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The Enzymic Release of Fatty Acids from Phosphatidylcholine in Green Peas (*Pisum sativum*)*

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ABSTRACT

'Phospholipid acyl-hydrolase' (PLAH), an enzymic activity releasing fatty acid from phosphatidylcholine (PC), has been identified and characterised in green peas. The K_m value for PC dipalmitoyl ester was 0·167 mM. The enzymic activity possessed a pH optimum of 5·6 and was stable for 20 min only at that pH value. The optimum temperature was 45 °C and thermal sensitivity was indicated by a 94% decrease in activity upon exposure of the enzyme to 55 °C for 3 min, and by an exponential decrease in activity upon storage at 4 °C for 1 week. The enzyme was optimally activated by 2·0 mM calcium chloride at pH 5·6, and the optimal concentration of sodium dodecyl sulphate was 0·75 mg ml^{-1}. Pea PLAH was non-competitively inhibited by sodium cyanide, EDTA and p-chloromercuribenzoate, with no activity in the presence of mercuric chloride. The results from this study are related to those of other workers on lipid-degrading enzymes in peas, and a pathway is proposed for the enzymic degradation of endogenous lipids in fresh or unblanched frozen peas during post-harvest storage.

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INTRODUCTION

Peas (*Pisum sativum* L.) are cultivated in many parts of the world, and in Canada the total area presently devoted to green pea production is 22 000–26 000 ha. The production of green peas in Canada increased from an average of 57 900 tonnes in 1969–73 to 67 300 tonnes in 1980, with a concurrent increase in farm value from $6·5 million in 1969–73 to $18·9 million in 1980 (Agriculture Canada, 1981). On a dry weight, whole seed, basis, the protein content of peas is 18 to 28 %, and the lipid content is 3 to 4 %. Peas are a significant source of dietary protein, but there is sufficient lipid to be implicated in flavour deterioration, to which a major contributing factor is lipid oxidation, in both fresh and processed peas. The percentages of total component fatty acids in pea lipids are: oleic acid, 27·6–34·9; linoleic acid, 41·5–48·1; linolenic acid, 7·4–8·3 (Haydar & Hadziyev, 1973).

Deterioration in the flavour of fresh or unblanched frozen peas during post-harvest storage may be partly due to the activity of lipid-degrading enzymes. Lipoxygenase was identified and biochemically characterised in peas by Eriksson & Svensson (1970) and by Haydar *et al.* (1975). Lipase in peas was studied by Wagenknecht *et al.* (1958) to account for the release of fatty acids from the triglyceride fraction (the major pea lipid component). Phospholipase D was identified in peas by Quarles & Dawson (1969).

The purpose of the investigation reported in this paper was the detection and biochemical characterisation in peas of an enzymic system releasing fatty acids from phospholipids. Bengtsson & Bosund (1966) provided evidence for phospholipid hydrolysis, with concurrent free fatty acid (FFA) production, in unblanched peas placed into frozen storage. Reichert & MacKenzie (1982) have reported that in peas the polar lipid content was approximately 40–50 % of the total lipids. Phospholipid may reasonably be expected to be a significant source of FFA for lipoxygenase action.

MATERIALS AND METHODS

Enzyme source

Green peas (*Pisum sativum* L. cvs. Lincoln and Lotus) were obtained from the Department of Plant Science, University of Manitoba. For this

study, the Lincoln variety, harvested in 1980, and the Lotus variety, harvested in 1981, were used. The peas were stored at −20 °C until required.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Enzyme preparation

Twenty grams of peas were homogenised in a Waring blender for 2–3 min with 20 ml of sodium phosphate buffer, 0·1 M, pH 5·6. The homogenate was allowed to stand for 15 min at 0 °C, and the decanted liquid was centrifuged twice at 20 000 × *g* for 30 min at 0 °C. The supernatant liquid (crude extract) was retained for enzyme studies. Crude extracts had to be used in this study since attempts at partial purification did not result in a significant increase in specific enzyme activity.

Substrate preparation

0·04 g of L-α-phosphatidylcholine (PC), dipalmitoyl ester (approximately 98% pure, synthetic, obtained from Sigma Chemical Co., St. Louis, Missouri, USA), 30 ml of 10 mM sodium dodecyl sulphate (SDS) and trace amounts of butylated hydroxytoluene were placed in a plastic centrifuge tube. The mixture was sonicated at 50 °C for 30 min (bursts of 15 s, about 15 s apart). The resulting 1·8 mM substrate (3·6 micro-equivalents of acyl ester per millilitre) was stored, until required, at −20 °C under nitrogen.

Enzyme assay

Activity was determined by the production of FFA from PC, using a modification of the rhodamine 6G method of Hirayama & Matsuda (1972). The reactions were carried out at 25 °C in 13 × 100 mm culture tubes with teflon-coated screw caps. The standard reaction mixture composition (total volume 1·0 ml) was: sodium phosphate buffer, 0·1 M, pH 5·6, 0·6 ml; the micellar solution of PC, 1·8 mM, 0·2 ml; enzyme preparation (8–10 mg protein per millilitre), 0·2 ml. The reaction was

terminated by the addition of 1·7 ml of a mixture of ethanol and 1·5M sulphuric acid (200:1, v/v), followed by vortex mixing, 3·3 ml of hexanes (pesticide grade) were added, the tube contents were mixed for approximately 1 min, and the phases were allowed to separate. After 1 h, a 2·0 ml aliquot of the upper hexane layer was withdrawn and placed into a 1-cm glass cuvette. The FFA in the hexane layer was determined by the addition of 1·0 ml of rhodamine solution, followed by gentle mixing. This solution was prepared immediately prior to use by mixing equal volumes of rhodamine 6G reagent (Chakrabarty *et al.*, 1969) and spectral grade benzene. After 20 min at room temperature, for pink colour development, the A_{515} was determined and related to a standard curve constructed with oleic acid. One unit of enzyme activity was equivalent to one nanomole of FFA released per minute at 25 °C.

RESULTS

A linear relationship was established between enzyme concentration and FFA production. The K_m value (pH 5·6, 25 °C) for L-α-PC dipalmitoyl was 0·167 mM. The enzyme was active within a pH range of 4·4–8·5, with a narrow optimum at pH 5·6 and with very low activity at pH values above 6·0. Control experiments demonstrated the absence of non-enzymic hydrolysis over the entire pH range studied. Complete stability of the enzyme for 20 min was demonstrated only at pH 5·6. The optimum temperature was 45 °C (pH 5·6; reaction time of 15 min), and a 94% loss of activity was demonstrated upon exposure of the enzyme to 55 °C for 3 min. There was an exponential decrease in the activity of the crude extract stored at 4 °C for 1 week, with a 50% decrease in activity during the first 24 h of storage.

The enzyme was activated by calcium chloride, with optimal activation at pH 5·6 in the presence of 2·0 mM $CaCl_2$, showing an approximately fivefold increase in activity compared with that in the absence of added calcium (Fig. 1).

Minimal calcium activation was observed at pH 7·0, while, at pH 5·2 and 6·2, increasing activation was demonstrated up to a concentration of 3·0 mM $CaCl_2$. Variations in SDS concentration (0·25–1·50 mg ml^{-1}) in the substrate preparation resulted in changes in the reaction rates. The optimum SDS concentration for enzyme activity was found to be 0·75 mg ml^{-1}.

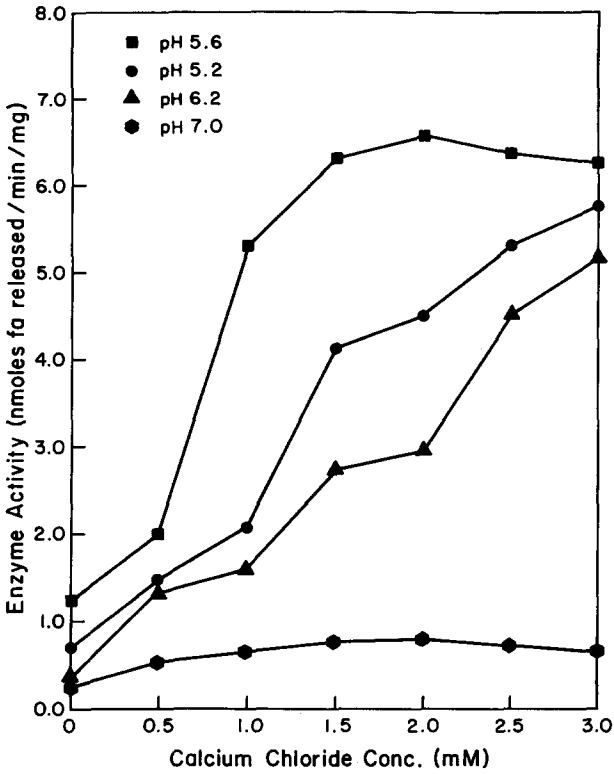

Fig. 1. Effect of calcium chloride on pea 'phospholipid acyl-hydrolase' activity.

The enzyme was non-competitively inhibited by 1·0 mM and 5·0 mM sodium cyanide, losing approximately 17% and 33%, respectively of its original activity; by 0·1 mM and 0·5 mM EDTA with respective losses of 50% and 78% of the original activity; and by 0·1 mM and 0·5 mM *p*-chloromercuribenzoate (*p*-CMB) with respective losses of 33% and 87% of the original activity. Enzyme activity was not demonstrated in the presence of 0·25 mM mercuric chloride.

DISCUSSION

The detection and biochemical characterisation of an enzymic system in peas releasing FFA from PC has been achieved. Crude extracts had to be used in this study owing to purification difficulties, possibly due to the close association of enzyme activity with a major protein component, extraneous to the enzyme itself (Galliard, 1971; Henderson *et al.*, 1981).

This study has demonstrated an overall enzymic reaction resulting in the production of fatty acids from PC. Individual enzymes have not been identified but the enzymic activity described in this paper may be given the generic term 'phospholipid acyl-hydrolase' (PLAH). The rhodamine method does not distinguish among the specificities of the enzymes belonging to this group.

The K_m value of 0·167 mM obtained in this study compares with the corresponding value of 0·18 mM from fababeans (Henderson *et al.*, 1981). These are apparent K_m values since the enzymic reaction occurs at the micelle–water interface in an emulsion system. The pH optimum of 5·6 was identical to that for the hydrolysis of PC in fababeans (Henderson *et al.*, 1981) and compares with other plant phospholipases, such as cucumber (Galliard *et al.*, 1976) and bean leaves (Matsuda *et al.*, 1979), which have acidic pH optima. The enzyme is very susceptible to pH denaturation, as demonstrated by both the pH profile and the pH stability experiments. Heat stability studies indicated that the enzyme is relatively heat labile. Galliard (1971) reported a 50% decrease in activity of potato tuber 'lipolytic acyl-hydrolase' upon exposure to 60 °C for 3 min, while exposure of bean leaf 'lipolytic acyl-hydrolase' to 65 °C for 20 min at pH 4·0 resulted in complete inactivation (Matsuda *et al.*, 1979). Advantage may be taken of the heat sensitivity of pea PLAH in the possible control of enzymic lipid degradation in raw unprocessed peas. Also, pea PLAH in crude extract form was unexpectedly unstable during storage at 4 °C for 1 week.

Results from this study have indicated an interaction between the effect of pH and the activation of pea PLAH by calcium ions. The observed activity in the absence of added calcium suggested that the crude extracts contained sufficient endogenous calcium to promote enzyme activity during routine assay. Raw immature green peas contain 26 mg of calcium per 100 g edible portion (Watt & Merrill, 1975). Activation of the enzyme by calcium may be due to the removal of undissociated fatty acids from the micelle–water interface as insoluble calcium soaps, or to interaction with ionisable groups on the enzyme, or to binding interaction between PC and the enzyme. Phospholipase A_2 from several sources has been shown to have a highly specific requirement for calcium, even compared with other divalent ions (Castle & Castle, 1981), so that pea PLAH may include phospholipase A_2. The interaction of calcium activation and pH effect may be explained by a possible excess of hydrogen ions repelling calcium ions at the PC micelle–water interface, or by protonation

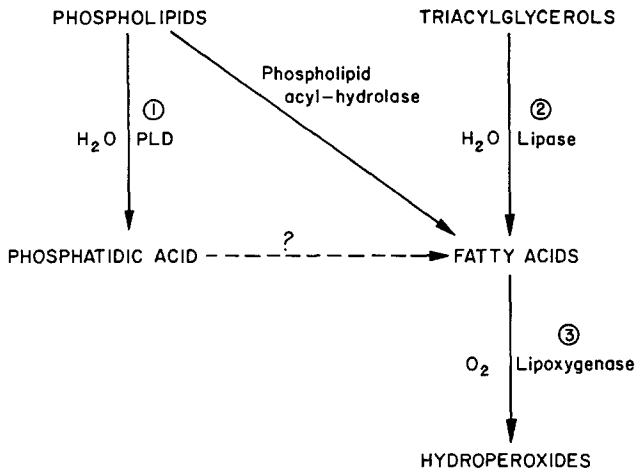

Fig. 2. Proposed pathway for the enzymic degradation of endogenous lipids of *Pisum sativum* during post-harvest storage. (1) Quarles & Dawson (1969); (2) Wagenknecht *et al.* (1958); (3) Haydar *et al.* (1975). PLD = phospholipase D.

interference with calcium binding at the appropriate ionisable groups on the enzyme (Ikeda *et al.*, 1981; Teshima *et al.*, 1981).

SDS was used in this study for the preparation of a stable PC emulsion. At concentrations above $0{\cdot}75\,mg\,ml^{-1}$, SDS was inhibitory to the enzyme, possibly due to a dilution effect on the availability of PC at the interface, or to an alteration in the characteristics of the mixed PC–detergent micelles, as was experienced with Triton C-100 (Dennis, 1973). The enzyme may require a specific PC–SDS mixed micellar surface, or there may be interaction at the micelle–water interface between calcium ions and the excess negative groups of SDS.

Inhibition by sodium cyanide and EDTA is supporting evidence for the requirement of pea PLAH for a metallic co-factor, and inhibition by *p*-CMB and mercuric chloride may indicate the participation of sulphydryl groups in the maintenance of enzymic structural integrity. However, the inability to demonstrate activity in the presence of mercuric chloride may have been simply due to enzyme denaturation or to interference with colour formation between rhodamine and FFA.

From the results obtained in this study, with results described elsewhere on lipid-degrading enzymes in peas, a sequential hydrolytic and oxidative enzymic pathway is proposed for the degradation of endogenous lipids in fresh or unblanched frozen ungerminated peas during post-harvest storage, leading to flavour deterioration (Fig. 2).

The pathway resembles those proposed by Galliard *et al.* (1977) for disrupted tomato fruits and by Henderson *et al.* (1981) for fababean tissue disruption. There is no convincing evidence for the precise manner in which FFA may be produced from phosphatidic acid. Confirmation of an active phosphatase in peas would enable a mechanism to be proposed for the complete enzymic degradation of PC into its five component parts. It is hoped that results from this study will help to emphasise the importance of lipid-degrading enzymes in the post-harvest degradation of endogenous lipids in peas, with special reference to the preservation and maintenance of an acceptable flavour in stored peas.

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REFERENCES

Agriculture Canada (1981). *Market commentary: Horticulture and special crops.* Ed. Communications Branch.

Bengtsson, B. & Bosund, I. (1966). Lipid hydrolysis in unblanched frozen peas (*Pisum sativum*). *J. Food Sci.*, **31**, 474.

Castle, A. M. & Castle, J. D. (1981). The purification and partial characterization of phospholipase A_2, a secretory protein of rabbit parotid gland. *Biochim. Biophys. Acta*, **666**, 259.

Chakrabarty, M. M., Bhattacharyya, D. & Kundu, M. K. (1969). A simple photometric method for microdetermination of fatty acids in lipids. *J. Amer. Oil Chem. Soc.*, **46**, 473.

Dennis, E. A. (1973). Kinetic dependence of phospholipase A_2 activity on the detergent Triton X-100. *J. Lipid Res.*, **14**, 152.

Eriksson, C. E. & Svensson, S. G. (1970). Lipoxygenase from peas, purification and properties of the enzymes. *Biochim. Biophys. Acta*, **198**, 449.

Galliard, T. (1971). The enzymic deacylation of phospholipids and galactolipids in plants. Purification and properties of a lipolytic acyl-hydrolase from potato tubers. *Biochem. J.*, **121**, 379.

Galliard, T., Matthew, J. A., Fishwick, M. J. & Wright, A. J. (1976). The enzymic degradation of lipids resulting from physical disruption of cucumber (*Cucumis sativus*) fruit. *Phytochem.*, **15**, 1647.

Galliard, T., Matthew, J. A., Wright, A. J. & Fishwick, M. J. (1977). The enzymic breakdown of lipids to volatile and non-volatile carbonyl fragments in disrupted tomato fruits. *J. Sci. Fd Agric.*, **28**, 863.

Haydar, M. & Hadziyev, D. (1973). Pea lipids and their oxidation on carbohydrate and protein matrices. *J. Food Sci.*, **38**, 772.

Haydar, M., Steele, L. & Hadziyev, D. (1975). Oxidation of pea lipids by pea seed lipoxygenase. *J. Food Sci.*, **40**, 808.

Henderson, H. M., Shambrock, D. L. & Eskin, N. A. M. (1981). The enzymic deacylation of *p*-nitrophenyl esters and phosphatidylcholine in *Vicia faba* minor. *Food Chem.*, **7**, 249.

Hirayama, O. & Matsuda, H. (1972). An improved method for determining lipolytic acyl-hydrolase activity. *Agr. Biol. Chem.*, **36**, 1831.

Ikeda, K., Sano, S. & Samejima, Y. (1981). pH dependence of the binding constant of Ca^{2+} to the phospholipase A_2 of *A. lalys blomhoffii*. Participation of ionisable groups with pK values of 5·16, 6·45 and 7·30. *J. Biochem.*, **90**, 1125.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265.

Matsuda, H., Tanaka, G., Morita, K. & Hirayama, O. (1979). Purification of a lipolytic acyl-hydrolase from *Phaseolus vulgaris* leaves by affinity chromatography on palmitoylated gauze and its properties. *Agr. Biol. Chem.*, **43**, 563.

Quarles, R. H. & Dawson, R. M. C. (1969). The distribution of phospholipase D in developing and mature plants. *Biochem. J.*, **112**, 787.

Reichert, R. D. & MacKenzie, S. L. (1982). Composition of peas (*Pisum sativum*) varying widely in protein content. *J. Agr. Fd Chem.*, **30**, 312.

Teshima, K., Ikeda, K., Hamaguchi, K. & Hayashi, K. (1981). pH dependence of the binding constant of Ca^{2+} to cobra venom phospholipases A_2. *J. Biochem.*, **89**, 13.

Wagenknecht, A. C., Lee, F. A. & Graham, R. J. (1958). The preparation of lipase from peas and the action of steapsin and pea lipase on crude pea lipid. *Food Res.*, **23**, 439.

Watt, B. K. & Merrill, A. L. (1975). Composition of foods—Raw, processed, prepared. *Agriculture Handbook No. 8*, p. 43. United States Department of Agriculture.