Phytochemistry, 1976, Vol. 15, pp. 1471–1474. Pergamon Press. Printed in England.

PURIFICATION AND PHYSIOLOGICAL PROPERTIES OF TWO LIPOLYTIC ENZYMES OF *SOLANUM TUBEROSUM*

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(*Revised received* 3 *April* 1976)

Key Word Index—*Solanum tuberosum* cv. Majestic, Solanaceae; potatoes; phospholipase; acyl hydrolase; purification; lysosomes; acid phosphatase; disease.

Abstract—Two lipolytic enzymes have been separated and partially purified from potato tubers. One enzyme of higher isoelectric value, possessed acyl hydrolase activity toward a number of *p*-nitrophenyl fatty acyl derivatives, the relative activity depending on the fatty acyl chain length. There was also some activity towards phosphatidyl choline. The other enzyme possessed phospholipase and galactolipase activity, but showed a low acyl hydrolase activity towards *p*-nitrophenyl fatty acyl derivatives. When applied to plant tissues, the enzyme with the greater acyl hydrolase activity caused rapid ion efflux from discs of potato tuber and beetroot, followed by reabsorption of ions by the tissues. The purified phospholipase did not produce this effect but induced acid phosphatase leakage from lysosome-enriched fractions of potato sprout tissue. No maceration of tissue or protoplast disruption was observed when either of the two enzymes were incubated with a variety of plant preparations.

INTRODUCTION

It has previously been found [1] that homogenisation of potato tuber tissue resulted in a rapid breakdown of endogenous lipid materials, due partially to enzymic action, including phospholipid acyl hydrolases. Following attempts to purify the enzyme complex [2] it was concluded that several activities were ascribable to a single enzyme. In a continuation of this work [3] it was further concluded that under appropriate conditions, the potato tuber enzyme, acting upon endogenous substrates, could be described as a phospholipase, a galactolipase or an acyl transferase/hydrolase. Other workers [4], using a different variety of potato, have isolated a lipolytic enzyme possessing both galactolipase and phospholipase activity. However, on modification of the purification procedure in the present study, it was possible to separate a further component of acyl hydrolase activity from that of the phospholipase and galactolipase activities. In addition, the two fractions were capable of different physiological effects when applied to various plant tissues and organelles.

RESULTS

In an initial experiment, a crude potato supernatant extract, taken after the homogenisation, filtration and centrifugation stages of purification, was subjected to isoelectric focussing. The major peak of acyl hydrolase was not separated from the phospholipase and it was only after the additional column chromatography stages, followed by re-electrofocussing of the purified extract, that it was possible to separate the enzymes (Fig. 1). A residual amount of acyl hydrolase activity always remained with the phospholipase. The mean pI values (from four similar purification procedures) for each activity were phospholipase, 4.24 ± 0.35 and acyl hydrolase,

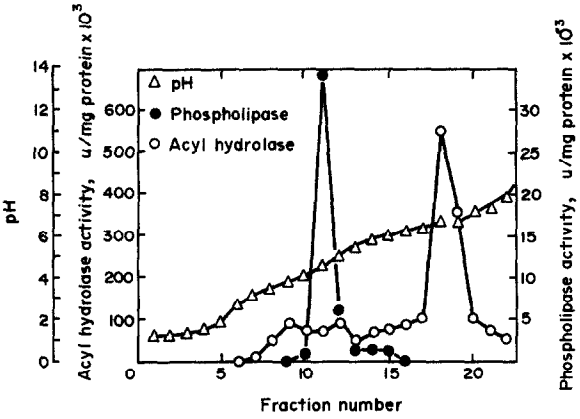

Fig. 1. Isoelectric separation of phospholipase from acyl hydrolase. See text for details.

7.29 ± 1.40. In addition, a hitherto undetected form of activity was found on lecithin plates in the form of clear as opposed to opaque haloes. These developed after prolonged incubation in the region of the major acyl hydrolase activity.

Whilst separation of phospholipase from a portion of acyl hydrolase was achieved by electrofocussing, the sp. act. was reduced. No pectolytic or polygalacturonate-splitting activity could be detected in either of the enzyme fractions.

Various properties of the two enzymes, when tested using different types of assay at two pH values, are summarised in Table 1. These results showed that whilst the phospholipase was of either type 'A' or 'B', adjudged by its acyl esterase activity with lecithin as substrate, the acyl hydrolase had neither of these two activities. It is noteworthy that the acyl hydrolase did, as stated

Table 1. Relative properties of acyl hydrolase and phospholipase. Assays were done in citrate buffer 0.05 M for pH 5 and Pi buffer 0.1 M for pH 7. The phospholipase (PLA) was taken as that enzyme with pI 4.24 and the acyl hydrolase (AH) as that enzyme with pI 7.29.

Assay procedure	Enzyme	Enzyme activity, units/ml pH 5.0	pH 7.0
Hydrolysis of *p*-nitrophenyl palmitate	PLA	816	1070
	AH	60	2090
Cup-plate (Lecithin substrate)	PLA	3470	1510
	AH	1260*	363*
Acyl esterase (Lecithin substrate)	PLA	1.22	0.083
	AH	0.000	0.000

* Clear haloes.

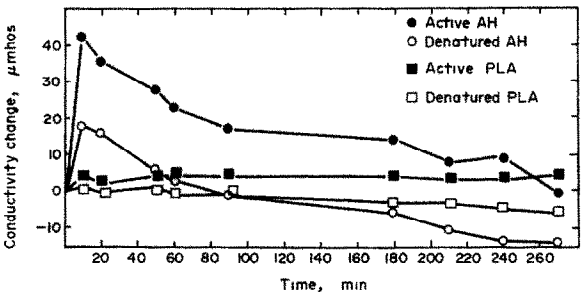

Fig. 2. Electrolyte release from washed beetroot discs incubated with acyl hydrolase (AH) or phospholipase (PLA). Conductivities of solutions before disc introduction were subtracted from subsequent values following addition of discs. Conductivity readings were further corrected against a control incubation containing discs in distilled water alone, the conductivity of which increased from 6 to 22 μmohs/hr over the time course studied. See text for further details.

above, have some reaction with lecithin in the cup–plate assays, although clear as opposed to the normal opaque haloes were produced. In addition, the time taken for clear haloes to develop was longer than for the opaque type. TLC failed to identify reaction products when the phospholipase was incubated with a variety of chromatographically homogeneous lipids and characterisation of the specificity of the enzyme beyond that of type 'A' or 'B' was not achieved. However, when 0.1 ml (0.33 mg) of digalactosyldiglyceride (DGDG) was incubated separately with equal volumes of the two enzymes for up to 5 hr at 30°, and the end-products of incubation subjected to TLC analysis, it was noted that the DGDG spot of Rf 0.42 was always absent from the phospholipase enzyme incubation, but not from the acyl hydrolase treatment. Thus the phospholipase was assumed to possess galactolipase activity. Hydrolysis of *p*-nitrophenyl palmitate occurred more rapidly at pH 7 than at pH 5 for both of the enzymes whilst lecithin digestion, by whatever mechanism, occurred more rapidly at pH 5 than pH 7.

The specificity of the acyl hydrolase enzyme was tested by comparing the activity with a variety of *p*-nitrophenyl fatty acyl derivatives of increasing acyl chain length. The following *p*-nitrophenyl fatty acid acyl derivatives were used with the activities (units/ml) in brackets: acetate (924), laurate (1230), myristate (4230), palmitate (14600) and stearate (464). The results indicated that the activity increased with fatty acid chain length up to that of palmitate (C_{16}).

The effects of the two enzymes on electrolyte release from plant tissue discs was tested after it had been confirmed that neither of the extracts contained pectolytic activity.

Six beetroot discs each (6.5 mm diameter), washed 18 hr in running tap H_2O, were introduced into media containing 9 ml of freshly dist H_2O together with 1 ml of reconstituted freeze-dried, purified phospholipase or acyl hydrolase. The enzymes had been previously dialysed against dist H_2O to reduce the conductivity of the solutions. Heat denatured enzyme controls were included, together with controls containing discs in dist H_2O alone. The conductivities of the incubation media were measured immediately before and after disc introduction and at regular intervals up to 280 min. The active acyl hydrolase caused a rapid electrolyte release from beetroot discs into the medium (Fig. 2) followed by a steady decrease of conductivity, presumably as a result of reabsorption of ions. The boiled acyl hydrolase (10 min) had a similar but much reduced effect whilst the phospholipase-treated discs showed no tendency toward electrolyte release greater than that observed in the controls containing dist H_2O only. When a similar experiment was done with washed potato tuber discs, acyl hydrolase again caused the greatest amount of electrolyte release.

No leakage of betacyanin from beetroot was found during treatment of discs with either of the enzyme preparations, nor was there any detectable leakage of nucleotides [5] from potato tuber tissue treated similarly. There was no evidence in these experiments of tissue maceration caused by either of the enzymes.

Cucumber protoplasts, viewed under the phase contrast microscope, were irrigated with either of the two potato enzymes or with commercial preparations of phospholipases 'A', 'C', or 'D'. There was no detectable sign of damage however, in any treatment except the commercial phospholipase 'A', which caused protoplasts to burst.

When lysosome-enriched extracts from potato sprouts were incubated for 30 min at 37° with the active phos-

Table 2. Release of acid phosphatase from potato sprout lysosomes incubated with purified phospholipase. Acid phosphatase was assayed in the supernatants of mixtures incubated for 30 min and containing equal volumes of fresh lysosome-enriched extract (pH 7.1) with isoelectrically focussed active or heat denatured (boiled for 1.5 hr) potato phospholipase. The nett release of acid phosphatase was calculated with reference to a control incubation containing dist H_2O instead of phospholipase. Incubations were at 37°

Treatment	Acid phosphatase activity in supernatant Units/ml Zero time	30 min	Activity increase
Water	8570	4970	(−) 3560
Denatured enzyme	8080	4810	(−) 3270
Active enzyme	8700	15400	6710
Nett water-control corrected increases			
Denatured enzyme			290
Active enzyme			11300

pholipase preparation, an increase of supernatant acid phosphatase could be demonstrated after this period (Table 2). This indicated that the enzyme was active in causing solubilisation of enzymes from lysosomes.

DISCUSSION

It has previously been stated [3] that under appropriate conditions, a lipolytic enzyme from potato tubers displays phospholipase, galactolipase and acyl transferase/hydrolase activity, and that these activities cannot be separated from one another. Other workers have also isolated a lipolytic enzyme from potato tubers possessing both galactolipase and phospholipase activities [4]. In initial experiments, we found, that acyl hydrolase and phospholipase activities could not be separated from each other in crude extracts owing to their closely corresponding pI values. However, following various column chromatographic techniques and further electrofocussing of the semi-purified extract, partial separation of these two activities was achieved. Phospholipase, containing galactolipase and residual acyl hydrolase activity, had a pI similar to that seen within initial crude extracts, but the major acyl hydrolase peak possessed a higher pI than previously encountered. Occasionally associated with the latter purified enzyme was a hitherto undetected form of phospholipase which produced clear as opposed to opaque haloes in cup–plate analysis. Because the acyl hydrolase enzyme could not remove acyl ester from a lecithin substrate, it was concluded that its phospholipase-like activity on cup–plates was of a different form to either 'A' or 'B' type specificity. Since the original phospholipase possessed acyl esterase activity with lecithin, it must be of either type 'A' or 'B'. A 'B' type phospholipase has been found in potato tubers of cv Benimaru [4].

It is difficult to state whether the purification procedures adopted here have separated different enzymes or whether structural modifications have resulted to portions of a single, non-specific enzyme, causing the generation of sub-units of altered isoelectric values and differing substrate specificities. The phospholipase/galactolipase enzyme of the present work appears to correspond to that found by other workers in a different variety of potatoes [4], except that the pI of our enzyme is lower.

The effects which the acyl hydrolase enzyme has on electrolyte leakage from washed beetroot and potato tuber discs indicates that it is active physiologically, although it is difficult to postulate its mode of action. Insofar as the discs were washed before use, the rapid electrolyte release upon incubation with the enzyme must originate from within the plasmalemmae and not the cell free space. However, it is possible that the slow subsequent conductivity decrease of the incubation medium results from a re-equilibration of released ions with this component of the tissue. The much smaller, continual efflux of ions from tissue discs incubated with dist H_2O only, is probably a measure of the loss of residual ions from the cell free space, this effect being masked by the much greater efflux in the acyl hydrolase incubation.

That the phospholipase is capable of liberating acid phosphatase from lysosome preparations from potato sprouts indicates that this enzyme is also capable of acting disruptively upon its own tissues when its cellular position is altered—in this case by extraction. These results agree, therefore, with the concept derived from previous work [1].

Although we have already provided evidence [6] that phospholipase of fungal origin can cause release of hydrolytic enzymes from lysosomes of higher-plants (*in vitro*), the present results suggest a further mechanism whereby cell disorganisation could occur in disease. Thus, phospholipases from the host or the parasite may both be responsible for the release of lysosomal enzymes which occurs in some diseased tissues [7–9]. Such an action, however, does not exclude the possibility that other substances in the host–parasite complex may also cause disruption of cell compartments.

EXPERIMENTAL

Organisms. The source and storage of potato tubers (*Solanum tuberosum* cv Majestic) was as previously described [6]. Cucumbers and beetroots were purchased locally.

Enzyme assays (a) Acyl hydrolase was assayed by a method similar to that of ref. [2] using *p*-nitrophenol derivatives of acetate, laurate, myristate, palmitate and stearate as the substrates. The final conc of substrate in the reaction mixtures was 0.835 mM except in the case of acetate (see below). Solubilisation of the substrates within the aq. reaction mixtures was assisted by the inclusion of Triton X-100 (2 g/100 ml substrate). The reactions were carried out at pH 7 in Pi buffer (50 mM) and stopped, except in the case of the acetate substrate, by the addition of 0.1 M glycine–NaOH buffer pH 9.5. One unit of acyl hydrolase activity was defined as that amount of enzyme causing the release of 1 μmol of *p*-nitrophenol in 15 min at 35°. In the case of the acetate acyl hydrolase assay, the final conc of substrate in the reaction mixture was 0.66 μmol/ml and the reaction was carried out at pH 7.4. Release of *p*-nitrophenol was assayed after 15 min incubation without addition of pH 9.5 buffer to stop the reaction. In this case, zero time blanks were prepared and read directly before the incubation mixtures. The amount of *p*-nitrophenol liberated was calculated from a separate calibration curve for the latter in pH 7.4 buffer. Acyl hydrolase was routinely assayed using *p*-nitrophenyl palmitate as the substrate. (b) Phospholipase was routinely assayed using the method of ref. [10] modified as described in ref. [6]. In addition, the hydrolysis of lecithin was measured by the decrease in acyl ester content of the substrate (refined soyabean phosphatidyl choline Sigma) over a given time when a 1% aq. emulsion was incubated with enzyme extracts at pH 5 in 0.1 M citrate buffer or pH 7 in 0.05 M Pi buffer. The method of ref. [11] was employed. (c) Acid phosphatase was assayed by the method of ref. [12], suitable zero time controls being included. (d) Polygalacturonase was assayed by the methods of ref. [13] or ref. [14]. (e) Pectin methyl *trans*-eliminase was assayed by the method of ref. [14] and pectin methyl esterase by the method of ref. [15]. (f) Protein was measured by the method of ref. [16].

Enzyme purification. A method similar to that of ref. [2] was used with modifications to the initial stages. Enzyme activity was routinely assayed by the modified cup–plate technique and the acyl hydrolase method with *p*-nitrophenyl palmitate as the substrate. All extraction procedures were carried out at 4°. 1.5 kg of potato tubers were washed, peeled and diced before homogenisation with two vols of $NaHSO_3$ (2×10^{-3} M). The homogenate was left to ppt starch for 30 min after which it was partially clarified by passage through two layers of muslin. Supernatant was subjected to centrifugation at 2500 *g* for 30 min. Protein precipitation of the supernatant (2600 ml) was carried out with $(NH_4)_2SO_4$ and the fraction of protein precipitating between 30–70% saturation was retained. The fraction was solubilised in a min vol of 50 mM Pi buffer pH 7 and then dialysed against several changes of this buffer for 16 hr to remove $(NH_4)_2SO_4$. The extract was reduced in vol by *ca* 33% using polyethylene glycol

(MW = 1000), followed by further dialysis to re-equilibrate with 50 mM Pi buffer pH 7. A *ca* 5-fold purification factor with respect to phospholipase activity was achieved by this procedure. The extract was freeze-dried in order to reduce its vol for gel filtration. This was carried out using Sephadex G 150, eluting with 50 μM Pi buffer pH 7 (10 ml/1 hr). The majority of phospholipase and acyl hydrolase activities were eluted together, and these fractions were combined. The purification factors at this stage were: phospholipase, 20.8-fold; acyl hydrolase, 10.5-fold. The combined fractions from gel filtration (250 ml) were subjected to ion exchange chromatography with DEAE cellulose (column 30 cm × 1.5 m) using NaCl gradient (0–0.5 M), in 50 mM Pi buffer and 40 fractions were collected (6 ml). Acyl hydrolase and phospholipase eluted together at *ca* 0.25 M NaCl concentration. The extract could be stored (−20°) at this stage following lyophilisation with no loss of activity over many months. To separate acyl hydrolase from phospholipase, a portion of the lyophilised extract (12 ml) containing 0.03 mg ml protein was subjected to isoelectric focussing following dialysis against 50 mM Pi buffer pH 7 (2 changes) and glycine (1%) over 12 hr. Focusing was carried out between the pH range 3.5–10.0. The details of the procedure were as described in ref. [6]. Following electrofocussing (72 hr) and fractionation, acyl hydrolase and phospholipase activities were partially separated. The two peaks were collected separately, lyophilised and stored at −20° after dialysis against 50 mM Pi buffer pH 7. All subsequent experimental work was carried out on preparations reconstituted to their original vols in Pi buffer pH 7 unless otherwise stated. In the lyophilised state the enzymes were stable and have been stored for up to a year without any detectable loss of activity.

TLC was done using Si with $CHCl_3$–MeOH–HOAc–H_2O (34:6:4:1) ref. [1]. Plates were visualized either by exposure to I_2 vapour or by charring with 50% H_2SO_4 at 180°. Chromatographically homogeneous lipid markers were used. Digalactosyldiglyceride (10 mg/3 ml of C_6H_6–EtOH 4:1 containing 50 μg/ml BHT as anti-oxidant) was a gift from Dr. T. Galliard, ARC Food Research Institute, Norwich.

Conductivity measurements. These were carried out as described in ref. [6].

Protoplast preparation. The preparation of cucumber protoplasts was similar to that described in ref. [17] as modified in ref. [6]. Lysosomal preparations from potato sprout tissue were produced as described in ref. [6].

Acknowledgements—This work was supported by a grant from the Agricultural Research Council.

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