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Research article

Characterization and partial purification of acyl-CoA:glycerol 3-phosphate acyltransferase from sunflower (*Helianthus annuus* L.) developing seeds

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ABSTRACT

The glycerol 3-phosphate acyltransferase (GPAT, EC 2.3.1.15) from sunflower (*Helianthus annuus* L.) microsomes has been characterised and partially purified. The *in vitro* determination of activity was optimized, and the maximum value for GPAT activity identified between 15 and 20 days after flowering. The apparent Michaelis–Menten K_m for the glycerol 3-phosphate was 354 μM. The preferred substrates were palmitoyl-CoA = linoleoyl-CoA > oleoyl-CoA with the lowest activity using stearoyl-CoA. High solubilisation was achieved using 0.75% Tween80 and the solubilised GPAT was partially purified by ion-exchange chromatography using a Hi-Trap DEAE FF column, followed by gel filtration chromatography using a Superose 12 HR column. The fraction containing the GPAT activity was analysed by SDS-PAGE and contained a major band of 60.1 kDa. Finally, evidence is provided which shows the role of GPAT in the asymmetrical distribution, between positions *sn*-1 and *sn*-3, of saturated fatty acids in highly saturated sunflower triacylglycerols. This work provides background information on the sunflower endoplasmic reticulum GPAT which may prove valuable for future modification of oil deposition in this important crop.

1. Introduction

Recently, interest in controlling and manipulating the fatty acid composition of vegetable oils has increased appreciably. Attention has primarily focussed on the realization that seeds can store a wide variety of different fatty acids in their triacylglycerols (TAG) and the fact that fatty acid composition and distribution in triacylglycerol molecules determines the physical, chemical and nutritional properties of these oils.

In oil seeds, by far the most common lipid is TAG. In higher plants, the biosynthesis of these TAGs is now known to occur by sequential acylations, via a number of different routes, in the endoplasmic reticulum of the cells. The central pathway of synthesis of TAG consists of four reactions, three of them being acylations of the glycerol backbone catalysed by acyl-CoA dependent acyltransferases. In the first step, a glycerol 3-phosphate acyltransferase (GPAT; EC 2.3.1.15) catalyzes the acylation in the *sn*-1 position of the glycerol 3-phosphate to form lysophosphatidic acid (LPA). In most plants e.g. safflower [16], this enzyme shows a particular preference for saturated fatty acids such as palmitate. Secondly, a lysophosphatidate acyltransferase (LPAAT; EC 2.3.1.51) transfers a fatty acid to the *sn*-2 position generating phosphatidate (PA). The third enzyme of this pathway is the phosphatidate phosphatase (EC 3.1.3.4), which hydrolyses phosphatidic acid to yield diacylglycerol (DAG), a substrate for phospholipid and glycosylglyceride synthesis. Finally, in the only step specific to TAG synthesis, diacylglycerol is acylated at the *sn*-3 position by a diacylglycerol acyltransferase (DAGAT; EC 2.3.1.20) [14,35].

The activity of a plant GPAT enzyme was first recognized by Barron and Stumpf [2] in a microsomal fraction from the mesocarp of avocado (*Persea americana*). Subsequently, additional GPAT activity was revealed in chloroplasts from spinach [17] and in mitochondria from the endosperm of castor bean (*Ricinus communis* L.) [32]. Nowadays it is known that there are three types of GPAT in plant cells; they are localized in plastids (including chloroplasts), in the endoplasmic reticulum and in mitochondria [9,26]. GPAT in chloroplasts is a soluble protein that is localized in the stroma [17,22]. By contrast, membrane-bound forms are found in mitochondria and the endoplasmic reticulum [9]. GPAT enzymes in the chloroplast, cytoplasm and mitochondria are different proteins and they differ also in their substrate specificities. The

Abbreviations: BSA, Bovine serum albumin; CHAPS, 3-[(3–cholamidopropyl) dimethylammonio]-1-propane sulfonate; DAF, days after flowering; DAG, diacylglycerol; DAGAT, diacylglycerol acyltransferase; GPAT, glycerol 3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidate acyltransferase; PA, phosphatidic acid; TAG, triacylglycerol; TLC, Thin-layer chromatography.

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soluble enzyme uses acyl-ACP as a substrate *in vivo*; whilst the membrane-bound GPATs use acyl-CoA thioesters as substrates [10,14].

Most studies have been concerned with the soluble form of the enzyme, which has been purified from several species. Bertrams and Heinz [4] partially purified GPAT from chloroplasts of pea (*Pisum sativum*) and spinach (*Spinacia oleacea*) by ion-exchange column chromatography and isoelectric focusing. The degree of purification, in terms of the specific activity, was about 1000-fold for the enzyme from pea and 200-fold for that from spinach. The preparation from pea chloroplasts contained two isoforms. In 1987, Nishida et al. [27] purified GPAT from the chloroplasts of squash (*Cucurbita moschata*) cotyledons by ACP-affinity column chromatography and ion-exchange column chromatography. Isoelectric focusing revealed the presence of three isoforms with isoelectric points of 6.6, 5.6 and 5.5, respectively. Each of these isoforms was purified to homogeneity with the degrees of purification being 24,000-fold, 40,000-fold and 32,000-fold, respectively. Finally, the crystal structure of the soluble chloroplast acyltransferase from squash was published by Turnbull et al. [33].

Partial purification of the endoplasmic reticulum GPAT was not accomplished until much later. In 1995, Eccleston and Harwood [8] partially purified (150-fold) this enzyme from a microsomal fraction of avocado mesocarp after solubilisation with 3-3-cholamidopropyl dimethylammonio-1-propane sulfonate (CHAPS) and by affinity column chromatography with glycerylphosphorylethanolamine. In 2000, Manaf and Harwood [23] partially purified the cytosolic GPAT from oil palm (*Elaeis guineensis*) tissues after solubilisation with CHAPS. Purifications of up to 70-fold were achieved with several protein bands being present in the final preparations. To date no membrane-bound GPAT from any plant species has been identified in the databases or purified to homogeneity. In *Arabidopsis*, eight putative GPAT genes, designated from AtGPAT1 to AtGPAT8, have been described [36]. Only AtGPAT1 and ATGPAT5 have been studied in detail and none of them have shown to play a clear role in seed TAG biosynthesis [3,18].

Sunflower seed oil ranks fourth in world terms and is a major product of Argentinian, Russian and European agriculture. Traditional sunflower oil contains about 60% linoleic acid with little or no α-linolenate. Two important variants have been obtained by traditional breeding that either contain high (70–80%) oleate or an intermediate level (60%) [13]. Because sunflower oil contains TAG molecular species highly enriched in linoleate [28], characterization of the biosynthetic acyltransferases is of special interest.

In this report, GPAT activity was studied using a microsomal membrane fraction from sunflower seeds (achenes). Solubilisation and significant purification were achieved. Such studies represent a first step in obtaining detailed knowledge of the sunflower endoplasmic reticulum GPAT in seeds. The understanding of this enzyme, including the possibility of cloning the gene (or genes) responsible for this activity, will be valuable in order to modify TAG biosynthesis in seeds and, thereby, manipulate TAG species in the oil, to improve its physicochemical properties and also open the possibility of increasing oil yields.

2. Results and discussion

2.1. Characterization of microsomal sunflower GPAT

Initially, GPAT activity in sunflower seeds was examined in high-speed particulate fractions (microsomes) as GPAT activity is associated with the endoplasmic reticulum and in previous studies [8,23] the majority of the GPAT activity was found in the 105,000-*g* pellets from fruits or seeds. Several parameters were tested in these fractions with the aim of optimizing GPAT activity determinations.

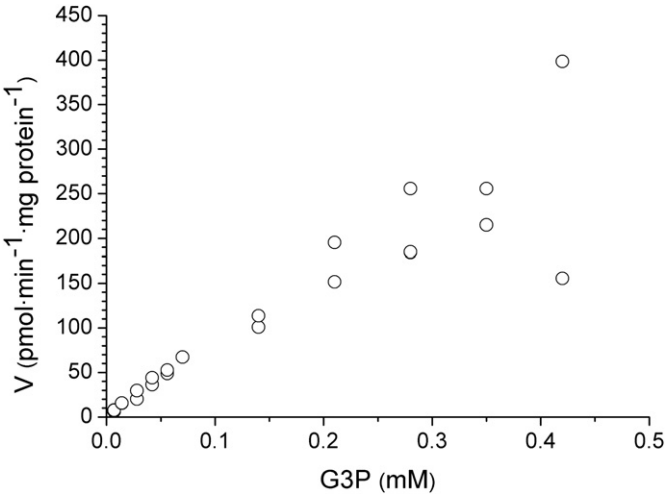

Fig. 1. Effect of glycerol 3-phosphate concentration on the incorporation of radioactivity into glycerolipids by microsomal GPAT from sunflower seeds. For the assays, 100 μM oleoyl-CoA was used as acyl donor.

Firstly, the apparent K_m for glycerol 3-phosphate in developing sunflower seed microsomes was established (Fig. 1). The results gave an apparent Michaelis–Menten K_m of about 350 μM and a maximum activity (V_{max}) of 2.08 pmol min^{-1} μg^{-1} of protein for the donor substrate. This K_m value can be compared to those estimated for other plant species. For example, the microsomal GPAT K_m of avocado was of 100 μM [8], olive 1370 μM [31], spinach chloroplast 700 μM [4], and palm callus 3400 μM [23]. In order to avoid glycerol 3-phophate becoming the limiting substrate, further GPAT assays were made using a concentration of 1.5 mM for the glycerol 3-phosphate.

Secondly, microsomal protein concentration and incubation times were evaluated. Assays with increased microsomal protein content, from 10 to 300 μg, were undertaken. The GPAT activity increased linearly from 10 to 60 μg of protein (Table 1), therefore a concentration of 50 μg protein in final assay volume of 100 μL was used in further experiments. Time course experiments were then carried out in which incubation times from 2 to 120 min were tested. Product formation increased linearly up to 20 min, hence an incubation time of 15 min was selected as optimum for assaying GPAT activity (Table 1) in sunflower microsomes.

When assaying GPAT activity it is important to consider that GPAT works only on monomeric substrates [15] and that acyl-CoAs are amphiphilic molecules, having a structure similar to surfactants. For this reason, molecules of acyl-CoA could form micelles in the aqueous solution when the acyl-CoA concentration is higher than the critical micellar concentration [1] causing very low availability of monomeric donor substrates for the microsomal GPAT. In previous studies, it was proposed that bovine serum albumin (BSA) could bind to acyl-CoAs thus avoiding the formation of micelles [16,31]. In order to determine any beneficial effect of adding additional BSA in sunflower GPAT assays, the incorporation of [^{14}C] glycerol 3-phosphate by microsomal GPAT was determined using different BSA concentrations (data not shown). It was found that

Table 1
Optimal incubation characteristics of GPAT from 15 DAF seed microsomes.

Microsomal sunflower GPAT	
Glycerol 3-phosphate K_m (μM)	354
Typical V_{max} (pmol min^{-1} μg^{-1} protein)	2.08
Protein dependence (mg ml^{-1})	Up to 0.06
Reaction linearity (min)	20
pH optimum	7.5