polyamines such as spermine and spermidine, reported to inhibit oat leaf proteases [13], could not be ascertained. A decrease rather than an increase in ninhydrin-positive material was recorded during incubation (Table III), suggesting that the polyamines contributing to the ninhydrin staining were modified. Moreover, in the presence of polyamines the pH used for measuring acidic protease activity shifted to higher values, thereby reducing the enzyme activity.

Table III. The effect of spermine on the pH and the absorbances at 570 nm of ninhydrin-stained leaf extracts upon incubation at pH 4.5 and 7.5 for 2 h with hemoglobin as the substrate.

	pH during incubation	A_{570nm} t=0	A_{570nm} t=2 h	t_2-t_2
phosphate/citrate buffer pH 4.5	4.5	0.086	0.403	0.317
phosphate/citrate buffer pH 5.7	5.7	0.098	0.214	0.116
phosphate/citrate buffer pH 4.5 + 10 mM spermine	5.7	0.555	0.447	-0.108
Tris-HCl buffer pH7.5		0.143	0.282	0.139
Tris-HCl buffer pH 7.5 + 10 mM spermine		0.558	0.319	-0.239

The finding of Veierskov and Thimann [11] that serine activates the acidic protease, could not be confirmed. Using concentrations up to 2 mM in the incubation mixture, no increase in activity of either the acidic or the neutral protease was observed. However, serine, as an amino acid, increased background ninhydrin staining and, thereby, reduced the sensitivity of the assay.

A different problem with the ninhydrin determination occurred when the stabilizing agent MCE was present in incubation media at pH 7.5. Under these conditions, staining increased whether proteases were present or not. This problem was circumvented by incubating blanks containing either no enzyme or heat-inactivated enzyme extracts.

ATP has been found to stimulate proteolysis in spinach leaves [14] and pea chloroplasts [15,16] but did not affect the activity of the oat leaf proteases *in vitro* (data not shown).

Discussion

Although the acidic and the neutral proteases in oat leaves differ in substrate preference, inactivation temperature, stability *in vitro* [1], and subcellular localization [2], they could not be separated by gel filtration and ion-exchange chromatography, indicating that they must have rather similar chemical properties. Only when extracts were made at pH 4.5, a slight difference in charge appeared to be introduced, suggesting a slight modification of the neutral enzyme at low pH. The neutral protease is relatively unstable at low pH and its charge alteration might reflect a change predisposing to inactivation. In fact, after the gel-filtration step, neutral-protease activity was readily lost.

This instability of the neutral protease might be the reason why its properties are different from the "neutral" protease described by Drivdahl and Thimann [9,10]. This latter enzyme was characterized as a sulphydryl type exopeptidase with pH optimum 6.6. In contrast, the neutral protease investigated in our study is an endopeptidase [1]. Although it was stabilized by MCE and inhibited by IAc and PMSF, but not by PCMPS, as the enzyme described by Drivdahl and Thimann, its nearly complete inhibition by *o*-phenanthroline characterizes it as a metallopeptidase. Furthermore, it was retained on DEAE-Trisacryl over a broad pH range, whereas the Drivdahl and

Thimann enzyme was not bound at pH 5.8. Since the yield of this latter enzyme was only a few percent, it appears that the purification procedure selected a minor protease, different from the major endopeptidase activity at neutral pH.

The acidic protease purified in the present study seems to be rather similar to the one described by Drivdahl and Thimann, except that it was not reproducibly bound to hemoglobin-Sepharose. The acidic protease activity present in barley leaves likewise could not be purified on such affinity columns [8]. In spite of shoulders in the pH-activity profile at pH 3.5 and 5.5 [1], only one type of acidic protease was recovered throughout gel filtration and ion-exchange chromatography. Since about 16% of the acidic-protease activity is localized in the intercellular space of the leaves, it must be identical to the enzyme localized in the vacuole. The partial inhibition encountered with sulphydryl- and serine-type enzyme inhibitors seems to suggest that different types of proteases are present in the partially purified protease peak after ion-exchange chromatography. However, such partial inhibition by different types of inhibitors has also been found for other plant proteases [6,17-19].

Using hemoglobin as a substrate, the activity of both the acidic and the neutral endopeptidases could be reliably determined. However, the ninhydrin staining method is sensitive to interference by various compounds and particular attention has to be given to the use of appropriate blanks. Also, the pH has to be carefully monitored in order to prevent anomalous effects. Storey has also draw attention to these problems [20].

In contrast to previous reports [13,11], effects of polyamines and serine on the activity of the enzymes could not be substantiated. Thus it is highly unlikely that the effect of these compounds on the rate of chlorophyll and protein loss during senescence is mediated by changes in the activity of the major proteases.

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CHAPTER 7

PROTEOLYTIC ENZYMES IN DEVELOPING LEAVES OF OATS (*AVENA SATIVA* L.)
II. AMINOACYL-2-NAPHTHYLAMIDASES

H.C.P.M. VAN DER VALK, M.I.A. VAN BENTUM and L.C. VAN LOON

Abstract

Using 2-naphthylamides of nineteen amino acids as substrates, five aminopeptidases and one arginine (Arg)-specific endopeptidase were identified in the first leaf of oats (*Avena sativa* L.) by activity staining after electrophoretic separation of the soluble proteins on polyacrylamide gels. The main aminopeptidase was largely unspecific but showed preference towards alanine, glycine and leucine. Two aminopeptidases were specific for arginine and lysine and one acted as an iminopeptidase specific for proline. The fifth was most active with methionine but also liberated some other N-terminal amino acids. All aminopeptidases were more active at low than at neutral pH. The activity of the main aminopeptidase strongly decreased in the course of leaf development. The Arg-specific endopeptidase was active at pH 4.5 and 7.5 and was inhibited at pH 7.5 by *o*-phenanthroline, suggesting it to be a metalloenzyme. Its activity also decreased during leaf development.

During storage of protein extracts in the cold, changes in the electrophoretic protein profile, indicative of protein degradation, were evident. Under such conditions, some of the Arg-specific endopeptidase was associated with the protein band containing ribulosebisphosphate carboxylase, suggestive of a possible role in protein breakdown during leaf senescence. The aminopeptidases may be involved in the rapid degradation of peptides generated by endopeptidase action.

Key words: *Avena sativa* L.; aminopeptidase; endopeptidase; leaf senescence; protease; protein degradation.

Abbreviations: B-Arg-2-NA, N-1-benzoylarginine-2-naphthylamide; EDTA, ethylenediamine tetraacetic acid (disodium salt); 2-NA, 2-naphthylamide; PMSF, phenylmethylsulphonylfluoride; Rubisco, ribulosebisphosphate carboxylase/oxygenase; Tris, Tris(hydroxymethyl)aminomethane.
Amino acids are indicated by the three-letter code.

Introduction

During natural development of the first leaf of oats, the soluble protein content decreases almost linearly from a very early stage onwards [1,2]. This gradual loss of protein occurs in the absence of increases in the activity of the two major proteases present in oat leaves. Both the "acidic" protease, with pH optimum at 4.5, and the "neutral" protease, most active at pH 7.5, decrease in activity throughout development. Only at the final stage of leaf senescence, when 70 to 80% of the protein has been degraded, the capacity of both enzymes for protein degradation increases. In extracts, the acidic protease readily degrades all soluble proteins present. However, in the leaf it is fully compartmentalized in the vacuole and in the intercellular space up to an advanced stage of senescence [3]. Thus, its role in the net breakdown of protein during development is questionable. The neutral protease seems to be far more selective as to the protein substrates degraded. Notably, *in vitro* ribulosebisphosphate carboxylase (Rubisco) is stable in extracts incubated at pH values above 6 [1], whereas *in vivo* it is among the proteins most readily lost at the onset of senescence [4,5]. These observations suggest that additional proteolytic activity must be present to account for the observed loss of protein. However, fractionation of extracts by gel filtration and ion-exchange chromatography have failed to reveal proteases different from the acidic and neutral proteases characterized previously [6].

A further way to differentiate enzymes involved in the hydrolysis of proteins consists of activity staining after electrophoretic separation. Enzymes splitting the peptide bond behind a given amino acid in a polypeptide can be visualized by adding the 2-naphthylamide derivative of the amino acid as a substrate. Upon hydrolysis, the 2-naphthylamine is liberated and trapped by a diazonium salt, yielding an insoluble, coloured product at the site of enzyme activity in the gel [7,8]. Using aminoacyl-2-

naphthylamides, aminopeptidases can be visualized, whereas a blocked N-terminal amino group in such substrates enables endopeptidases to be identified. By such procedures, Malik [9] identified five aminopeptidases in the leaves of 7-days-old oat seedlings. Three of these aminopeptidases were able to hydrolyse L-Met-2-NA, whereas the other two were active towards L-Arg-2-NA. Only one of the former could also cleave off glycine, alanine, serine and histidine. All enzymes had pH optima above 7, were inhibited by high concentrations of o-phenanthroline, and decreased upon incubation of detached leaves in darkness.

In the present investigation, additional substrates, both with a free and a blocked N-terminus, have been used to more fully characterize the peptidases present in oat leaves throughout their development. Surprisingly, all enzymes detected proved to be more active at acidic than at neutral pH values. It will be discussed as to how far these properties may contribute to a role in the turnover of oat-leaf proteins during development.

Materials and methods

Plant material

Oats (Avena sativa L. cv. Victory) were grown in a greenhouse as described previously [6]. Seeds were sown in containers of 50 x 60 x 10 cm in potsoil at a spacing of ca. 4 cm. The growing seedlings were supported by a frame of wire netting.

First leaves were harvested and the top 4.5 cm were used for all assays.

Preparation of enzyme extracts

Extracts were prepared by grinding 4 g of leaf apices in a mortar with pestle in extraction buffer containing 50 mM Tris-HCl, 0.2% (w/v) ascorbic acid, 0.1 M NaCl, 0.1% (v/v) 2-mercaptoethanol, 2 mM EDTA, pH 8.0, at 2°C. To facilitate grinding, a small amount of acid-washed sea sand was added. The homogenate was filtered through gauze and the filtrate brought to 15 ml with extraction buffer. The filtrate was then centrifuged, for 15 min at 15,000 g and 60 min at 30,000 g successively. To the resulting supernatant sucrose was added to a final concentration of 0.5 M and the extract was used immediately for electrophoretic analysis, unless stated otherwise.

Electrophoretic separation and activity staining of enzymes

Electrophoresis in 7.5% polyacrylamide gels was performed in glass tubes (7 x 0.7 cm) using the discontinuous system of Davis [10] at 2°C. Routinely, 200 l of extract were applied to the gel. Electrophoresis was performed for 20 min at 2 mA per gel, then at 4.5 mA per gel until the bromophenolblue tracking dye almost reached the bottom of the gel. After electrophoresis, gels were stained for either protein or enzyme activity. Proteins were visualized by overnight immersion of the gel in a saturated solution of Amido black in 5% trichloroacetic acid, followed by repeated washings with 7% acetic acid [11]. For enzyme staining, gels were incubated at room temperature, first for 30 min in buffer at the appropriate pH (0.2 M phosphate-citrate buffer pH 4.5, 0.2 M Tris-maleate buffer pH 6.0, or 0.2 M Tris-HCl pH 7.5), then in the same buffer containing 0.05% substrate and 0.04% of either Fast Red TR (at pH 4.5 or 6.0) or Fast Garnet GBC (at pH 7.5) as the coupling agent. As substrates, the 1-aminoacyl-2-naphthylamides were used unless only the DL-mixture was available. These substrates, as well as the benzoylated derivatives, were from both Koch-Light and Serva. The aminoacyl-2-naphthylamides and their benzoylated derivatives were first dissolved in 0.01 vol of acetone and of dimethylsulphoxide, respectively, before being diluted in the buffer solutions. When investigated, inhibitors were included both in the preincubation buffer and in the substrate solution. Incubations lasted from 1-20 h, depending on enzyme activity. Reactions were stopped with 7% acetic acid.

The electrophoretic patterns were scanned using a Chromoscan (Joyce, Loebl & Co. Ltd, Gateshead, England) and the resulting densitometer tracings were used in calculating R_f values and in quantitating enzyme activity of the bands by measuring the area under the peaks.

Results

Originally, protein extracts were prepared using a buffer containing 0.5 M salt [1], and subsequently dialysed at 2°C against electrophoresis buffer to avoid interference of the high salt concentration with the subsequent electrophoretic separation. However, during dialysis the pattern of the electrophoretically separated proteins was altered in that protein bands in

the upper part of the gel became less intense and two bands with R_f values around 0.31 and 0.50 became prominent. This phenomenon was not specific to any developmental stage of the leaf. The same phenomenon was observed when extracts were stored frozen (Fig. 1). Apparently, even at low temperature, some of the proteins were subject to degradation. Since lowering the salt concentration in the extraction medium to 0.1 M did not affect the protein pattern, the extraction buffer was modified accordingly.

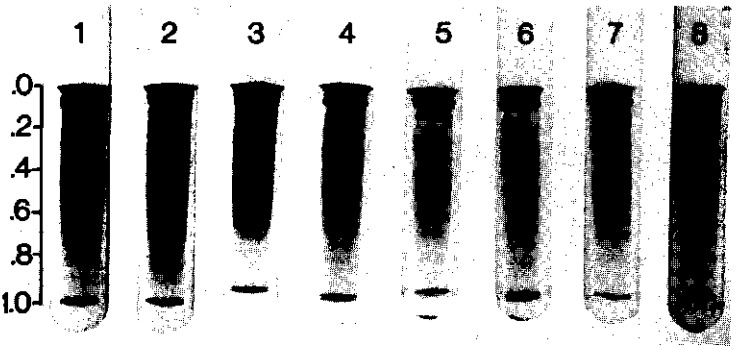

Fig. 1. Electrophoretic patterns in 7.5% polyacrylamide gels of soluble proteins from the first leaf of (lanes 1,2) 7-, (lanes 3,4) 14-, (lanes 5,6) 21- and (lanes 7,8) 28-days-old oat plants. The equivalent of 50 mg of fresh weight of tissue was applied to each gel. Extracts were subjected to electrophoresis either immediately after preparation (lanes 1,3,5,7) or after having been stored frozen for one week (lanes 2,4,6,8). Gels were stained with Amido black.

Incubation of gels after electrophoresis in each of nineteen different aminocyl-2-naphthylamides at pH 4.5, 6.0 and 7.5 revealed that in each case activity was most pronounced at pH 4.5. As shown in Fig. 2 for Gly-2-NA, activity at pH 7.5 was much lower than at pH 6.0, and this again substantially lower than at pH 4.5. The diazonium salt used as the coupling agent at pH 4.5 and 6.0, Fast Red TR, was not effective in trapping the liberated 2-naphthylamine at pH 7.5. Conversely, Fast Garnet GBC, employed at pH 7.5, did not react well at the lower pH values. Hence, the choice of the diazonium salt appeared decisive in characterizing the peptidase activities. Although with prolonged incubation times some diffusion of the coloured product did take place, weak bands remained sufficiently distinct to be clearly discernable even after 20 h of incubation.

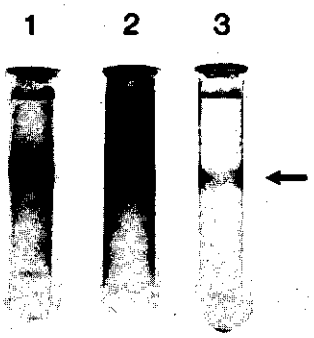

Fig. 2. Electrophoretic patterns in 7.5% polyacrylamide gels incubated with Gly-2-NA for 1 h at (lane 1) pH 4.5, (lane 2) pH 6.0 and (lane 3) pH 7.5. The arrow indicates the enzymatically active band. The band at R_f 0.11 as well as the weak bands at R_f's 0.29-0.31 and 0.44 are artifacts due to the precipitation of protein after addition of 7% acetic acid to stop the enzyme reaction. The equivalent of 50 mg of fresh weight of tissue was applied to each gel immediately after preparation of a protein extract from leaves of 10-days-old plants.

Fig. 3 shows the patterns obtained with selected aminoacyl-2-naphthylamides. A diagrammatic representation of the specificity of each of the aminopeptidases encountered is given in Fig. 4. A major aminopeptidase at an R_f value around 0.45 hydrolysed all nineteen substrates, be it at exceedingly different rates. Glycine, alanine and, to a somewhat lesser

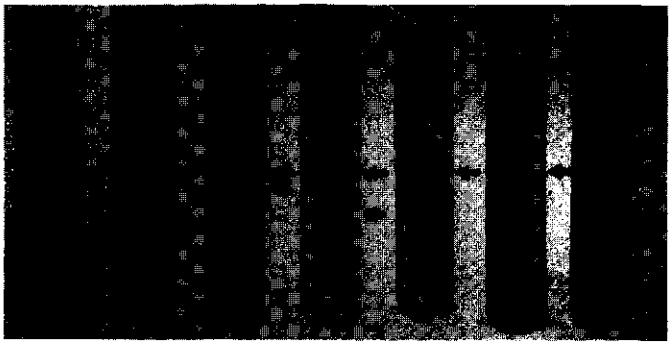

Fig. 3. Patterns of aminopeptidases upon incubation with (lane 1) Ala-2-NA, (lane 2) Pro-2-NA, (lane 3) Arg-2-NA, (lane 4) Phe-2-NA, (lane 5) Ser-2-NA, (lane 6) Glu-2-NA and (lane 7) Met-2-NA. Gels were incubated in substrate solution for 1 h, except the one in Pro-2-NA (lane 2) which was left overnight. Conditions as for Fig. 2.

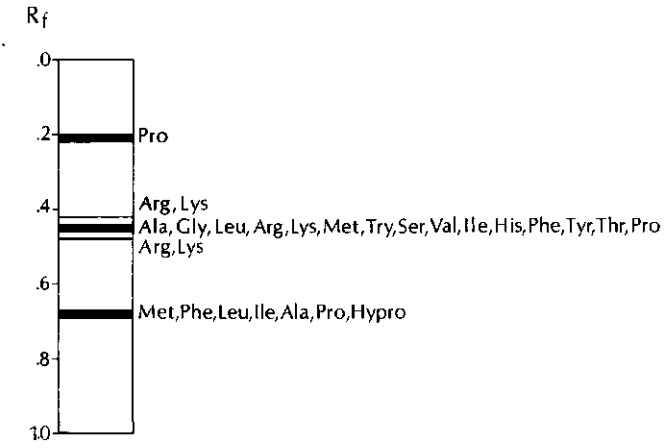

Fig. 4. Diagrammatic representation of the aminopeptidases present in first oat leaves and their specificity. For each enzyme band, amino acids are arranged in order of decreasing activity.

extent, leucine were preferred, whereas the derivatives of histidine, tryptophan, aspartic acid, glutamic acid, cysteine and hydroxyproline were hydrolysed only marginally. On the gel this aminopeptidase was flanked by two enzymes at R_f values of 0.42 and 0.48 that were substantially less active and showed preferential staining with Lys-2-NA and Arg-2-NA. An iminopeptidase specific to proline was revealed at R_f 0.20. At R_f 0.68 an aminopeptidase was present that also showed distinctive selectivity. It was most active with methionine but also reacted with phenylalanine, and to a small extent with leucine, isoleucine, alanine, proline and hydroxyproline. The relative activity of this aminopeptidase was enhanced when DL-Met-2-NA was used as the substrate instead of the L-form.

Using Met-2-NA as the substrate at the optimal pH of 4.5, both the aminopeptidase present at R_f 0.45 and that at 0.68 were strongly inhibited by 10 mM cupric acetate (45 and 55%, respectively), o-phenanthroline (45 and 65%) and phenylmethylsulphonylfluoride (PMSF) (55 and 85%).

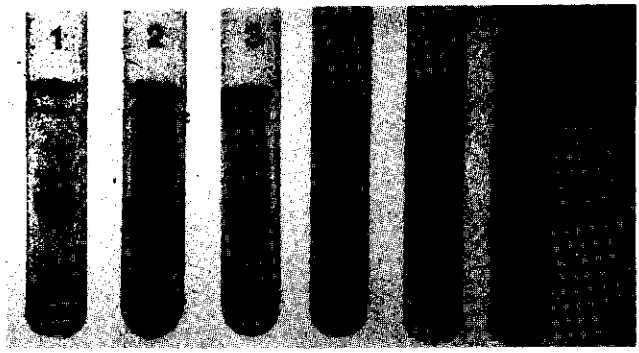

Fig. 5. Patterns of B-Arg-2-NA-specific endopeptidase activity upon incubation for (lanes 1,3,5) 1 h or (lanes 2,4,6) overnight at (lanes 1,2) pH 4.5, (lanes 3,4) pH 6.0 and (lanes 5,6) pH 7.5. Conditions as for Fig. 2.

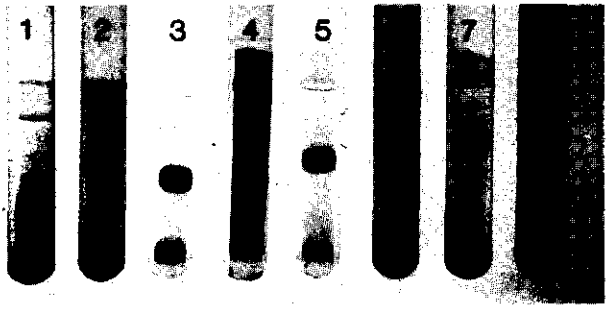

Fig. 6. Patterns of B-Arg-2-NA-specific endopeptidase upon electrophoresis in gels of (lanes 1,2) 6.25%, (lanes 3,4) 7.5%, (lanes 5,6) 8.75% and (lanes 7,8) 10% polyacrylamide and incubation at (lanes 1,3,5,7) pH 4.5 or (lanes 2,4,6,8) pH 7.5. Gels were incubated overnight in substrate solution.

Of five benzoylated derivatives (B-Ala-2-NA, B-Leu-2-NA, B-Arg-2-NA, B-Phe-2-NA and B-Tyr-2-NA), only B-Arg-2-NA was hydrolysed, indicative of the presence of a trypsin-like proteolytic activity. This activity was present as one distinct band at R_f 0.57 and no minor bands of activity were revealed upon prolonged incubation (Fig. 5). Although B-Arg-2-NA was readily hydrolysed both at pH 4.5 and at 7.5 but considerably less so at pH 6.0, only a single enzymic activity seemed to be involved: when extracts

were separated on gels ranging from 6.25 to 10% polyacrylamide, bands at identical R_f values were obtained upon incubation at either pH value (Fig. 6). The activity was not inhibited by 10 mM cupric acetate, PMSF or p-chloromercuriphenylsulphonate at either pH 4.5 or 7.5, nor by o-phenanthroline at pH 4.5. At pH 7.5, however, in the presence of 10 mM of the latter compound, the band intensity was reduced 45%.

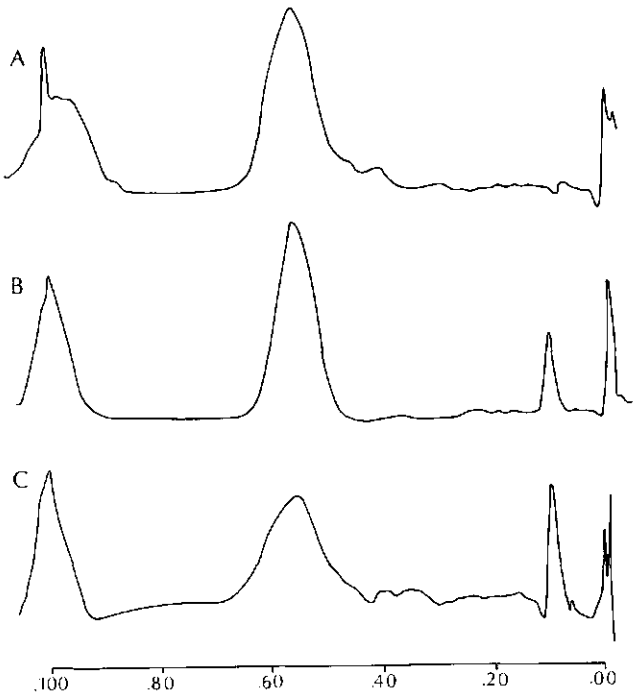

Fig. 7. Densitometer tracings of electrophoretic patterns of the B-Arg-2-NA-specific endopeptidase upon electrophoresis in 7.5% polyacrylamide gels of protein extracts (A) immediately after preparation and (B,C) after storage for one week at -20°C. Gels were incubated overnight at either (A,B) pH 4.5 or (C) pH 7.5.

When extracts were stored for at least one day and subsequently subjected to electrophoresis, and the gels were stained using B-Arg-2-NA as a substrate, besides the band at R_f 0.57 a sharp minor band of activity was revealed at R_f 0.11. This latter band occurred upon incubation at either pH 4.5 or 7.5 but was most conspicuous at pH 7.5 (Fig. 7). This activity coincided with the main protein band present in the gel (Fig.1), which contains Rubisco. Presumably, the activity at R_f 0.11 reflects association of the enzyme with substrate protein *in vitro*. Thus, the B-Arg-2-NA-hydrolysing activity might be (one of) the enzyme(s) responsible for the alteration of the protein pattern upon storage of the extracts (Fig. 1).

Representative substrates were selected to follow the pattern of the peptidases at weekly intervals during leaf development. As shown in Fig. 8, the activity of the main aminopeptidase around R_f 0.45 was highest in 7-days-old, emerging leaves and decreased throughout leaf maturity and senescence. The slight increase in the activity hydrolysing Arg-2-NA at day 14 appears to be due to the flanking aminopeptidases at R_f's 0.42 and 0.48, which decreased in activity only once senescence had been initiated. The proline-specific iminopeptidase at R_f 0.20 did not change significantly in activity throughout leaf development. In contrast, the aminopeptidase at R_f 0.68 increased significantly between days 14 and 21, and decreased thereafter. The endopeptidase hydrolysing B-Arg-2-NA (R_f 0.57) only decreased at a linear rate from 7 until 28 days of development. No additional aminopeptidases hydrolysing alanine, serine, methionine, arginine, glutamic acid, phenylalanine or proline were found to appear during the course of leaf development.

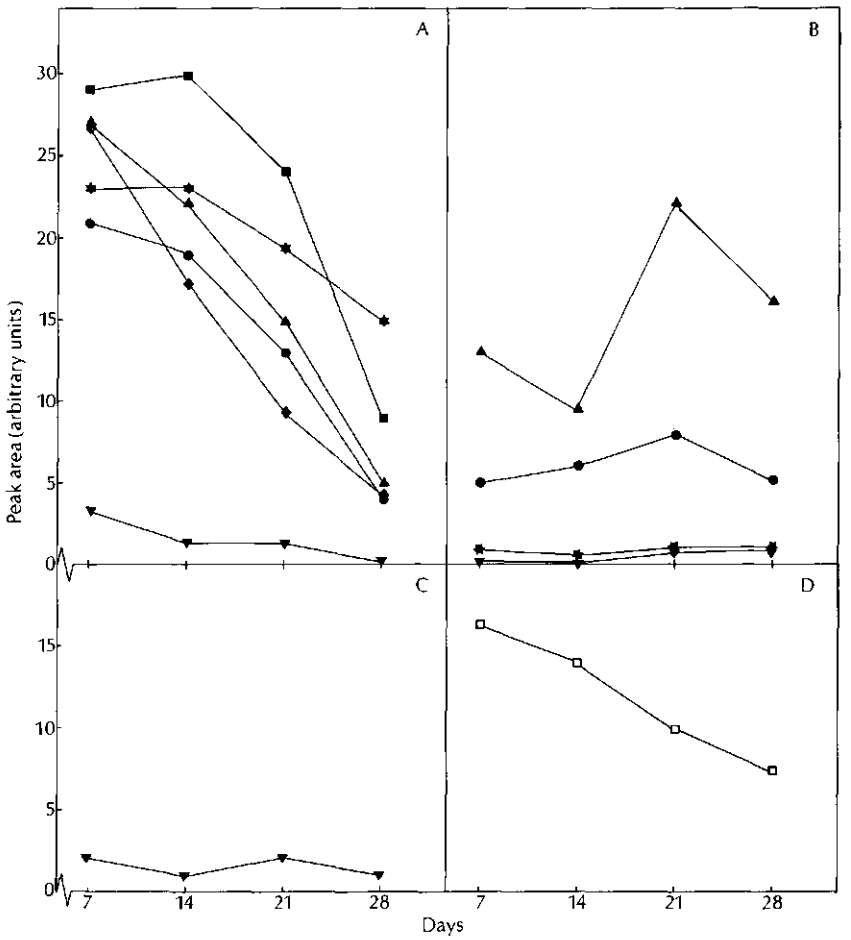

Fig. 8. Relative activities (peak areas) determined from densitometer tracings of electrophoretic patterns of (A) aminopeptidase at R_f 0.45, (B) aminopeptidase at R_f 0.68, (C) aminopeptidase at R_f 0.20 and (D) endopeptidase at R_f 0.57, after incubation in (x) Ala-2-NA, (■) Arg-2-NA, (▲) Met-2-NA, (◆) Ser-2-NA, (o) Phe-2-NA, (▼) Pro-2-NA or ( ) B-Arg-2-NA, as a function of oat leaf development. Staining was allowed to develop overnight, except in the case of Ala-2-NA (A) where incubation was for 1 h.

Discussion

Oat leaves were found to contain five aminopeptidases and one arginine-specific endopeptidase. The main aminopeptidase was largely unspecific but preferentially hydrolysed N-terminal alanine, glycine and leucine. Two aminopeptidases migrating slightly more rapidly and more slowly, respectively, were specific for the basic amino acids arginine and lysine. One enzyme was specific for proline and, thus, constitutes an iminopeptidase. The last aminopeptidase again split off various amino acids but methionine was strongly preferred. In contrast to the main aminopeptidase, this enzyme decreased only late in leaf development. It also differed in being present in extracts made at pH 4.5 [6] and in showing enhanced activity with D-amino acids, indicating different chemical and functional properties.

The aminopeptidases present at R_f's 0.42, 0.45, 0.48 and 0.68 can be equated with the enzymes AP 4, 1, 5 and 2, respectively, of Malik [9]. This author did not report activity of AP 1 with arginine, probably because his use of a gel with a higher percentage of polyacrylamide led to overlap of the three stained bands. On the other hand, an additional Met-2- NA-specific aminopeptidase, AP 3, was not encountered in our study, whereas an iminopeptidase not described by Malik [9] was found. Similar, high-molecular-weight iminopeptidases have been found to be predominant in wheat leaves [12] and apricot seeds [13]. In our study, all aminopeptidases displayed highest activity at low pH. The pH optima above neutrality determined by Malik [9] are presumably to be attributed to the use of a coupling agent unsuitable for trapping the liberated 2-naphthylamine at low pH. An attempt to determin the pH optimum of the enzyme in solution, using l-leucine-p-nitroanilide as a substrate, failed due to pH-influenced color development, and therefore absorbance values, of the product [14].

The arginine-specific endopeptidase is not identical to either the acidic or the neutral protease described previously [1,6] and, thus, constitutes a proteolytic enzyme not hitherto described for oat leaves. Using B-Arg-2-NA as a substrate, it showed high activity at both pH 4.5 and 7.5, and was not sensitive to inhibition by sulphydryl- or serine-type protease inhibitors but only to o-phenanthroline at pH 7.5, suggesting it to be a metalloenzyme. Intriguingly, some of the activity tended to associate with

the Rubisco protein band on gels upon storage of extracts. If it would have access to chloroplasts in vivo, it might play a role in the degradation of Rubisco. Such a role would not be inconsistent with its decrease during leaf development: as less protein substrate remains, less protease activity could suffice to degrade it.

Interest in the role of aminopeptidases in protein turnover has increased recently as a result of the finding that the in vivo half-life of a protein is a function of its amino-terminal residue [15]. Thus, the action of aminopeptidases may either reduce or prolong the life-time of a protein depending on the final N-terminal amino acid. It has been proposed that the in vivo degradation of long-lived proteins may be the result of a slow aminopeptidase cleavage that exposes a destabilizing residue, after which rapid degradation occurs. The life-time of the peptides generated by endoprotease action might depend similarly on the N-terminal amino acid exposed. Even though in our study the aminopeptidases were found to have low pH optima, their activity at pH 7.5 was still substantial. Furthermore, their specific activity does not change appreciably during leaf development. Hence, a role of the aminopeptidases in protein turnover seems reasonable. However, under conditions of accelerated protein breakdown, when acidification of the cytoplasm may occur, the aminopeptidases would be most active and readily degrade peptides generated by endoproteolytic cleavages. Thus, rapid proteolysis is likely to be the result of a concerted action of proteases with endo- and exopeptidase activities. Whereas the mechanism of normal protein turnover remains to be elucidated, the slow, steady decline of protein during natural development may be controlled by, on the one hand, compartmentalization of protease [3] and, on the other hand, by maintaining a pH removed from the optimum for protease action.

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General discussion

During the development of the first leaf of oats, the protein content reaches a maximum shortly after emergence and declines almost linearly over a period of 4 weeks until the then yellow leaf desiccates and dies off [1]. The regulation of the decrease in protein content could involve several proteolytic enzymes. Oat leaves contain two major proteases, an acidic and a neutral endopeptidase that differ in several properties. An additional endopeptidase with trypsin-like specificity has also been identified. Five more or less specific aminopeptidases were found by their action on aminoacyl-2-naphthylamides. Carboxypeptidase activity has also been found to be present. The synthetic substrate, N-carbobenzoxy-l-phenylalanine-l-alanine, was hydrolysed over a pH range from 5.0 to 6.5, but because the colour development of the product depended on the acidity, the properties of this enzyme could not be reliably determined. Combined, the endopeptidases and exopeptidases constitute a rather complete set of enzymes, capable of degrading the endogenous proteins in vitro within a few hours. During natural development of greenhouse-grown plants, the activities were highest in young and fully-grown leaves and decreased throughout the stage of leaf senescence.

In our experiments, the plants were routinely grown under controlled conditions and the leaves were harvested at defined physiological ages. Yet, the activities of the major endopeptidases could vary substantially. Notably, their activities tended to increase in plants kept in a growth cabinet, whereas they remained constant or decreased in greenhouse-grown plants. Possibly the conditions in the growth cabinet imposed some kind of stress similar to other conditions known to increase protease activity [2]. However, surprisingly little differences were found in the rates of decrease in protein content in leaves from plants grown under these different conditions. Thus, the protease activities measured with either hemoglobin or endogenous substrates were not correlated with the rate of protein loss. It can be concluded, therefore, that changes in protease activities are not the main regulatory mechanism for protein breakdown during senescence.

To investigate whether changes in the compartmentation of the proteases and substrates could be a mechanism for regulating protein degradation, the distribution and subcellular localization of the proteases were in-

vestigated. The major acidic protease was found to be located in the intercellular space and the vacuole and, thus, is spatially separated from the protein substrates in the cytoplasm and organelles. Vacuoles could be isolated up to an advanced stage of leaf development, but when about 50% of the protein had been degraded, pure vacuoles could no longer be obtained. The reason for this was that the protoplasts from which the vacuoles were prepared, became unstable and vacuoplasts were formed. Thus, at least up to this stage, the tonoplast appeared to remain intact. In agreement with this finding, the acidic protease was found to remain fully compartmentalized within the vacuole. Only at a late stage of senescence, when most of the protein has been degraded the tonoplast may rupture and the hydrolytic enzymes present in the vacuole may come into contact with the remaining substrates, allowing for a rapid degradation in the then acidic environment.

During natural leaf development in greenhouse-grown plants, the vacuolar protease activity decreased with leaf age. This might mean that vacuolar proteases could still be gradually released into the cytoplasm to function in the degradation of protein substrates. However, these enzymes are not active at a neutral pH and quickly loose activity under such conditions. Thus, such a mechanism seems implausible. Instead, it appears more rational to envisage a mechanism in which protein substrates to be degraded enter the vacuole. Recently, evidence has been presented that vacuoles are able to degrade proteins [3], and that transport of proteins over the tonoplast takes place [4]. In the cytoplasm proteins to be degraded would somehow be marked, both to select them for degradation, and for carrying them through the tonoplast. Distinguishing characteristics of readily degraded proteins are being sought for by H. Klerk in a parallel project at our laboratory. How protein degradation in membrane-bound organelles is controlled, is unclear. Recently evidence has been presented that chloroplasts and mitochondria contain (latent) proteolytic activities [5,6]. However, such activities could not be detacted in the present study.

The major neutral endopeptidase is absent from vacuoles and appears to be located in the cytoplasm. In contrast to the acidic protease , it does not seem to degrade all proteins and it could, thus, serve a more specific function. In spite of the use of various chromatographic technics, the two enzymes could not be separated and it must be concluded that they have very

similar molecular weights and charges. The possibility that the two enzymes might be related was considered, particularly since the benzoylarginine-2-naphthylamide-hydrolysing enzyme also exhibits activity at both acidic and neutral pH values. However, inhibition by o-phenanthroline characterized the neutral enzyme as a metallopeptidase whereas the acidic enzyme was insensitive. Furthermore, the properties of the enzymes were not changed by exposure to different pH's or to mercaptoethanol (unpublished data). The neutral protease was relatively unstable and lost activity at a progressively increasing rate during purification, suggesting that in the absence of stabilizing substrate, it is subject to self digestion. This hampers its isolation and further characterization but may allow recovery of relatively stable minor proteases active at neutral pH. Since during purification from extracts the acidic protease remained contaminated with neutral activity, but both enzymes are spatially separated in the plant, further purification and characterization of the acidic protease would be possible by using intercellular washing fluid or highly purified vacuoles. To further characterize the neutral protease, evacuolated protoplasts [7] might be used as the starting material.

Aminopeptidases are generally thought to act at neutral pH-values. These enzymes could, therefore, be of importance in regulating protein degradation inside the cytoplasm. Interest in their role in protein turnover has increased recently as a result of the finding that, *in vitro*, the half-life of proteins might be determined by their amino-terminal residue [8]. The aminopeptidases identified in oat leaves were found to have highest activities at low pH. However, at pH 7.5 their activities were still substantial. These aminopeptidases showed highest activities in young and fully-grown leaves, but when in the later stage of senescence acidification of the cytoplasm takes place these enzymes readily degrade peptides generated by endoproteolytic cleavage.

The finding of an additional endoproteolytic activity, not clearly revealed by the ninhydrin staining method, indicates that the spectrum of proteases in oat leaves is more complex than originally expected. This activity was observed towards benzoylarginine-2-naphthylamide and , thus, has trypsin-like properties. Only five benzoylated substrates were available for this study. Thus, it is possible that even more additional endopeptidases could be identified with other substrates. The demonstration

that some of the activity was associated with Rubisco, which, under these conditions, was rapidly degraded even in the cold, suggests that this enzyme may play a role in the regulation of the breakdown of this protein. However, the effect may be spurious. If the enzyme readily associates with protein substrates, in extracts most of the bound activity will be on Rubisco, as the latter is the protein present in by far the largest amount in leaf tissue.

In this study, insight has been gained into the properties of the proteases present in developing oat leaves. It has been demonstrated that the protease activities in the leaves can vary under different conditions without affecting the rate at which the protein content decreases. Thus, the process of protein degradation appears to depend more on the nature of the proteases present than on the level of their activities. Much research will be needed to further elucidate the mechanisms of (de)compartmentalization and transport of proteases and substrates in the course of senescence. Only when such mechanisms have become clear, it will be possible to manipulate the process of protein degradation to optimize the redistribution of amino acids into developing seeds.

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SUMMARY

The loss of chlorophyll during the senescence of leaves is preceded by a decrease in protein content. Proteases responsible for the degradation of the proteins have been implicated in the regulation of the senescence process. The first leaf of the seedling of oats (Avena sativa L.) demonstrates the typical pattern of leaf senescence in cereals and was chosen to study the properties and subcellular localization of proteases throughout leaf development.

In Chapter 1, a general introduction is given that indicates the significance of the breakdown of proteins in leaves for seed yield and quality in cereals. Also in this chapter, protease classification is documented, and the possible role of changing protease activities in the regulation of protein degradation is discussed.

Oat leaves contain two major proteases with pH optima at pH 4.5 ("acidic protease") and 7.5 ("neutral protease"). During natural development of greenhouse-grown plants both types of enzymes showed highest activities in young and fully-grown leaves, and decreased throughout the course of senescence. Also in detached leaves incubated in the dark and, therefore, subject to accelerated ageing, loss of protein was not accompanied by increases in protease activities. In the light, protease activities increased, but the rate of protein loss was greatly reduced. Thus, protein breakdown appears to be independent of the amount of protease present and additional synthesis of the major proteases is not required for protein loss during senescence (Chapter 1).

This apparent paradox could be resolved if proteases and their substrates were spatially separated and brought into contact by a controlled decompartmentalization. Therefore, the distribution and subcellular localization of the major proteases were determined. Protoplasts were prepared as a first step in the isolation of vacuoles. However, the cell wall-degrading enzyme mixtures used for protoplast isolation were seriously contaminated by proteases interfering with the determination of the endogenous protease activity in the isolated protoplasts. These extra proteases could be inactivated by heating the cell wall-degrading enzyme mixture at 50°C for 10 min at pH 6.5. This treatment did not impair the cell wall-degrading activity. Protoplasts isolated with heated enzymes showed similar

protease activities as washed protoplasts, isolated with untreated enzymes. This proved that contaminating proteases were effectively removed during protoplast washing, and that the protease activity measured in isolated protoplasts was derived from the protoplasts themselves. (Chapter 3).

Vacuoles were isolated by osmotically lysing the protoplasts in the presence of K_2HPO_4. The maximum concentration of phosphate by which lysis occurred decreased progressively with increasing leaf age. By using the appropriate phosphate concentrations, it became possible to isolate clean vacuoles from leaves up to an advanced stage of senescence, when the leaves had lost more than 50% of their protein. From leaves older than 17 days, only vacuoplasts (vacuoles with adhering cytoplasm, within a resealed plasma membrane) could be obtained. The integrity of both the plasmalemma and the tonoplast decreased in these older leaves and this phenomenon might be linked with increased decompartmentalization at a late stage of senescence (Chapter 4).

When the distribution of the proteases was determined in different subcellular fractions, on an average 16% of the acidic protease activity was washed out of the intercellular space of the leaves. The major part of the acidic activity was located within the vacuole. The neutral protease was absent from both these compartments and must, therefore, be cytoplasmic. During the course of leaf development, all of the acidic protease activity present in protoplasts was recovered in the vacuoles, as long as clean vacuoles could be isolated (i.e. up to 17 days). It seems most likely that protein degradation is controlled by import of protein substrates into the vacuole (Chapter 5).

The acidic and neutral proteases were partly purified by gel filtration and anion-exchange chromatography (Chapter 6). The enzymes hardly separated, indicating that they have similar molecular weights and charges. The neutral activity was stabilized by mercaptoethanol and inhibited by inhibitors of metallopeptidases, whereas the acidic one was not. Both activities were inhibited to varying extents by sulphydryl- and serine-type inhibitors. Both enzymes were endopeptidases. The instability of, in particular, the neutral protease seriously hampers its further purification and characterization.

Furthermore, by activity staining after electrophoretic separation and using aminoacyl-2-naphthylamides as substrates, five aminopeptidases and

one trypsin-like endopeptidase were identified (Chapter 7). The main aminopeptidase was largely unspecific. Two aminopeptidases showed preference for arginine and lysine. An iminopeptidase acted on proline. The fifth aminopeptidase was most active with methionine. All enzymes were active at pH 4.5, 6.0 and 7.5, but showed highest activities at low rather than at neutral pH. The trypsine-like endopeptidase was active at pH 4.5 and 7.5, was inhibited by o-phenanthroline and was not identical with either the acidic or the neutral protease described previously. Its activity decreased during leaf development. During storage of protein extracts in the cold this arginine-specific endopeptidase associated with ribulosebisphophate carboxylase, concomitant with a loss of this protein band. As to how far this enzyme has a function in protein breakdown *in vivo* has to be further elucidated.

The results of this study show that the levels of protease activities are not correlated with the rate of over all protein degradation. The acidic protease has no entry to the substrates to be degraded. In contrast, the neutral protease appears to be located together with protein substrates and organelles in the cytoplasm, but seems to selectively degrade only a few proteins. Whereas the mechanism of normal protein turnover remains to be elucidated, the slow, steady decline of protein during natural development may be controlled by, on the one hand, compartmentalization of the acidic protease in the vacuole and, on the other hand, by maintaining a pH removed from the optimum for exopeptidase action in the cytoplasm.

SAMENVATTING

De afname van het chlorofylgehalte tijdens de veroudering van bladeren wordt voorafgegaan door een daling van het eiwitgehalte. Veelal wordt verondersteld dat proteasen, verantwoordelijk voor de afbraak van de eiwitten, een rol spelen bij de regulatie van het verouderingsproces. Het eerste blad van haverkiemplanten (Avena sativa L.) vertoont het karakteristieke patroon van bladveroudering in granen en werd daarom gekozen om de eigenschappen en subcellulaire lokalisatie van proteasen gedurende de bladontwikkeling te bestuderen.

In Hoofdstuk 1 wordt in een algemene inleiding de betekenis van de eiwitafbraak in bladeren voor opbrengst en kwaliteit van geoogste graanzaden uiteengezet. In dit hoofdstuk wordt tevens de klassificatie van de diverse typen van proteasen beschreven. Daarnaast wordt de mogelijke rol van veranderingen in protease-activiteiten bij de regulatie van de eiwitafbraak besproken.

De voornaamste proteasen in haverbladeren zijn een "zure" protease met een pH optimium bij 4,5 en een "neutrale" protease met een pH optimum bij 7,5. Tijdens de natuurlijke ontwikkeling van planten opgekweekt in de kas, bleek de activiteit van beide enzymen het hoogst in jonge en juist volgroeide bladeren. Gedurende de veroudering namen de activiteiten af. Ook in afgesneden bladeren, die in het donker werden geïncubeerd en daardoor versneld verouderden, ging de afname van het eiwitgehalte niet gepaard met toenamen in de protease-activiteiten. In het licht namen de proteasenactiviteiten wel toe, maar het verlies van eiwit verliep veel trager. Klaarblijkelijk is de eiwitafbraak niet afhankelijk van de hoeveelheid aanwezige proteasen en is extra synthese van de voornaamste proteasen niet vereist voor de tijdens de veroudering optredende eiwitafbraak (Hoofdstuk 2).

Deze schijnbare paradox zou opgelost kunnen worden als proteasen en hun substraten ruimtelijk van elkaar gescheiden zouden zijn en slechts via een gecontroleerde decompartimentalisatie met elkaar in contact zouden kunnen komen. Daarom werden de verdeling en subcellulaire lokalisatie van de voornaamste proteasen onderzocht. Als eerste stap in de isolatie van vacuolen werden protoplasten bereid. Echter, de gebruikte celwandafbrekende enzymen bleken zelf ernstig verontreinigd te zijn met proteasen.

Deze proteasen zouden protease-bepalingen in de gelsoleerde protoplasten bemoeilijken. De proteasen in het enzymmengsel konden worden geïnactiveerd door het enzymmengsel gedurende 10 minuten bloot te stellen aan 50 °C bij pH 6,5. Na deze behandeling konden nog steeds protoplasten worden bereid met het enzymmengsel. Protoplasten, geïsoleerd met verhit enzym, vertoonden dezelfde protease-activiteiten als protoplasten die met onbehandeld enzym waren bereid. Dit bewijst, dat verontreinigende proteasen daadwerkelijk verwijderd werden tijdens het wassen van de protoplasten en dat de protease-activiteit, gemeten in geïsoleerde protoplasten, afkomstig was van de protoplasten zelf (Hoofdstuk 3).

Vacuolen werden geïsoleerd door protoplasten osmotisch te lyseren in aanwezigheid van K_2HPO_4. De hoogste concentratie fosfaat waarbij lysis plaats vond, nam af bij toenemende leeftijd van het blad. Door gebruik te maken van de juiste fosfaatconcentraties bleek het mogelijk schone vacuolen te isoleren uit bladeren, die in een vergevorderd stadium van veroudering verkeerden. Het eiwitgehalte van deze bladeren was dan meer dan gehalveerd. Van bladeren ouder dan 17 dagen konden slechts "vacuoplasten" worden verkregen. Dit zijn vacuolen met aanhangend cytoplasma, omgeven door een opnieuw gesloten plasmamembraan. De integriteit van zowel het plasmalemma als de tonoplast nam af in oudere bladeren en dit verschijnsel zou kunnen samenhangen met een toenemende decompartimentalisatie in een later stadium van de veroudering (Hoofdstuk 4).

De verdeling van de proteasen werd bepaald in verschillende subcellulaire fracties. Gemiddeld kon 16% van de zure activiteit worden uitgewassen uit de intercellulaire ruimte van de bladeren. Het grootste deel van de zure protease-activiteit was gelocaliseerd in de vacuolen. Neutrale protease-activiteit was niet aanwezig in deze beide compartimenten en lijkt daarom gelokaliseerd te zijn in het cytoplasma. Gedurende het verloop van de bladontwikkeling werd, zolang schone vacuolen konden worden geïsoleerd (dit is t.m. 17 dagen na het zaaien), alle in de protoplast aanwezige zure proteaseativiteit teruggevonden in de vacuole. Dit maakt het waarschijnlijk dat eiwitafbraak wordt gecontroleerd door import van eiwit-substraten in de vacuole (Hoofdstuk 5).

De zure en neutrale proteasen zijn gedeeltelijk gezuiverd door middel van gelfiltratie en anionenwisselaars-chromatografie (Hoofdstuk 6). De enzymen konden nauwelijks gescheiden worden, hetgeen erop duidt dat zij

overeenkomstige molecuulgewichten en ladingen bezitten. De neutrale activiteit werd gestabiliseerd door mercaptoethanol en geremd door remmers van metallopeptidasen, de zure activiteit werd dat niet. Beide activiteiten werden in verschillende mate geremd door remmers van sulfhydryl- en serinepeptidasen. Beide enzymen zijn endopeptidasen. De geringe stabiliteit van in het bijzonder de neutrale protease maakt verdere zuivering en karakterisering uiterst moeilijk.

Daarnaast werden, door kleuring op activiteit na scheiding door middel van elektroforese en gebruik van aminoacyl-2-naphthylamidasen als substraten, vijf aminopeptidasen en een trypsine-achtige endopeptidase aangetoond (Hoofdstuk 7). De voornaamste aminopeptidase was tamelijk aspecifiek. Twee aminopeptidasen waren specifiek voor arginine en lysine. Een iminopeptidase splitste proline af. De vijfde aminopeptidase was het meest actiefst met methionine. Alle aminopeptidasen waren actief bij pH 4,5, 6,0 en 7,5 maar vertoonden de hoogste activiteit bij pH 4,5. De trypsine-achtige endopeptidase was actief bij pH 4,5 en 7,5, werd geremd door o-phenanthroline en was niet identiek met de hierboven beschreven zure of neutrale protease. De activiteit van dit enzym nam af gedurende de ontwikkeling van het blad. Tijdens het bewaren van eiwitextracten in de kou associeerde dit arginine-specifieke endoprotease met ribulosebisfosfaatcarboxylase, terwijl de hoeveelheid eiwit in deze band op gels gelijktijdig afnam. In hoeverre dit enzym een functie heeft bij de eiwitafbraak in vivo zou verder onderzocht dienen te worden.

De resultaten van dit proefschrift tonen aan dat de hoogte van de protease-activiteit niet gecorreleerd is met de snelheid van de eiwitafbraak. De zure protease heeft geen toegang tot de eiwitten die afgebroken moeten worden. De neutrale protease is waarschijnlijk in het cytoplasma gelokaliseerd, samen met de eiwitsubstraten en organellen, maar lijkt slechts enkele eiwitten te kunnen afbreken. Hoewel het mechanisme van de normale eiwit-turnover nog dient te worden opgehelderd, zou de voortdurende afname van het eiwitgehalte tijdens de natuurlijke veroudering kunnen worden gereguleerd, enerzijds door compartimentalisatie van de zure protease in de vacuole en anderzijds door het handhaven van een niet optimale pH voor de werking van exopeptidasen in het cytoplasma.

CURRICULUM VITAE

Op 2 februari 1953 ben ik in Hillegom geboren. In 1970 werd het HBS-b diploma behaald aan het Triniteitslyceum te Haarlem en ging ik biologie studeren aan de Rijksuniversiteit te Leiden. In 1971 werd deze studie voortgezet aan de Landbouwuniversiteit te Wageningen. In 1980 werd het ingenieursexamen biologie, subspecialisatie organisme afgelegd met als hoofdvakken de plantenfysiologie, de celbiologie en de toxicologie. Van september 1980 tot april 1982 was ik als tijdelijk medewerker werkzaam bij de vakgroepen Experimentele Diermorfologie en Celbiologie, Plantencytologie en Morfologie, Dierfysiologie en Plantenfysiologie. Van april 1982 tot oktober 1983 heb ik de vervangende dienstplicht vervuld bij de vakgroep Plantenfysiologie aan de Landbouwuniversiteit. Van oktober 1983 tot november 1986 werd een promotieonderzoek verricht bij deze vakgroep, hetgeen resulteerde in dit proefschrift.

Sinds februari 1987 ben ik als plantecelbioloog werkzaam bij de stichting ITAL te Wageningen.