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Plant Physiology and Biochemistry 43 (2005) 535–547
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Characterization of microsomal and mitochondrial phospholipase D activities and cloning of a phospholipase D alpha cDNA from strawberry fruits

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Received 8 December 2004; accepted 7 April 2005

Available online 13 May 2005

Abstract

Phospholipase D alpha (PLD, EC 3.1.4.4)) is a key enzyme involved in membrane deterioration that occurs during fruit ripening and senescence. The biochemical and molecular characteristics of PLD was studied in strawberry (*Fragaria ananassa* Duch) fruits, which are non-climacteric fruits. PLD activity was primarily associated with the mitochondrial and microsomal fractions and showed increased activity during development. Optimal pH levels of activity were observed at 5.5 and 6.5 for mitochondrial PLD and at 5 and 7 for microsomal PLD. Calcium enhanced microsomal PLD activity at 1–40 μM levels. PLD activity followed Michaelis–Menten kinetics. Lineweaver–Burk analysis gave K_m values in the range of 114 and 277 μM using dipalmitoylphosphatidylcholine (DPPC) as substrate for mitochondrial and microsomal PLD, respectively. The V_{max} value for the microsomal PLD was nearly 12-fold higher than that of mitochondrial PLD. A 2874 bp full-length cDNA for PLD alpha was amplified from strawberry fruit mRNA using RT-PCR and 5′- and 3′-RACE encoding an 810 amino acid-polypeptide. The predicted strawberry PLD sequence showed the characteristic C2 domain and the phospholipase domains conferring calcium sensitivity and the enzyme activity, respectively. The strawberry PLD alpha showed a high degree of similarity to other PLD alphas from plants. The implications of PLD regulation during ripening of fruits are discussed.

Keywords: Calcium; *Fragaria ananassa* Duch; Membrane; Non-climacteric fruit; Phospholipase D

1. Introduction

Membrane deterioration is an inherent feature of senescence and fruit ripening. Investigations on several aspects of membrane properties during initiation and progression of senescence have shown that membrane deterioration is mediated by the tandem action of phospholipase D (PLD), phosphatidate phosphatase, lipolytic acyl hydrolase and lipoxygenase [23,28]. PLD is stimulated by low micromolar levels of calcium as well as low pH whereas phosphatidate phosphatase is stimulated by calcium and calmodulin [23]. These observations suggest that PLD action may be an integral part of the signal transduction events leading to senescence. Ethylene treatment of carnation flower petals resulted in an inhibition of ATP-dependent calcium uptake into membrane vesicles, noticeable within 5 h of ethylene treatment that may lead to potentially high cytosolic calcium levels [29]. A decline in ATP-dependent H^+ ATPase occurred during flower development that may in turn lead to a decrease in cytosolic pH [27]. Thus, PLD action may become stimulated during

Abbreviations: CTAB, cetyltrimethylammonium bromide; DMSO, dimethylsulfoxide; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid; EGTA, ethyleneglycol bis (β-aminoethyl-ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; IAA, indole-3-acetic acid; PA, phosphatidic acid; PIP_2, phosphatidylinositol bisphosphate; PLD, phospholipase D; PMSF, phenylmethylsulfonylfluoride; PVP, polyvinylpyrrolidone; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction.

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doi:10.1016/j.plaphy.2005.04.003

ripening and senescence through increased gene expression [32], increased cytosolic calcium or decreased cytosolic pH. An increase in PLD activity was observed during senescence of broccoli florets [7]. As well, increased membrane association of PLD has been observed in response to an exposure to chilling stress in corn leaf, corn kernel membranes [26,33], and during tomato fruit ripening [32]. In general, these studies point to the possibility that an irreversible increase in cytosolic calcium above the normal excited levels (>1 μM), and a decrease in cytosolic pH would activate PLD and membrane lipid degradation, eventually causing senescence.

PLD (EC 3.1.4.4) is a key enzyme that catalyzes the hydrolysis of membrane phospholipids into phosphatidic acid (PA) and a hydrophilic headgroup [11,14]. PLD in plants was originally proposed to be important in phospholipid catabolism, initiating a lipolytic cascade in membrane deterioration during senescence and stress [23,28]. PLD could also be involved in phospholipid turnover that maintain cell viability and homeostasis [6]. Recent studies in plants indicate that PLD action plays an important role in transmembrane signaling and cellular regulation [20,32]. Activation of PLD generates messengers such as PA and subsequently leads to physiological responses.

Several isoforms of PLD designated alpha, beta, gamma and delta, that are derived from a multigene family, have been implicated in developmental processes. Full characterization of PLD beta and gamma has been achieved only in *Arabidopsis* [35]. The distinction between the various isoforms was proposed on the basis of their stimulation by PIP_2 and calcium. However, sequence comparisons indicate a high degree of homology in the proposed phosphatidylinositol bisphosphate (PIP_2) binding sites (anion binding site) [32,35] flanking the C-terminal HKD motif, one of the two active site motifs in PLD. As well, PLD alpha, beta and gamma isoforms were stimulated to various degrees by PIP_2 [35]. Perhaps, a more distinct difference between these isoforms is in the C2 domain, the N-terminal ~150 amino acid long domain that imparts calcium sensitivity to PLD [41]. The C2 domains of the beta and gamma isoforms are more hydrophobic than the alpha isoform, which results in a high efficiency binding to the membrane. Previously characterized PLDs from several sources are stimulated at micromolar levels of calcium at physiological pH [23]. Considering the abundance of PLD alpha relative to beta and gamma isoforms, it is conceivable that PLD activity in these sources is potentially due to alpha isoforms. In terms of relative activity, PLD alpha activity is nearly 1000-fold higher than the activity due to PLD beta and gamma isoforms in *Arabidopsis* [12]. More detailed structural and functional details of PLD beta and gamma isoforms from other systems are needed before precise physiological roles can be assigned to the various isoforms. In general, PLD alpha appears to be the major isoform in several systems studied [32,35,39].

Studies in our laboratory on the expression and activity of PLD alpha in tomato [32] showed several interesting features. Even though PLD gene expression was inhibited at an early stage in tomato fruits expressing an antisense PLD alpha cDNA, PLD activity continued to be detected even at the ripening stage, albeit, at a reduced level. These results show a long half-life for PLD. Thus, in tomato fruits, which are climacteric fruits, PLD expression and activities are intimately linked to development and ripening. Strawberry fruits are non-climacteric and show characteristic features of growth and development including a lack of ethylene burst and respiratory climacteric. During early stages of fruit-set and expansion, the tissue is highly responsive to auxin, as deprival of auxin by the removal of achenes can result in the total arrest of fruit expansion [21]. Strawberry fruits are highly perishable and have a short postharvest life [36]. Catabolic breakdown of cellular structures such as the membrane and the cell wall, though inherently a part of the development of ideal organoleptic qualities, can lead to the deterioration of fruits if left uncontrolled. Preservation of membrane structure and compartmentalization may help maintain the quality of the strawberry fruits as in tomatoes [22,32]. However, no studies have been conducted to understand the PLD activity in strawberry fruits. In this study, we have investigated PLD activity in strawberry fruits to understand its regulatory aspects and how such properties may differ from that of climacteric fruits such as tomato. This will help design conditions for optimal handling and storage for minimizing membrane damage and increasing the storage quality of strawberry fruits.

2. Results

2.1. Changes in PLD during fruit development and ripening

Fruit softening in strawberries has been primarily coined to the activities of polygalacturonases (PG, EC 3.2.1.15) that result in cell separation [17]. To evaluate the potential role of PLD activity in fruit development and ripening, PLD activity was analyzed at various stages of development in two strawberry cultivars, Aromas and Seascape. The results are shown in Fig. 1A, B. PLD activity was analyzed by monitoring the liberation of radiolabeled choline from DPPC provided as an external substrate [28,33]. The strawberry fruits were homogenized, subjected to differential centrifugation and fractions corresponding to mitochondrial membrane, microsomal membrane and the soluble fraction separated [26]. The soluble fraction in strawberry fruits contained very little protein and PLD activity and was not used for further analyses. By contrast, the mitochondrial fraction (15,000 × *g*) and the microsomal fraction (105,000 × *g*) consistently showed detectable levels of activity. Various stages of strawberry fruit development are designated as 1- young immature (G1); 2- young expanding (G2); 3- mature white (W); 4- turning orange stage (T) and 5- firm ripened (R) stages. In general, PLD activity was higher in "Seascape" at all stages except the mature white stage (W). There was very little change in mitochondrial PLD activity during development of "Aromas" fruits. The microso-