Phytochemistry, 1976, Vol. 15, pp. 1475–1479. Pergamon Press. Printed in England.

MEASUREMENT OF LIPOXYGENASE ACTIVITY IN CRUDE AND PARTIALLY PURIFIED POTATO EXTRACTS

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(*Received* 6 *February* 1976)

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

Abstract—Two assay systems, one a spectrophotometric assay at 234 nm, the other based on the oxygen electrode, were compared as methods for the routine analysis of lipoxygenase activity in crude and partially purified potato extracts. The spectrophotometric assay was unsuitable for the analysis of crude extracts and only gave meaningful results under very restricted reaction conditions. The oxygen electrode provided a reliable method for routine analysis of lipoxygenase activity.

INTRODUCTION

The lipoxygenase (EC 1.13.11.12) catalysed oxidation of unsaturated lipids is a highly specific and complex process involving a soluble hydrophilic enzyme, a hydrophobic substrate, oxygen, and other enzymatic and non-enzymatic factors. Many workers have used a wide variety of iodometric [1, 2], manometric [1], polarographic [3], spectrophotometric [4–8] and cup-plate diffusion [9] assay techniques to establish the presence or absence of lipoxygenase activity in plant tissues. Measurement of the action of lipoxygenase depends on the reaction system employed, especially with regard to the choice of buffer medium [10–12], the dispersion of the substrate or substrates [7, 8, 11, 13–21] and the purity of the enzyme preparation itself [2, 5, 16, 22]. Although attempts have been made to express some of these assay methods in terms of each other [1, 8, 10, 15, 21, 23–25], a complete stoichiometric relationship between O_2 consumption, conjugated diene formation and pigment breakdown has not been reported.

Much of the early work on fatty acid peroxidation by crude plant extracts was hindered by the presence of endogenous pigment. Using etiolated wheat leaves to prepare active, pigment-free lipoxygenase extracts [26], preliminary experiments [27] based on pH profile [28] and specific inhibitor [26, 28, 29] studies demonstrated that the crocin [6], thiocyanate [5] and conjugated diene assays [7] could not be reliably used on their own to characterise fully the action of lipid oxidising enzymes in crude plant extracts. There are now two widely used methods for measuring lipoxygenase activity, one records by direct spectrophotometric analysis at 234 nm the conjugated diene chromophores formed [7], while the other involves the measurement of O_2 uptake by the oxygen electrode [3]. Preliminary studies with crude potato extracts failed to relate O_2 uptake and conjugated diene formation on a mole to mole basis. The work described in this report was designed to assess the relative merits of the polarographic and spectrophotometric assays as routine methods for measuring lipoxygenase activity with regard to later studies involving the effect of growth and storage on the lipoxygenase activity of crude potato extracts [30], and the characterisation of the substrate specificity of partially purified potato lipoxygenase [31].

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RESULTS AND DISCUSSION

The spectrophotometric assay

Many workers have reported that the formation and breakdown of linoleic acid hydroperoxide (measured spectrophotometrically) is catalysed by a wide variety of crude plant extracts [23, 32–38]. More recent studies with plant tissues have shown that endogenous enzyme factors like hydroperoxide isomerase [39–46] lipoperoxidase [47, 48] and epoxide formation [49] readily catalyse the degradation of unsaturated fatty acid hydroperoxides. Further work [50] has demonstrated that these hydroperoxide intermediates can be reduced by ferric ions in the presence of cysteine, and because lipoxygenase is now known to contain iron [51–53], it is possible that this metal is involved in a specific lipoxygenase/lipoperoxidase bifunctional complex [47, 48] which is active in plant extracts. With these points in mind, and using both TLC and ^{14}C techniques to analyse the breakdown products, the spectrophotometric assay was examined with regard to its value as a routine method for detecting lipoxygenase activity.

(a) *Crude lipoxygenase extracts*

This portion of the work was carried out with crude potato extract E_1, containing $Na_2S_2O_5$ to prevent the rapid darkening caused by phenolic oxidation during homogenisation of the tuber [54]. Under the reaction conditions described in Fig. 1, the breakdown of fatty acid measured radioisotopically was not proportional to the formation of total conjugated diene (hydroperoxide plus hydroxydiene) measured spectrophotometrically at 234 nm (Fig. 1a). For a given enzyme concentration under standard reaction conditions, the formation of

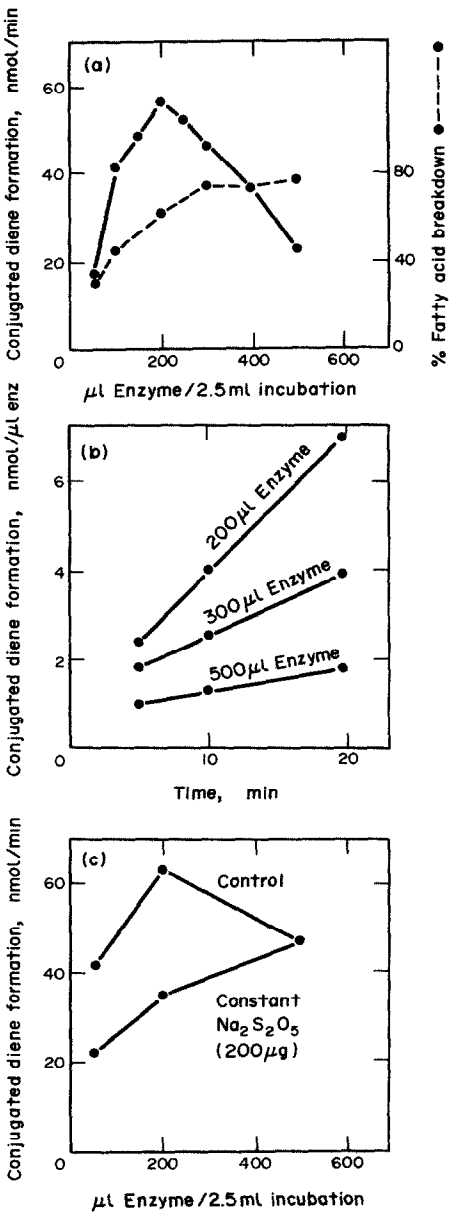

Fig. 1. Assessment of the spectrophotometric assay as a routine method for analysing lipoxygenase activity in crude potato extracts. Reaction system: ammonium linoleate substrate—10^{-3} M; 0.1 M acetate buffer pH 5.5; $Na_2S_2O_5$— 40 mg % in enzyme extract E_1; reaction time in experiments (a) and (c) 20 min. Conjugated diene formation was measured spectrophotometrically at 234 nm. Fatty acid breakdown was measured by radioisotopic analysis, and the % breakdown is expressed in terms of the total counts on the thin-layer chromatogram.

conjugated diene was linearly related to time, although the level of conjugated diene recorded was inversely proportional to enzyme concentration (Fig. 1b). TLC analysis of the products revealed that the ratio of hydroxydiene to hydroperoxide increased with enzyme concentration, and in experiment (a) above the level of 200 μl, an increase in enzyme concentration produced a corresponding loss in total conjugated diene accompanied by an accumulation of breakdown products at the origin of the chromatogram. In a further experiment involving both TLC and ^{14}C analyses, the actions of enzyme extracts with and without $Na_2S_2O_5$ were compared. It was found that, as opposed to the crude extract with $Na_2S_2O_5$, the enzyme preparation without reducing agent gave a conjugated diene product which was almost entirely in the form of the hydroperoxide, thus confirming that $Na_2S_2O_5$ was responsible for the reduction of hydroperoxide to its monohydroxy derivative. The results given in Fig. 1c show that when enzyme concentration was increased in a reaction system containing a constant level of $Na_2S_2O_5$, the pattern of total conjugated diene formation, as depicted by direct spectrophotometric analysis, was clearly influenced by the amount of reducing agent present in the reaction system. Further TLC analysis confirmed that the accumulation of breakdown products at the origin (as described earlier) was proportional to the concentration of $Na_2S_2O_5$, and suggested that $Na_2S_2O_5$ was also responsible for accelerating degradation of the conjugated diene products since conversion of hydroperoxide to its monohydroxy derivative only slightly affects *A* 234 nm. It was clear that the spectrophotometric assay was not suitable for routine analysis of crude extracts containing reducing agents, and was discounted as a likely method for the routine analysis of crude potato lipoxygenase extracts [30].

(*b*) *Partially purified lipoxygenase extracts*

Initial work established that, like the crude extracts without $Na_2S_2O_5$, the partially purified lipoxygenase fractions E_3 and E_4 produced conjugated dienes almost entirely in the form of their hydroperoxides. Preliminary spectrophotometric studies with lipoxygenase fraction E_3 suggested that there was a pH optimum 'plateau', while concurrent TLC analysis indicated a definite pH of 6.4–6.7 for fatty acid breakdown. Using linoleic acid [1-^{14}C] (Fig. 2) it was found that there was much better correlation between fatty acid breakdown and conjugated diene hydroperoxide formation in the reaction system using less enzyme and a shorter incubation period (Fig. 2b). Further work, using spectrophotometric analysis as a routine method for locating lipoxygenase activity in gel filtration eluate fractions, was also hampered by the "plateau" effect, and the peak of enzyme activity was only satisfactorily detected once linear conditions for hydroperoxide formation had been established. Many variables are clearly involved in assessing the correct conditions for valid measurement of conjugated diene formation at 234 nm, not only for crude extracts, but also the partially purified ones, and thus direct spectrophotometry should only be carried out under conditions where hydroperoxide formation is known to be an accurate description of fatty acid breakdown.

Based on the iodometric procedure for detecting lipoxygenase bands in polyacrylamide gels [2], Wallace [55] has recently developed a qualitative spot test for peroxide formation, and used it to locate lipoxygenase activity in fractions eluted from chromatographic columns. However preliminary work carried out by D. R. Phillips in this laboratory has established that unless the system is carefully monitored, accurate measurements of lipoxygenase activity by Wallace's method will be hindered by the same "plateau" effect as that described earlier for the spectrophotometric assay. Because of the difficulty in regularly obtaining a linear relationship between lipid

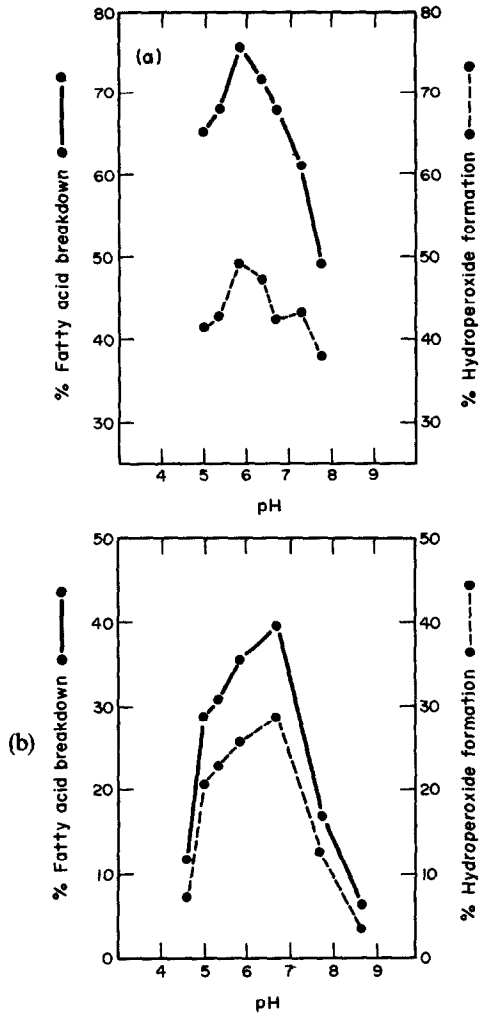

Fig. 2. Relationship between fatty acid breakdown and hydroperoxide formation as measured by the radioisotopic assay. Reaction system a: ammonium linoleate substrate—10^{-3} M; lipoxygenase extract E_3—0.2 ml/2.5 ml incubation; reaction time—10 min. Reaction system b: ammonium linoleate substrate—10^{-3} M; lipoxygenase extract E_3—0.1 ml/2.5 ml incubation; reaction time—5 min. The buffers were prepared according to the method of McIlvaine (1921). The values represent the % fatty acid breakdown or hydroperoxide formation, with respect to total counts on the thin-layer chromatogram.

breakdown and conjugated diene hydroperoxide formation it was felt that neither spectrophotometry nor iodometry could be used reliably as a routine method for detecting lipoxygenase activity in plant extracts. Since the reducing agent $Na_2S_2O_5$ did not interfere with the O_2 uptake assay of crude potato lipoxygenase extracts [54], it was decided to standardise this assay with regard to the routine analysis of both crude and partially purified lipoxygenase fractions.

The O_2 electrode assay

(a) *Standardisation*

Using potato lipoxygenase E_3 under substrate limiting conditions, and assuming that the concentration of O_2 in an air saturated solution at 25° is 240 μM and that each O_2 uptake reading represented complete oxidation of the substrate, it was found that a direct stoichiometric relationship existed between O_2 uptake and the amount of substrate broken down. This enabled O_2 uptake to be expressed on a precise molar basis.

The electrode itself was standardised using glucose and glucose oxidase [56] under substrate saturating conditions to measure a linear relationship between O_2 uptake and unit enzyme concentration. Linearity existed over an enzyme range of 0–1 mg/2.5 ml incubation, and the glucose/glucose oxidase assay was an accurate method for monitoring the response of the electrode.

(b) *Choice of substrate for crude lipoxygenase extracts*

Although unsaturated fatty acids form soap solutions at pH 9 or above, much lipoxygenase work is carried out at lower pHs where the substrate is not completely dissociated. Detergents lower the boundary tension at lipid–water interphases producing smaller mixed micelles which are more readily dispersed under aqueous reaction conditions, thus providing a better surface area of substrate for attack by lipoxygenase.

In an effort to eliminate another possible source of error in the routine analysis of crude potato extracts, viz. the variability of the fatty acid dispersion [1, 8, 11, 13–21], the rates of O_2 uptake were compared in two systems, one using the ammonium salt of linoleic acid [54] and the other using linoleic acid emulsified with Tween 20 in the ratio of 3.5 mg fatty acid:6.25 mg detergent [8]. Potato varieties Désirée, Majestic and Golden Wonder [30] were used as the sources of crude lipoxygenase extract E_1, and since no significant differences in lipoxygenase activity were detected, variation in substrate dispersion was not a problem in this study. Initial rates of O_2 uptake were consistently faster with the fatty acid substrate emulsified with Tween 20.

(c) *Choice of substrate for partially purified lipoxygenase extracts*

Although ammonium linoleate could be used for measuring lipoxygenase activity in crude extracts, it was obvious that detergent treatment would be necessary to emulsify the more complex lipids required for the subsequent substrate specificity studies [31]. Preliminary work established that Triton X-100 and Tween 20 were equally effective as agents for emulsifying lipid substrates.

Using ammonium linoleate as substrate, it has been reported [54] that partially purified potato lipoxygenase had a pH optimum of 5.5. As shown in Fig. 3a the effect of the Triton X-100 emulsified substrate on the pH profile of lipoxygenase extract E_4 was to spread out the peak of activity. This meant that minor variations in buffer conditions around pH 5.5 were less likely to affect results obtained with the Triton X-100 emulsified substrates. In addition, comparison between the sp. act. of the partially purified lipoxygenase fractions E_3 and E_4 was very difficult using ammonium linoleate as substrate, because the reaction rate versus enzyme concentration curves were not the same for both enzyme preparations. In order to find a better system, the relative activities with the ammonium salt and the Triton X-100 emulsified form of linoleic acid as substrates for estimating the sp. act. of lipoxygenase fraction E_4 was studied. The results in Fig. 3b show a trend which was a regular feature in all experiments dealing with the more purified enzyme fraction E_4. In the Triton X-100 system the relationship

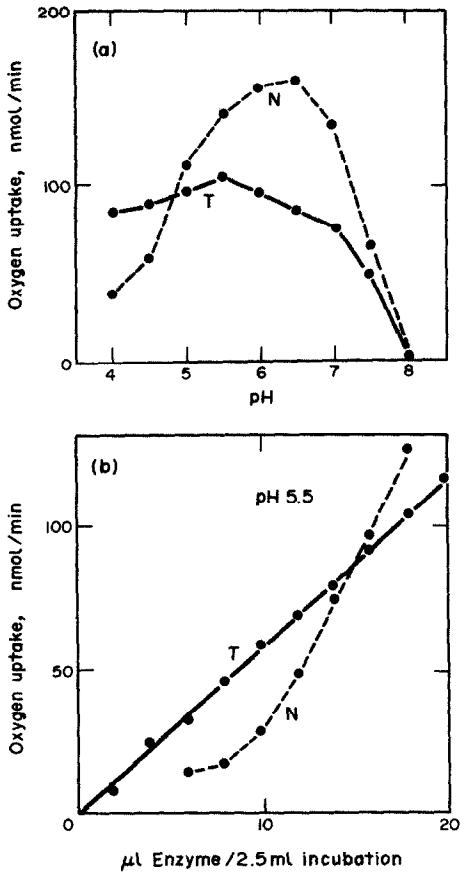

Fig. 3. Effect of pH and substrate emulsification on the activity of lipoxygenase extract E_4. In all reaction systems, substrate—5×10^{-4} M. Weight ratio of Triton X-100 to linoleic acid = 6.25:3.5. N = ammonium linoleate; T = Triton X-100-emulsified substrate. In experiment (a) N + 15 μl extract E_4; T + 10 μl extract E_4; and the buffers were prepared according to the method of McIlvaine (1921).

between O_2 uptake and unit enzyme concentration was linear over a larger range of enzyme concentrations, and sp. act. measurements from both substrate systems were only comparable above a certain enzyme level. As demonstrated by its "milky" appearance, ammonium linoleate exists in the form of large micelles at the low pH (5.5) [54] used to measure potato lipoxygenase activity, and the surface area may differ from preparation to preparation. Under the same reaction conditions, non-ionic detergents seemed to increase the effective concentration of linoleate which was available to the enzyme, and it is evident from Fig. 3b that the Triton X-100-treated substrate was more susceptible to attack by lipoxygenase extract E_4 at the lower enzyme concentrations. Further studies established that the sp. acts. of lipoxygenase fractions E_1, E_3 and E_4 could be satisfactorily compared using the Triton X-100-emulsified substrate.

CONCLUSION

Characterisation of lipoxygenase activity in plant extracts is hampered by the complex nature of the reaction system which involves a lipid substrate and an aqueous enzyme preparation. Although the polarographic assay is the most satisfactory method for routine analysis of both crude and partially purified lipoxygenase extracts, it is not sufficient to characterise fully the reaction mechanism. This requires TLC, radioisotopic or MS techniques to monitor product formation. In an accompanying paper, characterisation of the substrate specificity of partially purified potato lipoxygenase E_4 is described using a combination of O_2 uptake, spectrophotometry and TLC.

EXPERIMENTAL

Materials. Potato tubers Désirée, Majestic and Golden Wonder were grown locally at the Norfolk School of Agriculture, Easton, Norfolk, and were collected either during growth and used immediately for analysis, or at commercial harvest and stored at 5° until required.

Preparation of potato lipoxygenase extracts. A known wt of diced potato was homogenized at 1–2° with 2 vol of 50 mM KPi buffer pH 7, which contained $Na_2S_2O_5$ to prevent rapid darkening caused by polyphenolic oxidations [54]. Potato extract E_1 was the supernatant fraction collected after centrifugation of the crude homogenate at 17000 *g* for 30 min; potato extract E_3 was the sediment fraction resuspended after $(NH_4)_2SO_4$ fractionation between 30 and 50% saturation; and potato extract E_4 was the lipoxygenase fraction collected after filtration on Sephadex G-150.

Enzyme assays. All reactions were carried out in 0.1 M NaOAc buffer pH 5.5, in a final vol of 2.5 ml and at 25°. The O_2 uptake assay was performed as reported in ref. [58] and standardised as described in the main text. In the combined spectrophotometric/TLC assay the reaction was stopped either at zero time (substrate in the absence of enzyme) or after a preselected incubation period by first removing 1 ml reaction mixture into 4 ml 80% EtOH for subsequent spectrophotometric analysis at 234 nm [7] and then immediately adding 6 ml $CHCl_3$–MeOH (1:1) plus 1 ml 0.2 M NaOAc buffer pH 4 to the remaining 1.5 ml reaction mixture. Vigorous shaking of the latter mixture produced a biphasic Bligh and Dyer [59] separation; the lipid reaction products were taken from the lower $CHCl_3$ layer and analysed by TLC in petroleum (bp 60–80°): Et_2O: HOAc solvents [60], and identified using I_2 vapours [61] and specific sprays [62, 63]. The radioisotopic assay was as above except that linoleic acid (1-^{14}C] (0.1 μCi/mmol) was used and the TLC spots were measured as described [64].

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

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