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ISOENZYMES OF NAPHTHYL ACETATE ESTERASE IN SENESCING LEAVES OF *FESTUCA PRATENSIS*

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Key Word Index—*Festuca pratensis*; Gramineae; leaf senescence; enzymology; α-naphthyl acetate esterase; isoenzymes.

Abstract—Naphthyl acetate esterase (NAE) of leaves of *Festuca pratensis* had an apparent MW of 55000. Five major NAE isoenzymes were resolved by gel electrophoresis. During leaf senescence the proportions of these isoenzymes altered and two novel isoenzymes became active. Cycloheximide applied to leaves delayed and diminished the responses of NAE isoenzymes during senescence. The two novel NAEs were similar in MW and substrate affinity to pre-existing NAEs. Partially-purified NAE had no cholinesterase, carboxypeptidase, ethyl acetate esterase or ethyl butyrate esterase activity. Lack of inhibition by eserine, PCMB and organophosphorus insecticide classified these enzymes as acetylesterases.

INTRODUCTION

Leaf senescence results from the systematic destruction of cell components including fraction 1 protein, chloroplast ribosomal RNA and many anabolic enzymes of the cytoplasm and the chloroplast [1–3]. At the same time the activities of a number of hydrolytic enzymes show marked increases or qualitative modification [4–6]. The total activity of α-naphthyl acetate esterase (NAE) tends to decline slowly, but the number of NAE isoenzymes resolved by polyacrylamide gel electrophoresis increases during the senescence of *Festuca* leaves [7]. This paper presents a characterization of *Festuca* leaf NAEs as a prelude to investigations into the mode of activation of novel forms of these enzymes in leaf senescence.

RESULTS

Levels of chlorophyll and total soluble protein fell steadily during the senescence of excised leaves of *Festuca*. At the same time NAE activity of crude leaf extracts declined initially and then increased slightly before decreasing further (Fig. 1). Gel electrophoresis of soluble protein from senescent leaves resolved at least 9 bands of NAE activity. Table 1 gives changes in the activities of the 7 major isoenzymes over a period of 5 days after excision. Individual NAEs are numbered in order of increasing electrophoretic mobility. Non-senescent leaves and leaves after 1 day of senescence contained 5 major NAE isoenzymes. The most active of these, NAE-2, became progressively less prominent from day 1 to day 4, increasing again to day 5. NAE-3 intensified up to day 4 before falling away sharply. NAE-4 and NAE-5 increased in activity to day 2, then declined before increasing again at day 5. The activity of NAE-1 changed little during the experiment. A further two isoenzymes appeared at day 2. NAE-6 followed NAE-4 and NAE-5 while NAE-7 maintained a steady level. The pattern of total esterase estimated from the sum of the activities of

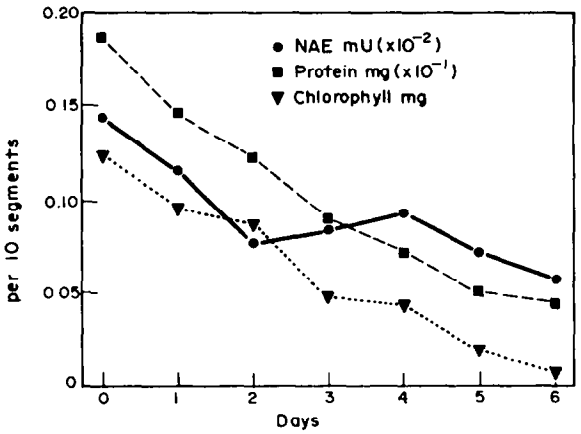

Fig. 1. NAE activity, protein and chlorophyll of excised leaf segments of *Festuca pratensis* senescing in the dark at 20°.

Table 1. Activities of NAE isoenzymes (arbitary units per 10 segments) of excised leaf segments senescing in the dark at 20°

Isoenzyme	Days after excision					
	0	1	2	3	4	5
NAE-1	5.8	9.9	10.5	7.2	17.5	13.2
NAE-2	56.6	60.5	28.0	24.0	13.0	40.4
NAE-3	43.0	44.6	64.6	38.5	24.3	51.1
NAE-4	33.0	51.7	63.7	53.0	31.4	76.5
NAE-5	28.6	32.5	50.4	59.4	60.6	31.2
NAE-6	0	0	72.9	57.8	47.3	67.8
NAE-7	0	0	17.5	23.2	23.4	21.0
Total NAE	167	199	308	263	217	301

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Table 2. Partial purification of NAE from non-senescent leaves of *Festuca pratensis*

	Volume (ml)	Total NAE (mU)	Specific activity (mU per mg protein)
Crude extract	49	210	4.1
45% ammonium sulphate	53.5	306	14.5
80% ammonium sulphate	5	45.5	193
Sephadex G-200 gel filtration	3	19.5	382
DEAE cellulose chromatography I	9	11.8	472
II	15	63.3	375

individual isoenzymes was inversely related to NAE estimated in crude extracts (Fig. 1).

NAE activity in this tissue was entirely soluble: no particulate NAE was detectable in leaf organelles fractionated by sucrose gradient centrifugation.

NAE was partially purified by ammonium sulphate precipitation, gel filtration and DEAE cellulose chromatography. Table 2 gives typical purification data for non-senescent leaves. Purifications of 95-fold maximum with yields of up to 36% were obtained. Isoenzymes of NAE were eluted from DEAE cellulose in order of increasing electrophoretic mobility, in two broad fractions (I and II). NAE from senescent leaves, when chromatographed on DEAE cellulose, gave a third fraction (III) containing the fast-running forms NAE-6 and NAE-7. Purified NAE had negligible cholinesterase and carboxypeptidase activity and did not hydrolyze ethyl acetate or ethyl butyrate.

The MW of NAE was determined by analytical gel filtration on Sephadex G-200 calibrated with standard proteins. There was no evidence of heterogeneity in MW: NAE eluted as a single peak of apparent MW 55000, within which there was no asymmetry of isoenzyme distribution. Qualitative alteration of isoenzyme pattern during senescence was not reflected in any shift in apparent MW.

K_ms of fractions I, II and III eluted from DEAE cellulose were determined for the substrate α-naphthyl acetate. Values of 1.80, 1.29 and 1.75 mM respectively were obtained.

Holmes and Masters [8] have described a classification scheme for animal esterases based on response to inhibitors. To determine whether different isoenzymes of NAE represented different classes of esterase, leaf extracts were electrophoresed and the gels incubated in 10^{-5} M eserine salicylate, 10^{-3} M PCMB or 10^{-4} M 'Malathion' (an organophosphorus insecticide) before being stained for NAE in the normal way. In no instance was there any qualitative or quantitative difference between the isoenzyme pattern of inhibitor-treated and control gels.

Table 3. Activities of NAE isoenzymes (arbitary units per 10 segments) of excised leaf segments treated with cycloheximide

Isoenzyme	Days after excision 1	2	3	4	5
NAE-1	6.3	6.6	2.2	3.6	0
NAE-2	47.0	36.9	47.5	15.0	5.4
NAE-3	28.6	18.5	39.9	21.6	4.7
NAE-4	40.5	31.5	39.2	26.4	17.0
NAE-5	39.2	39.0	38.3	39.9	22.3
NAE-6	0	0	0	44.0	32.3
NAE-7	0	0	0	6.0	0
NAE-8	0	0	0	3.6	11.2
NAE-9	0	0	0	1.4	0
Total NAE	162	133	167	162	93

Leaf senescence in grasses may be delayed by treating with inhibitors of cytoplasmic protein synthesis [3]. Cycloheximide (CH) at 10^{-3} M inhibits yellowing and hydrolase activity and delays protein breakdown in excised leaves of *Festuca* and *Lolium* [3,5]. Total NAE separated by gel electrophoresis altered little over the first 4 days of CH treatment (Table 3). Many of the changes in activity of individual isoenzymes occurring during senescence of control leaf tissue were delayed by one or two days and reduced in magnitude by CH. NAE-3, which increased to a maximum at day 2 in untreated leaves (Table 1) peaked at day 3 in CH-treated tissue (Table 3). Similarly, the sharp decline of NAE-2 over days 1 to 3 took place two days later in the presence of inhibitor. The appearance of NAE-6 and NAE-7 was delayed to day 4 and their activities greatly reduced. Two novel esterases became active in tissue exposed to CH for four and five days. One of these, NAE-8, which ran between NAE-6 and NAE-7, continued to increase after induction. The other, like NAE-7, appeared transiently at day 4.

DISCUSSION

Multiple forms of naphthyl esterase have been identified in many plant species, including tulip, cotton, pine, sugarcane, sweetcorn and barley [9–14]. In some instances [15,16] genetic studies have indicated a subunit structure for NAE, each subunit being controlled by up to 7 alleles. However, apart from observations of differences in NAE isoenzyme pattern between different tissues [17,18] there have been few attempts to relate variations in NAE isoenzymes to developmental events. Chen *et al.* [19] found an increase in the number and electrophoretic mobility of naphthyl butyrate esterases in *Xanthium* leaves of increasing age. In the present study the isoenzyme complement of *Festuca* leaf NAE was also observed to change with leaf age. The activation during senescence of novel NAEs of high electrophoretic mobility has been reported previously [7]. Their appearance is partially prevented by treatment with CH (Tables 1 and 3). In this respect they resemble other hydrolases that become active during leaf senescence [3]. It has been suggested that activation of catabolic enzymes in senescent leaves depends on the translation of stable messenger RNA present in the leaf prior to senescence [20]: CH-sensitive changes in NAE isoenzyme complement may be similarly controlled. This control may be exerted over turnover of NAE protein. It may also influence protein synthesis-dependent structural modification of these enzymes. MW heterogeneity was absent among NAE isoenzymes and this suggests that differences in electrophoretic mobility reflect charge differences. Variations in isoenzyme complement during senescence may be a result of changing interactions between differently-charged subunits, or modifications to the structure of existing esterases that cause relatively large changes in mobility without altering the MW sufficiently to allow resolution by gel filtration. It is well known that substitution of a single amino acid

may significantly alter the electrophoretic mobility of a polypeptide [21].

The *in vivo* function of NAE is unclear. Naphthyl esters are artificial substrates and are hydrolyzed by a number of wide-specificity hydrolases. Peptide hydrolases from germinated barley grains were found to hydrolyze naphthyl acetate [14]. Leaf peptidases with activities towards naphthylamide esters have been reported by Sopanen and Lauriére [22]. Other enzymes with naphthyl esterase activity include carbonic anhydrase, trypsin and cholinesterase. Partially purified *Festuca* leaf esterase was unable to hydrolyze choline esters, small aliphatic esters and the carboxypeptidase substrate benzoylarginine ethyl ester. The absence of response to eserine, PCMB and Malathion classified *Festuca* NAEs as acetylesterases in the scheme of Holmes and Masters [8]. However, plant esterases do not readily conform to schemes based on work with animal esterases [23]. Further studies on the properties of different plant esterases are needed before unequivocal classification is possible.

EXPERIMENTAL

Material. Meadow fescue (*Festuca pratensis* Huds. cv Aberystwyth Perdita) was grown under a 16 hr photoperiod at 20–25° to the 5- or 6-leaf stage. Youngest fully-expanded leaves were cut into 1 cm segments and allowed to senesce in darkness as described in refs [3, 7].

Methods. For chlorophyll, protein and enzyme determinations, 10 leaf segments were homogenized in 2 ml 50 mM Tris pH 7.5 containing 10 mM $MgSO_4$, 5 mM mercaptoethanol and 0.25 mM EDTA. The 12000 *g* pellet was used for chlorophyll measurement [24] and the supernatant for soluble protein and enzyme determinations. Protein was pptd with 10% TCA, solubilized in 0.1 N NaOH and determined by the procedure of ref. [25]. NAE activity was assayed by flourimetric measurement of the α-naphthol released in a reaction mixture consisting of 2.95 ml 0.1 M Pi buffer pH 7 containing 3.3×10^{-4} M α-naphthyl acetate, and 0.05 ml soluble protein extract. Fluorescence was measured in a Vitatron MPS instrument using an excitation wavelength of 330 nm and an emission wavelength of 460–470 nm. One unit of enzyme activity is equivalent to 1 μmol α-naphthol released per min. NAE isoenzymes were electrophoresed and visualized as described in ref. [26]. Gels were scanned at 480 nm in the MPS and individual isoenzymes quantified by determining the area of each peak of absorbance. NAE was partially purified following extraction of leaf tissue in 5 vols of 10 mM Pi buffer pH 7.5 containing 5 mM mercaptoethanol. NAE in the 20000 *g* supernatant was pptd between 45 and 80% $(NH_4)_2SO_4$ and redissolved in buffer. After gel filtration on Sephadex G-200 active fractions were pooled, pptd with 80% $(NH_4)_2SO_4$, redissolved in 10 mM Pi buffer and dialyzed against 100 vols of buffer for 18 hr. The extract was applied to a column of DEAE-cellulose (Whatman DE-52) and eluted with a linear gradient of 10–300 mM Pi pH 7.5. The MW of NAE was determined by gel filtration on Sephadex G-200 [27] calibrated with leaf fraction 1 protein, lactic dehydrogenase, glutamate pyruvate transaminase, hexokinase, haemoglobin and pancreatic ribonuclease.

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