*Phytochemistry*, 1976, Vol. 15, pp. 1481–1484. Pergamon Press. Printed in England.

# SUBSTRATE SPECIFICITY OF POTATO LIPOXYGENASE

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(*Received* 6 *February* 1976)

**Key Word Index**—*Solanum tuberosum*; Solanaceae; potato; lipoxygenase; substrate specificity.

**Abstract**—The substrate specificity of potato lipoxygenase was examined using a partially purified enzyme preparation from tubers of a potato variety with low lipolytic acyl hydrolase activity. Potato lipoxygenase is fully active only on free linoleic acid or linolenic acid, and only acts directly on more complex glyceride moieties in the absence of any significant endogenous lipolytic acyl hydrolase activity.

## INTRODUCTION

Homogenisation of potato tubers at 0° results in the enzymatic decomposition of the endogenous phospholipids and galactolipids producing free fatty acids (FFA) and their hydroperoxides, and lipolytic acyl hydrolase and lipoxygenase have been identified as the enzymes responsible [1].

$(NH_4)_2SO_4$ precipitation followed by gel filtration on Sephadex G-150 [2] has been used to separate lipoxygenase from the enzymes catalase and peroxidase which are also found in the particle-free supernatant of potato tuber extracts. The remaining traces of these haemoproteins were removed by subsequent DEAE-cellulose chromatography, but since purification of lipoxygenase beyond the second Sephadex G-150 stage resulted in an overall reduction in sp act i.e. the loss in activity was greater than the purification of the protein, this third stage was abandoned for routine preparations. An acetone preparation of lipolytic acyl hydrolase has been obtained completely free from lipoxygenase activity [3]; however so far, it has been impossible to prepare a potato lipoxygenase fraction free from all lipolytic acyl hydrolase activity.

During routine work in this laboratory on 23 varieties of potato, it was found that the Désirée variety had a relatively low lipolytic acyl hydrolase activity [4]. From previous work [5], it was clear that no single method was sufficient to fully characterise lipoxygenase activity, and thus, using a selection of polarographic, spectrophotometric and TLC chromatographic techniques, lipoxygenase isolated from the Désirée potato tuber was examined with regard to its substrate specificity.

## RESULTS

### *Fatty acids and their methyl ester or triacylglycerol derivatives*

The first set of experiments compared linoleic acid, linolenic acid and their methyl ester (ME) and triacylglycerol (TG) derivatives, as substrates for detecting lipoxygenase action. These substrates were investigated both polarographically and spectrophotometrically as shown in Table 1; the FFA and hydroperoxide (OOH) products were separated by TLC in petrol–$Et_2O$–HOAc (70:30:1 or 60:40:1) solvent systems [1] and identified with $I_2$ vapour [6] and specific hydroperoxide [7] and fatty acid [8] sprays. The polarographic assay revealed faster breakdown rates with the linolenate substrates, while the reverse was implied by spectrophotometric analysis. Further work which combined both spectrophotometric and TLC analyses, revealed that the hydroperoxides of linolenate compounds were less stable than their linoleate counterparts. This emphasised the danger of using the conjugated diene assay outside the limits of linearity between fatty acid breakdown and hydroperoxide formation [5]. TLC analysis suggested the following breakdown patterns: FFA yields FFA–OOH, ME yields ME–OOH (major spot) + FFA + FFA–OOH, TG yields TG–OOH + FFA + FFA–OOH.

### *Galactolipids*

Lipolytic acyl hydrolase and lipoxygenase have been identified as the enzymes responsible for the decomposition of endogenous phospholipids and galactolipids during homogenisation of the potato tuber [1], and the fol-

Table 1. Free fatty acids and their methyl ester and triacylglycerol derivatives as substrates for potato lipoxygenase $E_4$ at pH 5.5

| Substrate | Polarographic assay (% activity relative to 18:2) | Spectrophotometric assay $\Delta A_{234}$ (after 20 min) |
|---|---|---|
| Linoleic acid | 100 | 1.340 |
| Linolenic acid | 113.3 | 1.025 |
| Me linoleate | 36.4 | 0.528 |
| Me linolenate | 55.7 | 0.358 |
| Trilinoleoylglycerol | 9.9 | 0.188 |
| Trilinolenoylglycerol | 9.2 | 0.100 |

[1] Reaction system: substrate—$5 \times 10^{-4}$ M; lipoxygenase extract $E_4$—20 μg of protein/2.5 ml incubation.

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Table 2. Monogalactosyldiacylglycerol and digalactosyldiacylglycerol as substrates for potato lipoxygenase $E_4$ at pH 5.5

| Substrate | Polarographic assay (% activity relative to 18:2) |
|---|---|
| Linoleic acid control | 100 |
| MGDG | 13.3 |
| MGDG + trace linoleic acid | 51.0 |
| DGDG | 6.0 |
| DGDG + trace linoleic acid | 12.5 |

Reaction system: linoleic acid control—$5 \times 10^{-4}$ M; trace linoleic acid—$5 \times 10^{-5}$ M; MGDG and DGDG—$5 \times 10^{-4}$ M with respect to their fatty acid content; lipoxygenase extract $E_4$—22.4 μg of protein/2.5 ml incubation.

lowing study with the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) tried to assess the extent of direct lipoxygenase action on these polar glycerides.

The results from Table 2 show that the breakdown of both MGDG and DGDG was increased in the presence of trace amounts of linoleic acid which is in agreement with previously reported work [9]. The breakdown products were separated by TLC and identified with $I_2$ vapour and the appropriate spray reagents as described earlier, using a range of different solvent systems (a) $CHCl_3$–MeOH–7N $NH_4OH$–$H_2O$ (26:12:2:1) [10], to compare the relative breakdown of MGDG and DGDG; (b) $CHCl_3$–$Ac_2O$–$H_2O$ (15:30:1) [10], to analyse the glyceride breakdown products of MGDG; (c) petrol–$Et_2O$–HOAc (60:40:1) [1] to assess the formation of FFA breakdown products from MGDG and DGDG. Solvent system (a) confirmed that there was more MGDG broken down than DGDG, which was expected from the $O_2$ electrode results (Table 2); solvent system (b) revealed that MGDG breakdown produced a hydroperoxide which had the same $R_f$ value or degree of polarity as that previously reported for monogalactosylmonoacylglycerol hydroperoxide (MGMG–OOH) [10]; solvent system (c) demonstrated firstly that the breakdown of galactolipid on its own produced FFA–OOH, and secondly that in a reaction system containing galactolipid and traces of linoleic acid, the FFA–OOH produced from either MGDG or DGDG was greater than that produced by traces of linoleic acid on its own, i.e. FFA–OOH was being formed from both the galactolipid and traces of linoleic acid. This TLC investigation thus revealed the following breakdown products, MGDG yields MGMG–OOH (minor spot) + FFA + FFA–OOH (major spot), DGDG yields FFA + FFA–OOH.

*Monolinoleoylglycerol*

Up to this point, the results from the substrate specificity studies seemed to indicate that the major action of potato lipoxygenase $E_4$ on the triacylglycerol and galactolipid substrates involved a hydrolysis followed by oxidation of the FFA. Monolinoleoylglycerol (ML) is highly susceptible to attack by lipolytic acyl hydrolase [3] and was a convenient substrate for assessing the actual participation of lipoxygenase in glyceride breakdown by the partially purified potato extract $E_4$.

The $O_2$ electrode results (Table 3) indicate that ML and Me linoleate were oxidised at similar rates, although

Table 3. Monolinoleoylglycerol as a substrate for potato lipoxygenase $E_4$ at pH 5.5

| Substrate | Polarographic assay (% activity relative to 18:2) |
|---|---|
| Linoleic acid | 100 |
| Monolinoleoylglycerol | 25.3 |
| Methyl linoleate | 32.1 |

Reaction system: substrate—$5 \times 10^{-4}$ M; lipoxygenase extract $E_4$—22.4 μg lipoxygenase extract of protein/2.5 ml incubation.

compared to linoleic acid neither was a particularly good substrate for potato lipoxygenase $E_4$. Subsequent TLC analysis using the solvent systems petrol–$Et_2O$–HOAc (60:40:1) [1] to measure FFA–OOH production, and $CHCl_3$–$Ac_2O$–MeOH–HOAc (73:25:1.5:0.5) [10] to measure ML–OOH production, suggested the breakdown pattern, ML–OOH (faint spot) + FFA + FFA–OOH (major spot). This result seemed to confirm that the main action of the lipoxygenase-containing fraction $E_4$ on the glyceride moieties was hydrolytic in nature.

Previous work [4] revealed that crude extracts from the potato varieties Désirée and Duke of York possessed

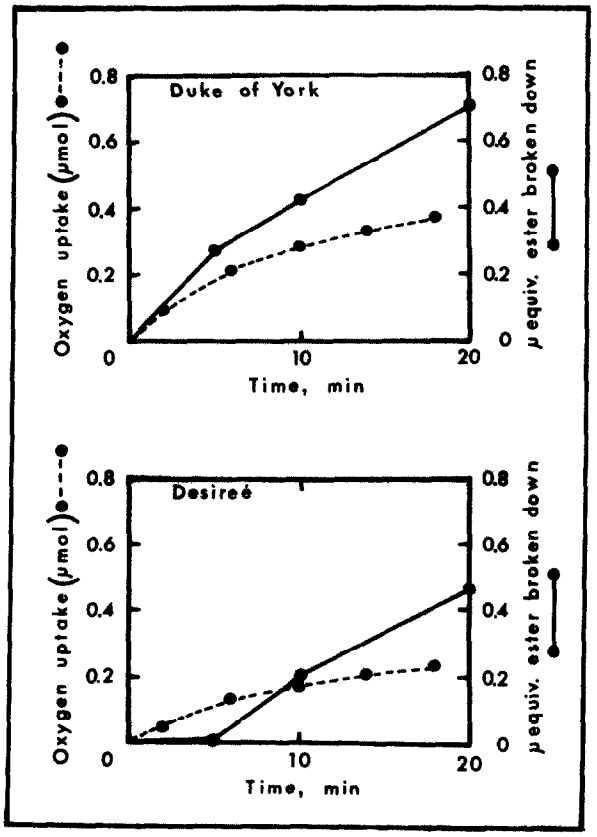


Fig. 1. Assessment of the hydrolase/lipoxygenase action in crude potato extracts at pH 5.5. Reaction system: monolinoleoylglycerol substrate—$10^{-3}$ M; crude enzyme extract—20 μl/2.5 ml incubation. Lipoxygenase activity was measured polarographically, and hydrolase activity was measured according to the method refs [11,12].

similar lipoxygenase activities (measured polarographically), but that Duke of York extracts had much higher lipolytic acyl hydrolase activity than Désirée extracts. However, as stated earlier in this paper, TLC analysis of the action of potato lipoxygenase $E_4$ demonstrated that partially purified Désirée extracts still possessed significant hydrolase activity. Thus, using crude enzyme preparations from both Désirée and Duke of York, a comparison was made between the activities of lipoxygenase, measured by $O_2$ uptake, and lipolytic acyl hydrolase determined by the quantitative estimation of acyl esters [11–12] in an attempt to assess the actual hydrolytic activity of Désirée extracts. The results (Fig. 1) demonstrate that with Désirée there was a lag phase in ester breakdown which was not encountered in the experiments with Duke of York. Concomitant TLC analysis confirmed that while the Duke of York extracts only produced FFA–OOH, the crude enzyme from Désirée produced both FFA–OOH and a small amount of ML–OOH which was formed during the hydrolytic lag phase. This experiment showed that lipoxygenase only acted directly on ML when the hydrolase activity was low. Although the Désirée lipolytic acyl hydrolase activity, determined spectrophotometrically [4], was very low, the glyceride breakdown studies described in this paper clearly demonstrated that hydrolysis was a controlling factor in the action of the partially purified extract $E_4$.

## DISCUSSION

The potato is a good source of lipoxygenase [13,14], and although attempts have been made to purify and characterise this enzyme [2,15] potato lipoxygenase has not yet been isolated completely from the influence of lipolytic acyl hydrolase. To determine whether lipoxygenase alone could attack the membrane-bound lipids in the tuber, or whether fatty acid release by hydrolase action was a prerequisite for oxidative breakdown, a range of lipids were studied as substrates for lipoxygenase fraction $E_4$.

Initial polarographic studies revealed that the triacylglycerols, trilinoleoylglycerol and trilinolenoylglycerol (Table 1) and the galactolipids, MGDG and DGDG (Table 2) were poor substrates, and subsequent TLC analysis showed the major product to be FFA–OOH. Me linoleate or linolenate (Table 1) and ML (Table 3) gave measurable $O_2$ uptakes after an initial lag phase, but TLC analysis demonstrated that, whereas the main product from the Me ester was its own hydroperoxide, the main product from ML was FFA–OOH, accompanied by small quantities of its own hydroperoxide. It has previously been demonstrated that ML is a good substrate for lipolytic acyl hydrolase whereas the Me ester is a rather poor one [3]. This may explain why, with a slight hydrolase contamination, potato lipoxygenase $E_4$ had a better chance of reacting directly with the Me ester of the fatty acid as opposed to its monoacylglycerol derivative. Direct polarographic and spectrophotometric analyses [15] indicated that the potato lipoxygenase isoenzymes A and B were more active on linoleic acid than on its Me ester and glyceride derivatives. The substrate specificity results described in this report, confirm these preliminary findings because potato lipoxygenase $E_4$ was only fully active on the free acid, and only directly oxidised the more complex glyceride moieties, e.g. monoacylglycerols, in the absence of significant endogenous hydrolytic action. The direct action of potato lipoxygenase on membrane lipids can only be properly assessed once lipoxygenase and lipolytic acyl hydrolase activities have been completely separated. However, the results presented here confirm that the extensive lipid degradation that occurs on disruption of potato tuber tissue is due to the combined effect of the hydrolytic and lipoxygenase enzymes.

## EXPERIMENTAL

The potato varieties, Désirée and Duke of York were grown, harvested and stored as described previously [5]. Galactolipids were isolated from spinach leaves [16]. Column chromatography (silicic acid) and TLC (Si gel CT) were used to purify the lipid substrates, and all organic solvents with the exception of $Et_2O$ were redistilled before use.

*Enzyme preparation.* Lipoxygenase extract $E_4$ was isolated from potato homogenates by $(NH_4)_2SO_4$ precipitation and Sephadex G-150 filtration [5], and its purity was checked by disc gel electrophoresis [17] applying a technique to identify the activity of lipoxygenases possessing neutral pH optima [18]. This isolation procedure produced a reasonably homogeneous protein similar to that prepared by chromatography on Ecteola-cellulose [15]. Free flow electrophoresis [19] as a single purification technique failed to remove the final traces of lipolytic acyl hydrolase from the Désirée preparations, and the characterisation of substrate specificity was carried out with partially purified potato lipoxygenase $E_4$.

*Substrate preparation.* The 3.5:6.25 mg fatty acid:detergent ratio selected from emulsifying linoleic acid in the work described in the accompanying paper [5] was found to be unsuitable for the more complex substrates under review in this present report. At the above concentration Triton X-100 inhibited mono- and triacylglycerol and glycolipid breakdown, while a 3.5:1 ratio was satisfactory for all the substrates. In the $O_2$ electrode studies the Me ester and glyceride derivatives of the FFA and glycolipids displayed varying degrees of lag phase, which was lessened when Triton X-100 emulsification was enhanced by ultrasonication for 30 sec. On further investigation it was revealed that the lag phase was inversely proportional to the eventual linear rates of $O_2$ uptake. Thus all substrates used in this study were emulsified by Triton X-100 treatment plus ultrasonication, and the results were expressed in terms of these ultimate rates.

*Assays.* The polarographic, spectrophotometric and TLC analyses were carried out as described previously [5]. Protein content was determined by the method of ref [20]. For the quantitative determination of acyl esters, the lipid reaction products were collected via the Bligh Dyer extraction procedure [21], and the resultant lipid residue was used to determine ester breakdown by the method ref [11] as modified in ref [12].

*Acknowledgements*—This work was supported by a grant from the Potato Marketing Board.

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