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Induction and localization of a lipoxygenase from *Fusarium proliferatum*

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Abstract

Biomass of the fungus *Fusarium proliferatum* was produced in liquid medium and used to obtain a crude enzymatic extract. Several methods were tested to obtain this extract: sonication, cryofracture and protoplasts disintegration. A lipoxygenase activity was found in this crude extract, which was highly induced when the fungus was grown in a medium containing soya oil rich in linoleic acid. Studies on the subcellular localization were performed using differential centrifugation techniques which allowed separation of the main organites. Homogeneity of separated fractions was ensured by measuring specific enzymatic activities, succinate dehydrogenase for mitochondrial fractions and NADH-cytochrome C reductase for microsomal fraction. The results of subcellular fractionation suggest that lipoxygenase is mainly distributed among microsomal and mitochondrial fractions.

Keywords: *Fusarium proliferatum*; Lipoxygenase; Subcellular localization; Marker enzyme; 1-Octen-3-ol

1. Introduction

Lipoxygenase has been found among plants, animals, higher mushrooms and also microorganisms. Volatile metabolites biosynthesized by fungi are mainly derived from C16 to C22 unsaturated fatty acids which are enzymatically converted into corresponding hydroperoxydes which are in turns converted into carbonyl compounds and alcohols. These natural flavor compounds derived from bioconversion by fungal biomass are particularly relevant for food industry. Although the end-products of the lipoxygenase catalysed reaction have been studied, little information is available in the literature concerning characterization and localization of lipoxygenase in fungi. The economic interest of lipoxygenase is particularly relevant for food industry. They are at the basis of biochemical pathway of flavor volatiles formation. In a previous study, *Fusarium proliferatum* lipoxygenase has been partially purified [1].

The purpose of the present work was to characterize induction and subcellular localization of *F. proliferatum* and to investigate the potentiality of a crude extract of the fungus to

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produce 1-octen-3-ol, characteristic mushroom flavor.

2. Experimental

2.1. Strain, media and culture conditions

F. proliferatum (Matsushima) Nirenberg (CBS 181.31) was cultured as already described [1] with 25 ml of commercial soya oil (50–58% linoleic acid, 5–9% linolenic acid) or glucose 10 g/l.

2.2. Homogenate preparation

Three methods for homogenate obtention were used.

(1) Sonication: 10 g of fungal biomass were resuspended in 60 ml of phosphate buffer (50 mM, pH 7) containing 0.6 M KCl (buffer A) and sonicated 3 times 5 min, 250 W, pulsed mode (Vibra cell apparatus, Sonics and materials, USA).

(2) Cryohomogenate: 10 g of fungal biomass were placed in a mortar and grinded with liquid nitrogen. The powder was suspended in 60 ml of phosphate buffer (50 mM, pH 7).

(3) Protoplasts obtention: 10 g of fungal biomass were incubated with 60 ml phosphate buffer (66 mM, pH 5.6 containing 0.6 M KCl (buffer B) and 10 mg/ml of enzymatic complex from *Trichoderma harzianum* (novozym 234, Sigma, France) containing cellulase, chitinase and protease. The medium was shaken at 140 rpm for 5 h at 30°C. The protoplast formation was checked by optical microscope. Protoplasts were then centrifuged ($3000 \times g$, 10 min, 4°C) resuspended in 60 ml of buffer A and disintegrated with a potter teflon homogenizer (220 rpm, 10 min, 4°C), (Bioblock scientific, France).

2.3. Subcellular fractionation

Fractionation was performed by differential centrifugation according to Shechter and Grossman [2].

2.4. Specific enzymatic activities measurements

Succinate dehydrogenase (SDH) was used as marker enzyme for mitochondrial fraction. SDH is strongly linked to mitochondrial inner membranes. SDH measurements were done according to Singer et al. [3]. Specific SDH activity was expressed as μmol of succinate consumed/min/mg of proteins.

NADH-cytochrome C reductase is an enzyme specific for endoplasmic reticulum. NADH-cyt. C reductase measurements were done according to Paliyath and Thompson [4]. Specific activity of NADH-cyt. C reductase was expressed as μmol of cytochrome C reduced/min/mg proteins.

Lipoxygenase activity was measured polarographically using a Clark O_2 electrode. Reactional medium was previously described [1]. Specific lipoxygenase activity was expressed as μmol O_2 consumed/min/mg of proteins.

2.5. 1-Octen-3-ol production

1-Octen-3-ol was produced and extracted from the fungal biomass and analysed by gas chromatography (GC) using the method of Wurzenberger and Grosch [5]. About 2.5 g of mushroom were blended with 15 ml 0.1 M sodium phosphate buffer containing 0.03% Tween 20, pH 6.5 with and without 1 mM linoleic acid. Extraction of volatiles was performed by shaking the mushroom homogenate with 5 ml dichloromethane and centrifuging at $10\,000 \times g$ for 10 min at 4°C. About 1 μl of the organic phase was analysed by GC.

3. Results

3.1. Induction of lipoxygenase activity

Specific lipoxygenase activity and biomass growth of *F. proliferatum* during culture (13 days) in soya oil and glucose supplemented

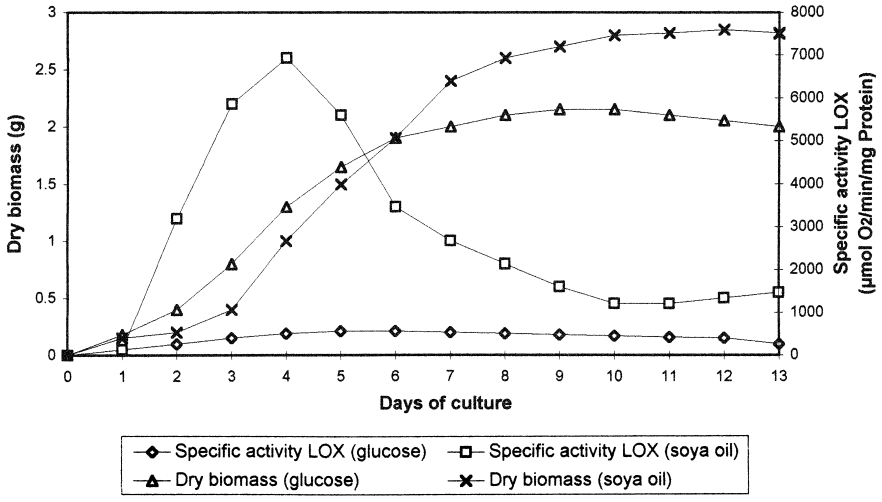

Fig. 1. Biomass and specific Lox activity of *F. proliferatum* in glucose and soya oil supplemented medium.

media was measured every day. Lipoxygenase activity was induced after 2 days although growth had already started since the first day of culture. In liquid soya oil culture medium, biomass growth is largely enhanced until 8 days, then reached a plateau until 13 days (Fig. 1). The maximum lipoxygenase activity was reached at day 4 with 6500 nmol O_2 consumed/min/mg and decreased until day 10 to reach 1054 nmol O_2 consumed/min/mg proteins. It has been demonstrated in our laboratory that *F. proliferatum* has a lipolytic activity which degrades oil and liberates fatty acids necessary both for growing and for lipoxygenase induction. In glucose supplemented medium, the maximum activity was observed between day 5 and day 7 (560 nmol O_2/min/mg proteins). This slight activity then decreased until day 13. Biomass increased at approximately the same rate as in soya oil medium but stopped growing at day 6 and stayed constant (2 g dry weight per flask) until day 13. The biomass of *F. proliferatum* was 1.4 times more important at day 8 in the medium containing soya oil than in glucose containing medium. Induction of lipoxygenase activity (13 folds) was clearly much stronger in medium containing soya oil than in glucose containing medium.

3.2. Subcellular fractionation and lipoxygenase distribution

Fungal homogenates obtained by three methods: sonication, cryohomogenate and protoplast disintegration, were used. Specific activities for marker enzymes such as succinate dehydrogenase (SDH) for mitochondria and NADH-cyt. C reductase for microsomes were measured with fractions S1, S2, C2, S3 and C3 obtained by differential centrifugation of homogenates (Table 1).

SDH activity, specifically associated with mitochondria, measured with homogenates obtained by sonication appeared to be distributed among all fractions. This method, too destruc-

Table 1
Specific activity of marker enzymes and lipoxygenase in the subcellular fractions of *F. proliferatum*

	Cryohomogenate		Protoplast disintegration		
	SDH	NADH-cyt. C red	SDH	NADH-cyt. C red	LOX
S1	0.019	0.115	0.010	0.150	0.420
S2	0.018	0.145	0.008	0.120	0.300
C2	0.045	0	0.055	0	1.020
S3	0.015	0.045	0.007	0.180	0.200
C3	0.016	0.275	0.022	0.550	2.850

Specific activities expressed as μmol substrate consumed/min/mg protein.

tive to keep organelle integrity, has been rejected for lipoxygenase localization. With the two other methods, SDH activity was principally localized in the mitochondrial fraction C2 and NADH-cyt. C reductase principally in the microsomal fraction C3. However, it is clear by comparing enzymatic marker activities that cryohomogenate is more destructive for intracellular organelle membranes. Moreover, enrichment of specific activities of marker enzymes in C2 and C3 fractions is higher with protoplasts disintegration than with cryohomogenate; 6.95 for SDH, marker enzyme of mitochondria in C2 fraction and 4.29 for NADH cyt. C reductase, marker enzyme of endoplasmic reticulum in C3 fraction against 2.81 and 2.59, respectively. If we consider that 100% of specific SDH activity is found in S1 cytosolic fraction obtained after protoplasts disintegration, 84% of SDH activity is recovered in C2 fraction and 4% in C3 fraction. These results indicate that C2 contains mainly mitochondria and that C3 is slightly contaminated with mitochondria or mitochondrial fragments. About 10.1% of lipoxygenase specific activity was recovered in microsomal fraction C3 which contains only 1.5% of total proteins from S1. Protoplasts disintegration method showed important LOX activity enrichment in microsomal fraction C3 (6.86) and also in mitochondrial fraction C2 (2.45).

3.3. 1-Octen-3-ol production

The formation of 1-octen-3-ol by a mycelial homogenate of *F. proliferatum* at two different culture times in soya oil medium was studied (Table 2). About 30.4 μg of 1-octen-3-ol per g of biomass was produced with 1 mM linoleic acid at day 4. The homogenate control without linoleic acid showed 6.7 μg of 1-octen-3-ol/g of biomass. This bioconversion of linoleic acid in 1-octen-3-ol was correlated with the maximum lipoxygenase activity found during culture (6500 nmol O_2 consumed/min/mg proteins). At 10 days of culture, 5.8 μg of 1-octen-3-ol/g of biomass was produced. This decrease of 1-octen-3-ol production is correlated and proportional (6 folds) with the decrease of lipoxygenase activity (1 054 nmol O_2 consumed/min/mg proteins).

4. Discussion

Our results show a clear induction of *F. proliferatum* lipoxygenase activity (13-fold) in liquid medium containing 48 mM linoleic and linolenic acid as compared to a medium containing 10 g/l glucose as carbon source. The induction of lipoxygenase by soybean oil has been previously described in *F. oxysporum* [6]. The authors observed a 3.6-fold increase in LOX activity on soybean oil culture medium compared to glucose supplemented medium. Other authors [7] also observed that adjunction of linoleic and linolenic acids in culture medium increases 16 fold the 1-octen-3-ol and caused an 8-fold increase of 1,5-octadien-3-ol production in *Penicillium caseicolum*. It was also reported that liquid mycelial culture of *Pleurotus pulmonarius* contained very little 1-octen-3-ol [8]. The authors showed that the presence of soya oil in the culture medium gave similar 1-octen-3-ol production level than in higher mushrooms. They concluded that this was due to induction

Table 2
1-Octen-3-ol production and lipoxygenase activity from *F. proliferatum* cultivated in soya oil medium

	Control	*F. proliferatum* + 1 mM linoleic acid	
		4 days cultured	10 days cultured
1-Octen-3-ol (μg/g Biomass)	6.7	30.4	5.8
Lox activity (μmol O_2/min/mg Protein	6500	6500	1054

of lipoxygenase synthesis by fatty acids present in the culture medium.

We have investigated the subcellular localization of *F. proliferatum* lipoxygenase by subcellular fractionation using differential centrifugation. The preparation of homogenate was very important to obtain relatively pure cellular organelle fractions. It should break the mycelial external membrane keeping as intact as possible intracellular organelles. Two mechanical methods (sonication and cryohomogenate) and one enzymatic method for the lysis of *F. proliferatum* external membrane were tested. This last method combined with Potter disintegration seemed to be more adapted to recover pure fractions. Lipoxygenase subcellular distribution was therefore studied using the last method of protoplasts disintegration followed by differential centrifugation. SDH and NADH-cyt. C reductase activities were measured to check whether each fraction was pure and specific of one type of organelle and that contaminations with other subcellular particles were slight.

The important relative activity in mitochondria (32%) is in accordance with studies previously performed using plant cells [9,10]. This mitochondrial localization has also been reported in *Saccharomyces cerevisiae* [2]. These authors also reported a cytosolic localization of lipoxygenase. We have also found a slight specific activity (0.2 μmol O_2/min/mg) in the cytosolic fraction S3. In our study, the cytosolic lipoxygenase localization could be attributed to partial liberation of membrane-associated enzymes during cell disintegration. Mitochondrial and microsomal marker enzymes (SDH and NADH-cyt. C reductase) were also present in S2 and S3 fractions.

The microsomal lipoxygenase localization represents 10% of the total activity of S1 (100%). This fraction is characterized by an important enzymatic enrichment (6.86) compared to S1 (1). The microsomal localization has also been found in tomatoes [11] and in senescing carnation petals [12]. Nevertheless, in our work, part of the specific activity can be attributed to mitochondrial contamination of the C3 fraction (4%). Total separation of mitochondria seems to be very difficult to perform and for that reason, it is very important to check the purity of each fraction by measuring marker enzymes.

Preliminary results of linoleic acid bioconversion to 1-octen-3-ol by a crude homogenate of *F. proliferatum* seemed to be at least correlated to LOX activity. An enriched LOX fraction will be very useful to characterize the intermediates in the flavor pathway and to improve 1-octen-3-ol yield. The potentiality of this fungus to produce economically interesting natural flavor will be investigated in the near future.

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