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LIPID-DEGRADING ENZYMES FROM POTATO TUBERS

DENNIS A. WARDALE

Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, NR4 7UA, U.K.

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Abstract—Three varieties of potato were used to investigate the activity of lipolytic acyl hydrolase, (LAH) and lipoxygenase, (LOX), for a short period after harvest. Both enzymes displayed very low levels of activity during the first few days, followed by an increase in later storage, with the hydrolase activity of Désirée tubers remaining low. An inverse relationship was found between the total LOX activity and the percentage of activity obtained in a particulate form. Only when the total LOX content was below 0.7 units (μmol/g/min fr. wt), was it possible to obtain a highly active particulate fraction. LAH particulate activity was dependent upon both enzymes remaining low. Protoplasts were isolated by the use of cell-degrading enzymes. When the total LOX activity in the tubers was low, 50% of this activity could be obtained in intact protoplasts. Once the LOX concentration in the tubers had risen, fewer intact protoplasts were isolated. No particulate activity of either enzyme was found when these protoplasts were lysed. The two lipid-degrading enzymes were not located in the amyloplasts.

INTRODUCTION

The first two steps in a sequence of lipid-degrading reactions in potato are caused by the action of a lipolytic acyl hydrolase (LAH) enzyme [1], which liberates free fatty acids from the endogenous membrane lipids and a lipoxygenase (LOX) enzyme [2], which converts linoleic acid and linolenic acid to their 9-hydroperoxide derivatives. The subcellular localization of these enzymes in potato tissue has been especially difficult, not only because of the degradative action of these enzymes on the membrane structure, but also because of the effect starch grains may have in rupturing the organelles. Previous work on potato shoots [3] located LAH activity in a lysosomal particle or small vacuole, but failed to achieve a high LOX particulate activity. The present work was undertaken to ascertain (1) whether there were any changes in enzyme activities immediately after harvesting and (2) whether protoplasts could be used as the first step in the localization of the enzymes. It was thought that organelles would be subject to less damage from starch grains when released from lysed protoplasts, than after the tissue had been macerated. It was also of interest to discover whether amyloplasts contained any lipid-degrading enzymes.

RESULTS

LAH activity

Previous work [4] revealed high LAH values for 23 varieties of potato at maturity, with the exception of Désirée. Enzyme changes during storage [5] were studied in 6 varieties, where consistent levels of LAH activity were maintained throughout their life cycle. The variation of LAH activity for 3 varieties, over a much shorter period after harvesting, is illustrated in Fig. 1. During the first week of storage both Désirée (DES) and Pentland Javelin (PJ) showed very low levels of activity, with Maris Peer (MP) 10 times higher. All varieties displayed an increase over the next few days; DES showed a slight increase, followed by a return to the previous low level, whereas more pronounced increases were observed with the other two varieties. This higher level was maintained, but the activity in the PJ tubers was always 10 times lower than in MP.

High LAH particulate activity was only possible when the total activity in the tubers was low. About 16% of total homogenate activity was recovered in a pellet form (38 000 g for 20 min), from both DES and PJ tubers, when the total activity was *ca* 0.025 units (μmol/g/min fr. wt) and negligible when the activity reached 0.06 units. This is in agreement with the inverse relationship between total activity and particulate reported in earlier work on potato shoots [6]. A further factor in the production of high particulate activity appeared to depend on the level of lipoxygenase (LOX) in the tissue. It was possible to achieve 16% particulate activity when the total LOX content of the DES tubers remained low, 0.33 units (μmol/g/min fr. wt). After a few weeks of storage, only 8% particulate activity was obtained from tubers where the total LAH content was unchanged, but where LOX had risen to 6 units. Previous work on the

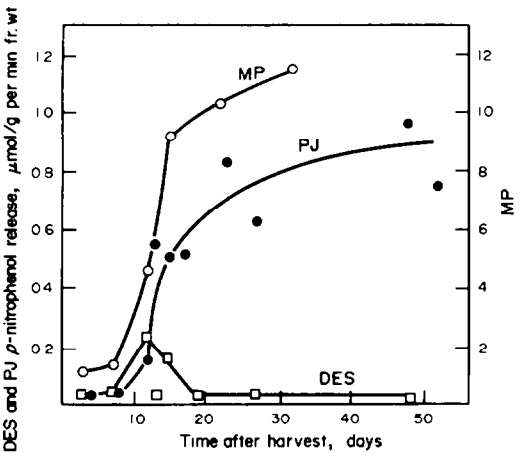

Fig. 1. Changes in lipolytic acyl hydrolase activity after storage at 7°. The varieties were Désirée (□–□), Pentland Javelin (●–●), and Maris Peer (○–○).

distribution of lipid-degrading enzymes in potato tubers [7] showed high LAH activity at the bud end and high LOX in the cortical tissues. In an attempt to obtain samples with a low LOX concentration, material from the bud end of tubers was macerated. Although the total LOX activity was lower, 3.4 units and the LAH activity 0.018 units, only 10% particulate activity was obtained. The addition of bovine serum albumin (BSA) (1%) to the buffer before maceration had no effect on the amount of LAH particulate collected.

High particulate recoveries had been achieved from potato shoots [6] where the LAH activity was below 0.01 units and the LOX from 2.5 to 3 units. This suggests that the effect of LOX on the isolation of an active LAH particulate fraction is more critical as the level of LAH in the tissue increases.

LOX activity

All 3 varieties showed very low LOX activities during the first few days after storage (Fig. 2), followed by a gradual increase with MP, and a sharp rise and gradual fall with DES and PJ tubers.

Previous work with potato tubers [6] and potato shoots [3] produced negligible LOX particulate recoveries. The present work showed that it was possible to obtain active LOX particulate from potato tubers, provided that the total activity was below 0.7 units. In experiments using all 3 varieties, over 40% particulate LOX activity was obtained when the total LOX remained at, or below, this level. In PJ and DES tubers the LAH activity was also low, but MP had a higher level (1.34 units) and it appeared that the concentration of LAH was not important. Potato sprouts, which had a total LOX content of 1 unit, gave only 6% particulate activity. This means that the level of LOX in the tissue is extremely critical in determining whether one obtains a particulate or soluble enzyme on disruption of the tissue.

It has been shown in potato tubers [2], that at an alkaline pH, there was very little LOX activity. Slices from stored DES tubers were therefore macerated with phosphate buffer, pH 8 and also after the slices had been evacuated with the alkaline buffer beforehand. No differences were observed in the total LOX or LAH activities between these and the previous results and only slight increases were noted in the amount of particulate activity recovered.

No resolution of the crude pellet was carried out, as the period available for obtaining an active LOX and LAH particulate fraction was very limited.

Protoplasts

Potato slices were incubated with the cell-degrading enzymes and the protoplasts collected by sedimentation as centifugation tended to rupture them. The protoplasts so obtained were of two types. The smaller type had homogeneous sized grains and the larger protoplasts, which were more numerous, had variable sized starch grains. After staining the protoplasts with neutral red it was noted that the large types were more easily ruptured. When the small protoplasts were lysed, cytoplasmic strands were released.

Initial work was carried out on stored DES tubers, where slices were incubated for 10 hr. No differences were found between the LAH or LOX activities in extracts produced after degradation or after maceration. The few intact protoplasts that were isolated contained *ca* 20% of the total LAH and LOX activities. After the addition of the pH 8 Tris–HCl buffer, the plasma membrane ruptured and little lipid-degrading enzyme activity was recovered in the released organelles. This confirmed the difficulties, mentioned earlier, in obtaining active LAH or LOX particulate from macerated tissue, which had a high LOX content.

It was thought that if the protoplasts were being lysed by high LOX activity, then the lipid-degrading enzymes may be present in the released organelles. However, when a degraded extract, after removel of intact protoplasts at 2 hr, was centrifuged at 38 000 g for 15 min, only slight (5%) LAH and LOX activities were found in the pellet. After 5 hr digestion, when further intact protoplasts were removed, no activity

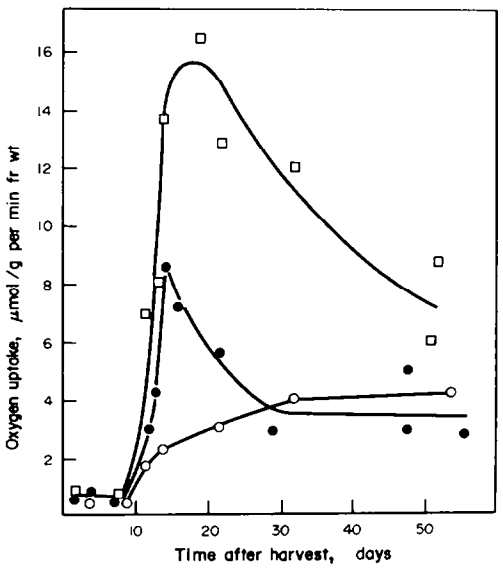

Fig. 2. Changes in lipoxygenase activity after storage at 7°. The varieties were Désirée (□–□); Pentland Javelin (●–●); and Maris Peer (○–○).

was present in the centrifuged particulate fraction. The addition of BSA (1%) to the degrading mixture did not increase the number of intact protoplasts.

Although the recovery of protoplasts was low from the stored DES tubers, a method was developed in which the slices were incubated with the cell-degrading enzymes in a funnel attached to a test-tube. The protoplasts floated through a 300 μm mesh nylon cloth and were collected at the bottom of the tube. Thus, no physical disruption of the tissue took place.

In order to prevent the destruction of the protoplasts, the short period of digestion was favoured, using freshly harvested PJ tubers, with a low LOX activity. After a 2 hr and a further 3 hr period, the material at the bottom of the tube was examined. Light microscopy examination showed that the first fraction contained a few intact protoplasts and a large number of starch grains, whereas the second contained a much higher proportion of protoplasts. Similarly, only 5% of the total LOX activity was associated with the fraction collected after a 2 hr digestion period, and over 50% (0.13 units) from the material deposited after a further 3 hr. It is known [8] that 40% of the endogenous membrane lipid is lost within minutes of cutting potato slices. Thus initially, during the degradation process, damaged protoplasts and organelles were being released from the cut potato surfaces.

It was therefore possible to obtain half the total enzyme activity in intact protoplasts from freshly harvested tubers. After 24 hr, only 25% of the activity remained, of which half was in a soluble form. In order to separate protoplasts from starch grains, a colloidal silica (Ludox) gradient [9] was used. After a 4 hr digestion period, the filtered extract was added to the top of a discontinous gradient and the protoplasts and starch grains allowed to pass down the column. Fractions were separated and the highest LOX activities were localized in the 5% w/w Ludox fraction (0.62 units) and at the bottom of the 20% fraction (0.27 units). Light microscopy examination revealed intact protoplasts and a few large starch grains at the bottom of the 20% fraction, smaller grains in the 20 and 10% fractions, and a dense mass in the 5% layer. Evidence presented in the next section indicated that the activity in the 5% layer came from the soluble enzyme binding with the colloidal silica.

Effect of Ludox on homogenates

When Ludox (5% w/w) or Percoll (colloidal silica, coated with polyvinylpyrrolidone) was added to potato homogenates from stored PJ tubers, the LOX activities were of the same order as control homogenates (Table 1). Extracts from potato slices macerated with buffer containing Percoll also contained similar LOX activity, but homogenates prepared with buffer and Ludox contained up to 60% more enzyme. Particulate fractions, isolated from control or Percoll-treated homogenates, were similar (4% LOX activity), whereas 19% particulate activity was recovered from homogenates treated with Ludox and 33% from a fraction isolated from homogenates extracted with Ludox. The two latter fractions were stable for 24 hr. It was also noted that when freshly harvested tubers were used the addition of Ludox, prior to maceration, increased the particulate recovery from 50 to 90%. Recovery of LAH particulate activity was also increased by Ludox (Table1). The control homogenate, with a high LAH content, gave 4% particulate recovery after centrifugation. Ludox added after extraction gave 29% and when added before extraction gave 42% particulate recovery. Boiled samples had no LOX or LAH activities.

The reason for the increased particulate recovery from homogenates extracted with Ludox, may be due to (a) binding with organelles that are released during the maceration, or (b) binding with free enzyme protein. The latter possibility appears more likely, because when Ludox was added to a homogenate after 6 hr, the activity present in the centrifuged fraction was still high—16% LOX and 22% LAH.

Amyloplasts

In other tissues slight LOX activity has always been associated with chloroplasts, although the possibilty that this was due to contamination by other organelles cannot be excluded. Potato amyloplasts, however, can be isolated in a relatively pure state [10]. After potato cubes from stored DES tubers had been shredded in the buffer medium with 12% sucrose, amyloplasts, starch grains and membrane particules passed through a nylon cloth, down a column of 15% w/w sucrose, and were collected in an attached tube. No LOX activity was found in this fraction or in the bottom half

Table 1. Effect of Ludox and Percoll on potato homogenates and particulate fractions

	LOX activity			LAH activity		
Enzyme localization	Homogenate units*	Particulate units*	%	Homogenate units*	Particulate units*	%
Homogenate	1.88	0.07	4	0.98	0.04	4
Homogenate + Ludox	1.66	0.31	19	0.83	0.24	29
Homogenate + Percoll	1.70	0.06	3	—	—	—
Ludox before maceration	2.91	0.96	33	1.27	0.53	42
Percoll before maceration	1.88	0.09	5	—	—	—

*Enzyme units refer to μmol substrate reacted/min/g fr. wt of tissue (O_2 for LOX and *p*-nitrophenylpalmitrate for LAH). Potato homogenates were prepared from stored PJ tubers and Ludox 5% w/w or Percoll 5% w/w added. Ludox or Percoll were also added to the buffer before maceration. Particulate fractions were obtained by centrifugation of the homogenates at 38 000 g for 20 min.

of the 15% sucrose solution. There was also no acid phosphatase or LAH activities in these two fractions. Most of the LOX activity was located in the 12% sucrose fraction, with 10% activity at the top of the 15% sucrose solution. After centrifugation of the combined active fractions at 38 000 g for 15 min, only 4% LOX and acid phosphatase activites were found in the particulate fraction. This is in agreement with the results obtained after maceration of stored tubers, and taking into consideration the time involved between shredding and examination of the tissue.

DISCUSSION

Organelles are often damaged during their extraction and isolation from potato tubers, with the resultant production of soluble enzymes. This damage has often been associated with the presence of starch grains in the tissue. In this present work slices have been carefully shredded, macerated or degraded to reduce damage and thus obtain an active LOX particulate. An inverse relationship between total activity and the percentage of activity obtained in a particulate form was shown to apply to LOX, as well as LAH. Only for a very short period after harvest was the total enzyme activity low enough to enable particulate LOX to be recovered after maceration of the tissue. It was also possible, at this stage, to obtain *ca* 50% of the LOX activity in intact protoplasts. After the LOX concentration in the stored tubers had risen, fewer intact protoplasts could be isolated and less than 20% LOX activity recovered. No LOX activity could be found, at this period, in organelles from lysed protoplasts or from macerated tissue.

LAH activity could be obtained both in a pellet form after maceration and from intact protoplasts, provided that the total activity of the two enzymes was low. It was impossible to produce active LAH particulate or many intact protoplasts from DES tubers once the LOX activity had increased during storage, even though the total LAH concentration remained low.

Localization of the lipid-degrading enzymes from a particulate fraction or from protoplasts has not been carried out. The available evidence points to a labile organelle, easily ruptured, especially when the tissue has a high LOX concentration.

In potato shoots [3], the subcellular location of LAH activity appeared to be a lysosomal particle or small vacuole. However, it was impossible to obtain any significant particulate LOX activity. From the results presented above, this can now be explained by the high total LOX content of the shoots. It is difficult, therefore, to visualize both enzymes being present in the same organelle, although in this present work on tubers, their activities have been closely parallel. Pitt and Galpin [11], in their work on potato shoots, found two peaks of acid phosphatase activity, located on Ficoll gradients, at 1.10 and 1.07 g/cm^3. These were called heavy and light lysosomal fractions. Our work on shoots located AP and LAH activity in the heavy fraction peak, but we were unable to locate the second peak. However, if this fraction of lysosomes had been damaged, due to the high LOX activity in the shoots, both LOX and AP activity would be present in the soluble fraction.

Matile [12] found 9 hydrolytic enzymes in maize root tips and isolated them into main sub-fractions by sucrose density gradients. These heavy and light lysosomal fractions differed not only in their relative densities in sucrose, but also in their enzyme content.

It is therefore suggested that in potato tubers and shoots the two lipid-degrading enzymes are in organelles, such as vacuoles or lysosomes, but which are probably different in size and composition.

Degradative enzymes can digest membrane lipids and with the potato acyl hydrolase this digestion is stimulated by free fatty acids [13]. However, the main factor in the recovery of intact vacuoles appears to be the concentration of LOX. This enzyme catalyses the production of fatty acid hydroperoxides and although their effects on membrane structures of mitochondria [14], microsomes [15] and lysosomes [16, 17] of mammalian tissue are known, little information has been reported for plant tissue. Most lipolytic enzymes and LOX have a pH optima in the range 5–6. This means that during the cell degradation process at pH 5.5, any soluble lipid enzyme was at optimal activity. The incubation also occurred over a much longer period than when the tissue was macerated at the higher pH of 7. It is also known [18] that increases in LOX activity in tubers, in response to wounding and infection, were similar to increases in peroxidase and phenol oxidase, whereas LAH activity was unaffected.

EXPERIMENTAL

Potato tubers (*Solanum tuberosum*) cv Désirée, Maris Peer and Pentland Javelin were grown locally, harvested by hand in August when not fully mature and stored at 7°.

Tissue extraction. The medium contained 25 mM K-Pi–citrate buffer, pH 7, 0.5 M mannitol and 2 mM 2-mercaptobenzothiazole. Cubes of potato tuber were homogenized in a blender for 2 × 5 sec bursts. The homogenate was filtered through muslin and centrifuged after sedimentation of starch grains.

Isolation of protoplasts. Potato discs 30 g, 1 mm thick, were incubated for 5 hr, + 20°, in 100 ml 0.5 M mannitol, 25 mM K-Pi–citrate buffer, pH 5.5, containing 15 mg/ml cellulysin and 5 mg/ml macerase. The digested material was filtered through muslin and the protoplasts collected by sedimentation. The protoplasts were lysed by the methods ref. [19].

Isolation of amyloplasts. Essentially by the method of ref. [10]; 400 g potato cubes evacuated for 5 min in 300 ml 0.3 M K_2HPO_4, 10 mM $MgCl_2$, 10 mM EDTA, 0.2% BSA and 12% (w/w) sucrose. The cubes were then shredded into the above buffer contained in a funnel, which was connected to a long column filled with buffer and 15% (w/w) sucrose. The shredded pieces were collected on nylon gauze above a 300 μm mesh nylon cloth, both being immersed in the buffer. Debris was removed at intervals from the gauze and particles passed through the cloth. The amyloplasts passed down the column and were collected in an attached tube.

Density gradients. These were w/w Ludox solns made up to 100% with 25 mM K-Pi–citrate buffer, pH 7 and 0.5 M mannitol. Discontinuous gradients were composed of 20 ml 20% Ludox, 30 ml 10% Ludox and 20 ml 5% Ludox. The degraded extract after 4 hr was added to the top of the column and left to settle for several hr before fractions were collected.

Chemicals. Cellulysin and macerase were obtained from Calbiochem.

Enzyme assays. Lipoxygenase was assayed by a polarographic method [4] using ammonium linoleate containing Triton X-100 (0.25%) as substrate and acetate buffer, pH 5.5. Lipolytic acyl hydrolase activity was determined using *p*-nitrophenylpalmitate as substrate by a continuous recording spectrophotometric method [1]. Acid phosphatase was determined as described in ref [6].

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