Pergamon

PII: S0031-9422(96)00810-2

Phytochemistry, Vol. 45, No. 8, pp. 1587–1590, 1997

0031-9422/97 $17.00+0.00

SOLUBILIZATION OF MEMBRANE BOUND Δ^{12}- AND Δ^{6}-FATTY ACID DESATURASES FROM BORAGE SEEDS

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(*Received in revised form* 21 *October* 1996)

Key Word Index—*Borago officinalis*; Boraginaceae; borage; solubilization; microsomes; Δ^{12}-desaturase; Δ^{6}-desaturase.

Abstract—Solubilization of two membrane-bound enzymes (Δ^{12}- and Δ^{6}-desaturases) involved in the biosynthesis of polyunsaturated fatty acids (linoleic 18 : 2 and γ-linolenic acids γ-18 : 3, respectively) was performed using borage seed microsomes. Of the three detergents Triton X100, sodium deoxycholate and CHAPS, the latter was found to be the most efficient for solubilization and maintaining the two desaturase activities. Solubilization was optimal with 1% CHAPS at a detergent–membrane protein ratio equal to one. Under these conditions, only 55% of the microsomal proteins were solubilized. These results are promising for further purification of the two desaturases. © 1997 Elsevier Science Ltd. All rights reserved

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INTRODUCTION

Higher plants contain various types of fatty acids differing in their degree of unsaturation [1]. Several acyl-desaturases are successively involved in the biogenesis of unsaturated fatty acids. In leaves of higher plants, acyl-desaturases were found to be located either in the plastids or in the endoplasmic reticulum (ER) [2–4]. The first desaturation step of 18-carbon fatty acids is catalysed by a chloroplast soluble desaturase that inserts a double bond at the $\omega 9$ position of stearic acid (18 : 0) bound to ACP and leads to oleoyl-ACP [5]. Stearoyl-ACP Δ^{9}-desaturase is the only soluble desaturase which has been purified so far from several plants such as safflower or avocado [5–7]. Other plant desaturases are membrane bound and introduce a second and a third double bond into acyl chains previously acylated on polar membrane lipids. Within the plastid, 18 : 1 fatty acyl chains can be further desaturated to 18 : 2 and α-18 : 3 by the successive action of n-6 and n-3 desaturases, these desaturations requiring NADH, ferredoxin and oxygen as cofactors [8–11]. On another hand, part of the 16 : 0 and 18 : 1 fatty acids synthesized in the plastid can be exported into ER. When the synthesis of phospholipid is completed in ER, 18 : 1 fatty acids are further desaturated to 18 : 2 and α-18 : 3 residues by the Δ^{12} and Δ^{15}-desaturases, respectively. There are striking similarities between the plastidial and microsomal (ER) bound desaturases, including stereochemical specificity, cyanide sensitivity, CO resistance and requirement for exogenous reductant (NADH) and molecular oxygen. Δ^{12}-desaturase is included in an enzymatic complex where cytochrome-b_5 and NADH-cytochrome-b_5 reductase are implied as electron donors [12, 13]. Some of these desaturases are absent in several mutants of plant or algae. A good biological model is *Arabidopsis thaliana* in which mutants altered in the fatty acid composition of their membrane lipids have been characterized [1, 4, 14]. In ER, mutations in two loci, *fad* 2 and *fad* 3, affect Δ^{12}- and Δ^{15}-desaturases [15–18].

In the past few years, the cloning of several cDNAs encoding plant membrane bound desaturases was successively achieved. Genes encoding the microsomal Δ^{15}-desaturase of *A. thaliana* [19–21] and tobacco [22] or the Δ^{12}-desaturase of *A. thaliana* [18], rapeseed and soybean [23] have been characterized. In contrast to the genetic approaches, biochemical methods to purify membrane bound desaturases were rather unsuccessful. A few years ago, in an attempt to solubilize a Δ^{12}-desaturase from plant microsomes, various detergents such as *n*-octylglucoside, Triton X100 or cholate were used [24, 25]. These detergents were found to inhibit Δ^{12}-desaturase activity, even after detergent removal. Two components of the enzymatic desatu-

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ration complex, cytochrome-b_5 and NADH-cytochrome-b_5 reductase, were successfully isolated by detergents from plant microsomes [26–28]. The first solubilization of a membrane bound desaturase was achieved from spinach chloroplast membranes using Triton X100 as detergent [29]. This Δ^{12}-desaturase has been successfully purified from chloroplast membranes [30]. The sequence of the cDNA encoding this desaturase, compared with other desaturases, revealed three histidine boxes highly conserved in all membrane-bound desaturases. This plastidial Δ^{12}-desaturase exhibited about 45% homology with homologous enzyme from cyanobacteria [30].

Another desaturase, Δ^6-desaturase, is able to introduce an additional double bond at C-6 of linoleic acid, leading to γ-linolenic acid biosynthesis. This fatty acid presents a growing interest for nutritional and pharmacomedical uses [31]. In animal microsomes, γ-18 : 3 is synthesized from 18 : 2 by a membranous Δ^6-desaturase which, as Δ^{12}-desaturase, is included in an enzymatic complex [32]. A cDNA encoding a Δ^6-desaturase in *Synechococcus* has also been isolated [33]. Among plants, one of the major sources of γ-18 : 3 is the oil of common borage seeds (*Borago officinalis* L.). Δ^6-desaturase activity in borage seeds and isolated microsomal membranes has been well studied. The *in vitro* biosynthesis of γ-18 : 3 was effective from radioactive 18 : 1-CoA or 18 : 2-CoA precursors [34].

In order to isolate and characterize the Δ^{12} and Δ^6-desaturases from borage seeds, it was necessary to study the effects of detergents on their activities. The present paper reports the effects of three detergents, Triton X100, sodium deoxycholate and CHAPS, on the activities of the two desaturases.

RESULTS AND DISCUSSION

In this work, the Δ^{12}-desaturase activity was studied by following the formation of radioactive 18 : 2 from [1-^{14}C]18 : 1-CoA. The Δ^6-desaturase activity was followed in parallel by measuring the radioactive γ-18 : 3 formed from newly synthesized 18 : 2, under the same conditions of incubation used as those with microsomes [34]. The effect of various detergents on the Δ^{12}- and Δ^6-desaturase activities and protein solubilization were studied on borage seed microsomes. Three detergents were selected, Triton X100 (non-ionic), sodium deoxycholate (anionic) and CHAPS (zwitterionic).

Using increasing concentrations of detergents, it was shown that CHAPS was the most efficient detergent to solubilize the Δ^{12}-desaturase activity at concentrations between 0.25 and 1% (Fig. 1A). Only Δ^{12}-desaturase activity was recovered with very low concentrations of Triton X100 and the activity rapidly decreased with higher concentrations. Sodium deoxycholate was less efficient. In the case of Δ^6-desaturase activity (Fig. 1B), CHAPS was found to be the most efficient detergent at a concentration of 1%. Δ^6-desaturase activity was higher in the solubilized fractions containing 1% of CHAPS than in the native microsomes. Triton X100 and sodium deoxycholate weakly solubilized the Δ^6-desaturase activity. Figure 1C shows the effect of detergents on protein solubilization. CHAPS and sodium deoxycholate had similar effects on the extent of solubilization of total proteins from microsomes. The optimum was obtained with 1% of detergent leading to a solubilization of about 55% of total proteins from microsomal membranes.

The use of various detergent–protein ratios showed that CHAPS was also the most efficient detergent in solubilizing and maintaining the two desaturase activities. Efficient solubilization of both desaturases was obtained with a ratio equal to one (data not shown). Finally, the concentration of 1% CHAPS with a detergent/protein ratio of one gives the optimal conditions to solubilize Δ^{12}- and Δ^6-desaturases from borage seed microsomes. No Δ^{15}-desaturase activity was recovered, in agreement with our previous results [34]. The effects of CHAPS are similar to those found with NADH-cytochrome-b_5 reductase or cytochrome-b_5 from potato microsomes [26–28]. In contrast, the Δ^6-desaturase from rat liver and the Δ^{12}-desaturase from spinach chloroplasts were solubilized with Triton X100 [29, 32]. Our results indicate that Triton X100 and sodium deoxycholate are either less efficient in solubilizing the enzymes or that they inactivate the enzymes more than CHAPS.

Increasing the centrifugation time up to 2 and 3hr confirmed that the enzyme proteins were actually solubilized and that the activities in the supernatant were not due to residual membrane vesicles. The Δ^{12}- and Δ^6-desaturase activities were well conserved and stable in the solubilized fraction. The solubilized fraction could be frozen for few days at -80° with only a small decrease of their activity. These results are in contrast to those found for the spinach plastidial Δ^{12}-desaturase which rapidly lost activity [30]. Such a high stability of the two microsomal desaturases from borage seeds is promising in order to perform further purification steps. It is of interest to note that when microsomes isolated from *Brassica napus* seeds were solubilized with the same detergent, no activity of either Δ^{12}- or Δ^{15}-desaturase was recovered (data not shown). These observations indicate that, in contrast with borage seed microsomes, membranes isolated from *Brassica napus* are not suitable for desaturase purification.

In further experiments, we tried to purify Δ^{12}- or Δ^6-desaturase from borage microsomes. In order to decrease the concentration of CHAPS before the purification steps, gel filtration (Sephadex G-25) was first used. Unfortunately all the desaturase activities were lost. In another set of experiments, the solubilized proteins were loaded onto a DEAE trisacryl column. The Δ^{12}- and Δ^6-desaturases were recovered mainly in the run-through fraction with a purification factor of 5 and 17, respectively. From this fraction, 25–30% of the microsomal proteins were recovered.

In conclusion, successful solubilization of Δ^{12}- and

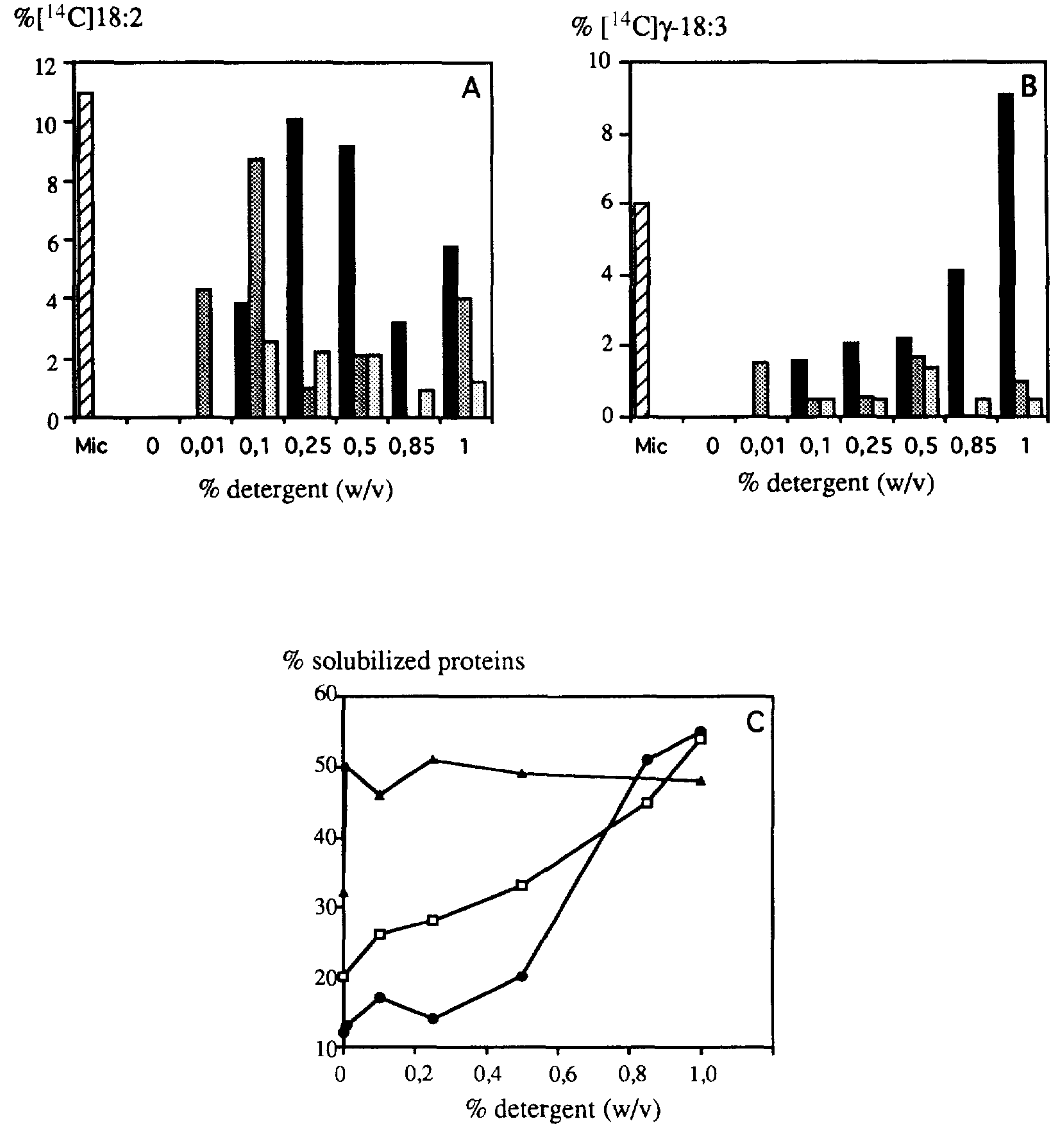

Fig. 1. Effect of increasing concentrations of CHAPS, Triton X100 and sodium deoxycholate on the Δ^{12}-, Δ^6-desaturase activities and on the protein recovery in solubilized fraction. Microsomes (▨) were solubilized in the presence of increasing concentrations of each detergent (■ CHAPS, ▩ Triton X100, ▤ sodium deoxycholate). After centrifugation at 100 000 × *g* during 90 min, the supernatant contained the solubilized proteins. The *in vitro* incubation was performed according to Galle *et al.* [34]. Results were expressed for the Δ^{12}-desaturase activity: (A) as % of radioactive 18 : 2 + γ − 18 : 3 *in vitro* synthesized/radioactive 18 : 1 + 18 : 2 + γ − 18 : 3 and for the Δ^6-desaturase; (B) in % of radioactive γ-18 : 3 *in vitro* synthesized/radioactive 18 : 1 − 18 : 2 + γ − 18 : 3 recovered in the solubilized fraction. The % of total proteins recovered in the solubilized fraction in presence of increasing concentration of detergents is shown in (C) (●— CHAPS, ▲— Triton X100, —□— sodium deoxycholate).

Δ^6-desaturases from borage seed microsomes was achieved using CHAPS as detergent. Borage microsomes are thus a highly suitable material in regards to the stability of the Δ^{12}- and Δ^6-desaturase activities recovered in the solubilized fraction. First experiments of purification of the enzymes with a DEAE-trisacryl column were promising. However, it is clear that, as established by Okayasu *et al.* [32] and Schmidt *et al.* [30], the best purification step would be an affinity column. In order to purify a Δ^{12}- or Δ^6-desaturase from borage seeds, we plan to prepare a plant cytochrome-b_5-sepharose column.

EXPERIMENTAL

Materials. *Borago officinalis* L. and *Brassica napus* seeds were obtained from the Institut National de Recherche Agronomique (I.N.R.A., Versailles, France). [1-^{14}C]oleoyl-CoA (sp. radioactivity 2Gbq mmol^{-1}) was purchased from Amersham. CHAPS, Triton X100 and sodium deoxycholate and all the other reagents were obtained from Sigma.

Solubilization of membranes. Preparation of microsomal membranes, lipid extraction and fatty acids analysis were carried out as previously described in ref. [34]. Microsomes were resuspended in buffer to final concentration of 18 mg total protein ml^{-1} phosphate 70 mM buffer, pH 7.2. In the first assay, solubilization was carried out in presence of varying amounts of detergent to reach a final concentration from 0 to 1% (w/v). Other experiments were performed with an amount of detergent calculated to obtain a detergent–total proteins ratio (w/w) from 0 to 4. After gentle stirring at 5°, 30 min, the unsolubilized membranes sedimented by centrifugation, 100 000 ×*g* 90 min. The Δ^{12}- and Δ^6-desaturase activities were then recovered in the supernatant containing solubilized proteins. Enzymic Δ^{12}- and Δ^6-desaturase activities were assayed using the same *in vitro* conditions as described in ref. [34]. Results expressed in % of [^{14}C]linoleic acid or [^{14}C]γ-linolenic acid/total [^{14}C]oleic + [^{14}C]linoleic acid + [^{14}C]γ-linolenic acids recovered. Protein concentration was measured according to Lowry *et al.* [35].

Purification. The supernatant fraction obtained after solubilization was diluted to decrease CHAPS concentration ×2 with buffer (Tris–HCl 20 mM pH 7.5 10% glycerol, 3 mM sodium azide, 10 mM β-mercapto EtOH). The diluted solubilized fraction was loaded onto a column of DEAE-trisacryl (3 × 16 cm) (Pharmacia). Elution was performed with 1 M NaCl in same buffer.

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