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## PROTEOLYTIC ACTIVATION OF A LIPOLYTIC ENZYME ACTIVITY IN POTATO LEAVES

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(Received September 23rd, 1987)

(Revision received November 22nd, 1987)

(Accepted December 30th, 1987)

Potato (*Solanum tuberosum* L.) leaves were shown to contain a lipolytic enzyme activity which is stimulated by treatment with purified trypsin, pronase, and to a lesser degree by chymotrypsin. This protease-stimulated activity was stable over a wide range of pH values. Lipolytic enzyme activity also appeared to be regulated by pH, with a pronounced stimulation at pH  $6.0 \pm 0.5$  and a subsequent inactivation at pH 8.0–9.0. This pH stimulation was slightly enhanced by ethylene diamine tetracetic acid (EDTA), and was inhibited by  $\text{Ca}^{2+}$ . Although leupeptin slightly inhibited the pH stimulation, two other protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor showed no effect. While some of the lipolytic enzyme activities in potato leaves (those detected by 1-acyl-2-[6-[(7-nitro-2,1,3 benzoxadiazol-4-yl) amino]-caproyl] phosphatidylcholine ( $\text{C}_6$ -NBD-PC) hydrolysis) are stimulated by protease or pH treatment, others (those detected by 4-methylumbelliferyl laurate (4MUL) hydrolysis) are inactivated by them. The possible physiological significance of this apparent proteolytic activation is discussed.

*Key words:* *Solanum tuberosum*; phospholipase; proteolytic activation; protease; regulation; phospholipids.

### Introduction

Previous studies from our laboratory [1] and others [2,3] have demonstrated that potato leaves contain high levels of lipolytic enzyme activities. One or more of these enzyme activities appeared to be regulated by calmodulin and by protein phosphorylation and dephosphorylation [4]. In addition, we recently reported that trypsin treatment also stimulated the rate of hydrolysis of  $\text{C}_6$ -NBD-PC in potato leaf extracts [5]. In this study, the proteolytic activation of a lipolytic enzyme was studied in more detail, and other investigations were carried out to determine if this enzyme activity is regulated by endogenous proteases.

### Materials and methods

#### Materials

Seed potato tubers (*Solanum tuberosum* L. cv. Kennebec or Russett Burbank) were planted

in 15-cm clay pots in commercial potting soil and watered three times/week with complete nutrient solution [6]. Plants were grown at 20°C under continuous illumination (20–25 W m<sup>-2</sup>) provided by fluorescent growth lights (Philips-Westinghouse Corp., 40 W Agro/Lites). Trypsin, chymotrypsin, pronase, leupeptin, and soybean trypsin inhibitor were obtained from Sigma.  $\text{C}_6$ -NBD-PC was obtained from Avanti Polar Lipids, Birmingham, AL, U.S.A. All other reagents were of the highest purity commercially available.

#### Preparation of soluble extracts containing lipolytic enzymes

Potato leaves (5 g of leaves 1–2 cm in length from 4–5-week-old plants) were rinsed with distilled water and homogenized in a chilled mortar and pestle with 20 ml of a solution containing 0.3 M sucrose, 0.1 M *N*-2-hydroxy-ethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 5 mM dithiothreitol, and 5 mM

$\beta$ -mercaptoethanol. The homogenate was filtered through two layers of cheesecloth and centrifuged at  $100\,000 \times g$  for 50 min. The supernatant fractions were used as the source of enzyme. All procedures were conducted at  $0-4^{\circ}\text{C}$ .

#### *Time-course studies*

For studies of the effect of proteases on the enzyme activity 0.2 ml of enzyme preparation was placed in a test tube ( $12 \times 75$  mm) and shaken at 120 rev./min in a water bath at  $25^{\circ}\text{C}$ . The commercial proteases ( $10\ \mu\text{l}$ ) were added at various times providing concentrations of 10 to  $100\ \mu\text{g}/0.2$  ml in the reaction mixture. When studying the effect of pH on enzyme activity 4 ml of enzyme preparation was placed in a small beaker and the pH was monitored constantly with an Orion model 701A pH meter equipped with a microelectrode. The pH was adjusted with 6 N HCl or 6 N NaOH. In both types of time-course experiments phospholipase activity was measured at 10-min intervals by removing small samples ( $10\ \mu\text{l}$ ) and assaying as described below.

#### *Enzyme assays*

Phospholipase activity was assayed using  $\text{C}_6$ -NBD-PC as a substrate by a technique which we recently described in detail [4]. The reaction mixture (2 ml) contained  $5\ \mu\text{M}$   $\text{C}_6$ -NBD-PC, 50 mM HEPES buffer (pH 7.0), and 10–20  $\mu\text{l}$  of enzyme sample. Relative fluorescence was measured continuously with a Sequoia-Turner Model 450 Fluorometer equipped with an excitation filter ( $460 \pm 5$  nm) and an emission filter ( $> 535$  nm). Esterase activity was measured using 4-methylumbelliferyl laurate (4MUL) as a substrate as previously described [6]. The reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 8.0), 40  $\mu\text{l}$  of 20 mM 4MUL in ethylene glycol monomethyl ether, and 1–2  $\mu\text{l}$  of enzyme sample. The fluorometer was equipped with an excitation filter ( $360 \pm 5$  nm) and an emission filter ( $> 415$  nm).

#### *Effect of trypsin on the rate of autolysis of phosphatidylcholine*

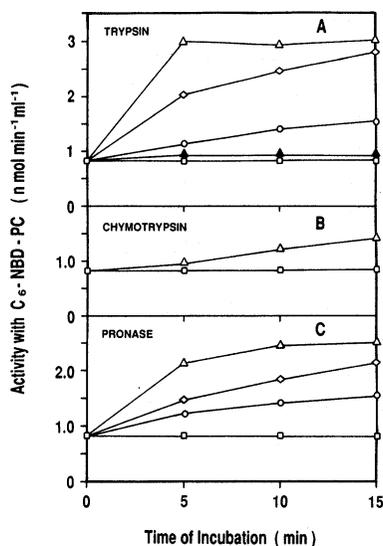
The effect of various treatments on the rate

of autolysis of phosphatidylcholine in leaf homogenates was measured as described previously [1,7]. A leaf homogenate was prepared and filtered as described above and divided into 4-ml aliquots. Trypsin (200  $\mu\text{l}$  of a solution containing 10 mg/ml stock) was added to two of the aliquots and they were incubated along with controls lacking trypsin at  $4^{\circ}\text{C}$  or  $25^{\circ}\text{C}$ . After 4 h or 0.5 h, respectively, the reactions were stopped by adding acetic acid, the lipids were extracted and separated by thin layer chromatography (TLC), and phosphatidylcholine was measured as previously described [1].

## Results

In the previous report the addition of 25  $\mu\text{g}$  of trypsin to a 1.1-ml enzyme mixture caused a 3-fold stimulation of the rate of hydrolysis of  $\text{C}_6$ -NBD-PC in the  $100\,000 \times g$  supernatant fraction of potato leaves [5]. In the current study three concentrations of trypsin were compared for their ability to stimulate  $\text{C}_6$ -NBD-PC phospholipase activity in a 0.2-ml enzyme mixture (Fig. 1A). The highest concentration, 100  $\mu\text{g}$ , caused approximately a 4-fold stimulation after 5 min. This elevated level of activity was maintained for the remainder of the experiment. This activation was prevented by adding a 2-fold weight excess of soybean trypsin inhibitor. Treatment of the enzyme mixture with chymotrypsin (also 100  $\mu\text{g}$ ) caused a much slower stimulation (Fig. 1B) of activity, similar to that caused by 10  $\mu\text{g}$  trypsin (Fig. 1A). Pronase treatment of the enzyme (Fig. 1C) caused a degree of stimulation that was slightly less than that with trypsin, but significantly higher than with chymotrypsin. Control experiments revealed that the three purified proteases were not contaminated with lipolytic enzyme activity (data not shown). Other experiments showed that the protease-stimulated activity was stable over the pH range of 5–9 (data not shown).

In the next experiment the effect of trypsin on the rate of autolysis of phosphatidylcholine (PC) in potato leaf homogenates was measured (Table I). Previously,  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -calmodulin were shown to stimulate the rate of autolytic



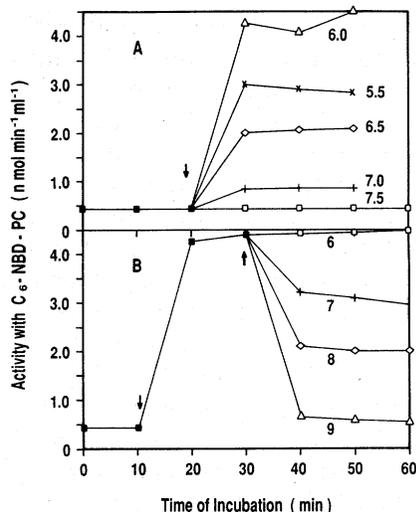
**Fig. 1.** Effect of protease treatment of the  $100\,000 \times g$  supernatant of potato leaves on subsequently-measured phospholipase ( $C_6$ -NBD-PC) activity. For each treatment the protease was added immediately after the initial (0 time) sample was removed. (A) Trypsin present in 0.2 ml: 0  $\mu g$  ( $\square$ ), 10  $\mu g$  ( $\circ$ ), 30  $\mu g$  ( $\diamond$ ), and 100  $\mu g$  ( $\Delta$ ); 100  $\mu g$  trypsin with 200  $\mu g$  soybean trypsin inhibitor per 0.2 ml ( $\blacktriangle$ ). (B) Chymotrypsin concentration: same symbols as in A. (C) Pronase concentration: same symbols as in A.

breakdown of PC by approximately 45% and 80%, respectively [1]. In this experiment trypsin stimulated this autolytic rate by approximately 20% at 4°C and 30% at 25°C. This experiment demonstrates that trypsin not only stimulated the rate of hydrolysis of an artificial substrate,  $C_6$ -NBD-PC, but it also stimulated the rate of hydrolysis of a natural membrane component, PC.

**Table I.** Effect of trypsin on the rate of hydrolysis of endogenous phosphatidylcholine (PC) in potato leaf homogenates during incubations at two different temperatures.

Treatment	% of original PC hydrolyzed during	
	4 h at 4°C	0.5 h at 25°C
Control	30.0 <sup>a</sup> ± 2.4	41.2 ± 1.7
Trypsin-treated (0.5 mg/ml)	35.8 ± 1.1	53.7 ± 3.2

<sup>a</sup>Data are means of at least 4 observations ± S.D. 100% represents 162 nmol PC/ml of homogenate.



**Fig. 2.** Effect of pH on phospholipase ( $C_6$ -NBD-PC) activity in the  $100\,000 \times g$  supernatant fractions of potato leaves. (A) Effect of various pH treatments on enzyme activity. Initial pH was 7.5 ( $\blacksquare$ ), and in the control sample the pH was maintained at 7.5 ( $\square$ ). In various treatments HCl was added (at arrow) to shift the pH to 7.0, 6.5, 6.0, and 5.5 (as indicated in the figure). (B) After an initial increase in enzyme activity by shifting the pH to 6.0 (first arrow), the pH was then either maintained at 6.0 as a control ( $\square$ ), or adjusted (second arrow) to 7.0, 8.0, or 9.0 (as indicated in the figure). For each of these pH treatments the original volume of  $100\,000 \times g$  supernatant was 4.0 ml and the pH was adjusted with 6 N HCl or 6 N NaOH. The enzyme activity was calculated after correcting for the small change in volume caused by HCl or NaOH.

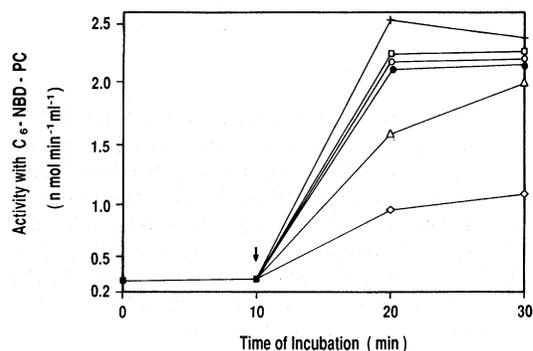
While conducting the above experiments it was observed that the enzyme activity also appeared to be modulated by pH. The next experiment (Fig. 2) was designed to verify this observation. Although the enzyme activity (always measured at pH 7.0) was very stable at pH 7.5 (which coincidentally was the pH of the

homogenization buffer) it was significantly stimulated by treatment at lower pH values (Fig. 2A). By adjusting the pH of the enzyme preparation to 7.0, 6.5, 6.0, and 5.5, the activity was stimulated approximately 2-, 4-, 10-, and 6-fold, respectively. After the pH shift, the degree of stimulation which was observed after 10 min was nearly unchanged at the 20- and 30-min samplings. Other time-course experiments revealed that the stimulation induced by a pH shift occurred within the first 2–3 min (data not shown). In the next experiment (Fig. 2B) phospholipase activity was first stimulated by dropping the pH to 6.0 (first arrow) and the effects of a second pH shift (second arrow) on the activity were investigated. Although the induced activity was stable at pH 6.0, it was lowered by approximately 30% at pH 7.0, and 60% at pH 8.0. When the induced activity was shifted to pH 9.0 the phospholipase activity was reduced to a level which was approximately equal to that of the original enzyme sample (before pH 6.0 stimulation). Other experiments revealed that an enzyme sample that was cycled through one pH 6.0-induced stimulation and one pH 9.0-induced lowering of activity could not be restimulated by a second pH 6.0 shift (data not shown).

Because the level of phospholipase activity appeared to be sensitive to pH, the effect of the pH of the homogenization medium on the levels of phospholipase activity in the resulting  $100\,000 \times g$  supernatant fractions was determined (Table II). Leaves of two varieties of potato plants were either homogenized in normal homogenization medium (pH 7.5) or homogenization medium that contained all of the nor-

mal ingredients except buffer. Without buffer, the final pH of the homogenate was  $6.0 \pm 0.1$ . In the supernatant fractions from both cultivars the enzyme activity in the pH 6 sample was approximately 3-fold higher than in the pH 7.5 sample. The results of this experiment (Table II) are consistent with those observed in Fig 2A, although the levels of stimulation are somewhat lower.

In order to determine whether the pH 6-induced stimulation could be attributed to the presence of endogenous acid protease(s) the effect of protease inhibitors was investigated (Fig. 3). In the control treatment the pH was



**Fig. 3.** Effect of various compounds on the stimulation of phospholipase ( $C_6$ -NBD-PC) activity induced by a shift to pH 6.0. The control sample (■) was incubated at pH 7.5 for 10 min and then the pH was adjusted to 6.0 (□) (arrow). For the other treatments the following compounds (final concentrations) were added to the  $100\,000 \times g$  supernatant fraction (0.5 ml) at the beginning of the experiment and the pH was similarly shifted to 6.0 after 10 min (arrow): 0.20 mM leupeptin (△), 1.0 mM PMSF (○), 100  $\mu$ g/0.5 ml soybean trypsin inhibitor (●), 5 mM  $CaCl_2$  (◇), and 5 mM EDTA (+). The enzyme activity was calculated after correcting for the small change in volume caused by addition of each compound tested.

**Table II.** Effect of pH of the homogenization buffer on the activity of  $C_6$ -NBD-PC phospholipase activity in the resulting  $100\,000 \times g$  supernatant fractions.

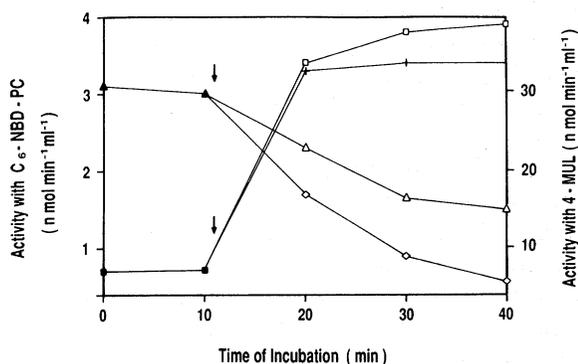
Cultivar	pH of homogenate	Enzyme activity (nmol min <sup>-1</sup> ml <sup>-1</sup> )
Kennebec	6.0	2.35
	7.5	0.69
Russett Burbank	6.0	1.32
	7.5	0.48

adjusted to 6.0 after 10 min, resulting in an approximately 9-fold stimulation of enzyme activity. The addition of either PMSF or soybean trypsin inhibitor had no effect on the stimulation. However leupeptin, a tripeptide inhibitor of many plant proteases [8], significantly inhibited the pH effect. This experiment indicates that an endogenous leupeptin-sensitive acid protease appears to be responsible for at least some of the stimulation induced by the pH 6 shift. The pH-induced stimulation was also slightly enhanced by EDTA and was severely inhibited by  $\text{Ca}^{2+}$  (Fig. 3). In contrast, the presence of 5 mM  $\text{Mg}^{2+}$  had no effect on the pH-induced stimulation (data not shown).

We have previously reported [7] that in addition to catalysing high rates of hydrolysis of natural phospholipids and  $\text{C}_6$ -NBD-PC, potato leaves also contain an enzyme activity which hydrolyzes another synthetic esterase sub-

strate, 4-methylumbelliferyl laurate (4MUL). The next experiment (Fig. 4) was designed to investigate whether the rate of hydrolysis of 4MUL is proteolytically activated in a manner similar to that observed with  $\text{C}_6$ -NBD-PC. This experiment revealed that instead of stimulating the rate of hydrolysis of 4MUL, trypsin rapidly inactivated the enzyme activity. Whereas trypsin caused approximately a 5-fold stimulation of phospholipase ( $\text{C}_6$ -NBD-PC) activity during the 40-min time-course, it inhibited esterase (4MUL) activity by approximately 80% during the same time period. When the enzyme sample was adjusted to pH 6.0 the rate of hydrolysis of  $\text{C}_6$ -NBD-PC was also stimulated approximately 5-fold and the rate of hydrolysis of 4MUL was inhibited by approximately 50%.

There are at least two possible explanations of these results. If both activities were catalyzed by one enzyme, then trypsin changed the substrate specificity of the enzyme. However if the two activities are catalyzed by separate enzymes, then one was stimulated by the protease and one was inactivated. The next experiment was designed to determine which explanation is correct. When the  $100\,000 \times g$  supernatant fraction was further fractionated by ammonium sulfate precipitation (Table III), most of the esterase (4MUL) activity (94%) was found in the 0–45% precipitate, and its activity was inhibited several-fold by trypsin. Although the phospholipase ( $\text{C}_6$ -NBD-PC) activity was distributed nearly equally in the three ammonium sulfate fractions, most of the trypsin-stimulated  $\text{C}_6$ -NBD-PCase activity (67%) was found in the 55–90% precipitate. This experiment demonstrates that potato leaves contain at least two lipolytic enzymes. One prefers 4MUL, is precipitated by 45% ammonium sulfate, and is inhibited by trypsin. The other hydrolyzes  $\text{C}_6$ -NBD-PC, is precipitated in the 55–90% ammonium sulfate fraction, and is stimulated by trypsin. Our laboratory is in the process of further purifying these two enzymes and determining their specificity towards natural lipid substrates.



**Fig. 4.** A comparative study of the effect of trypsin treatment and shift to pH 6.0 on the rates of hydrolysis of  $\text{C}_6$ -NBD-PC and 4MUL in a  $100\,000 \times g$  supernatant fraction from potato leaves. The initial rate of hydrolysis of  $\text{C}_6$ -NBD-PC was measured (■), and then after 10 min (arrow) either trypsin ( $500\ \mu\text{g/ml}$ , final concentration) was added (□), or the pH was shifted to 6.0 (+). The initial rate of hydrolysis of 4MUL was measured (▲), and then after 10 min (arrow) either trypsin ( $500\ \mu\text{g/ml}$ ) was added (◇), or the pH was shifted to 6.0 (△).

**Table III.** Separation of lipolytic enzyme activities by ammonium sulfate precipitation. The void volume of the Sephadex G-50 column was subjected to sequential ammonium sulfate precipitation.

Fraction	Protein (mg)	Substrate (Enzyme activity)			
		C <sub>6</sub> -NBD-PC(nmol min <sup>-1</sup> ml <sup>-1</sup> )		4MUL(μmol min <sup>-1</sup> ml <sup>-1</sup> )	
		-trypsin	+trypsin	-trypsin	+trypsin
100 K supernatant	84	21.4	96.3	487	195
Gel Filtration (G-50 void vol)	75	18.7	106.6	615	246
Ammonium sulfate:					
0–45% ppt	26	8.0	12.8	718	122
45–55% ppt	28	4.16	17.0	40	12
55–90% ppt	8.0	7.84	61.9	2.8	1.7

## Discussion

Many enzymes from mammalian sources have been shown to be stimulated by proteolytic cleavage. However, previous to this report, only two other plant enzymes have been reported to be activated in a similar manner: isofloridoside-phosphate synthase (IFP synthase) from a golden brown alga [9], and glucan synthase from soybean suspension cells [10]. In addition to being stimulated by proteolytic activation, IFP synthase was also shown to be stimulated by calmodulin, by protein phosphorylation [11], and by a shift to pH 6.0 [9]. The lipolytic enzyme activity discussed in this paper and IFP synthase both appear to be regulated by the same four mechanisms: calmodulin, protein phosphorylation, proteases, and pH.

A likely explanation for the stimulation of phospholipase activity at pH 6.0 is activation of the lipase caused by the presence of endogenous protease(s). The degree of stimulation caused by trypsin treatment and pH 6.0 treatment were comparable. The lowering of the rate of pH 6.0-stimulation by the protease inhibitor, leupeptin (Fig. 3), is probably the most convincing evidence for stimulation by endogenous proteases. Although the trypsin-stimulated activity was stable at pH 5–9, the pH 6.0-stimulated activity was rapidly inactivated at pH 8–9 (Fig. 2B).

This could either be explained by the presence of a second endogenous protease with an alkaline pH optimum, or by instability of the pH-activated form of the enzyme at higher pH values.

The proteolytic activation of an analogous mammalian enzyme, pancreatic phospholipase A<sub>2</sub>, has been well documented [12]. The enzyme is secreted as an inactive zymogen and is activated by the proteolytic removal of a heptapeptide from the amino terminal end. Other enzymes like the Ca<sup>2+</sup>-ATPase from erythrocytes are stimulated several-fold by both proteolytic enzymes and by calmodulin but are not synthesized as an inactive zymogen [13]. Further studies are now underway in our laboratory to purify the zymogen of this lipolytic plant enzyme and determine the exact mechanism of proteolytic activation by trypsin and by endogenous proteases. Our preliminary studies have led to the partial purification of a zymogen of about 100 kDa which is stimulated by trypsin treatment but not by pH treatment.

Further work is required to determine the physiological role and subcellular localization of this highly regulated lipolytic enzymatic activity in leaves. The increase in membrane degradation during senescence and infection may involve proteolytic activation of lipolytic enzymes. During senescence proteases have

been shown to catalyze the rapid breakdown of many proteins, especially ribulose biphosphate carboxylase [14]. It is conceivable that one of these proteases may also activate the lipolytic enzyme(s) that we have identified. Indeed, one of the other processes which occurs during senescence is the rapid breakdown of membrane phospholipids and galactolipids [15]. A proteolytically-activated lipolytic enzyme could easily explain these phenomena. Several endogenous proteases have been identified in potato leaves [16]. During the infection of plants by fungal pathogens there is a similar destruction of membrane lipids [15]. A proteolytically-activated lipolytic enzyme may also be involved in this process.

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