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EXPERIMENTAL ARTICLES

The Effect of the Preservative Sorbic Acid on the Lipid Composition of the Ascomycete Fungus *Penicillium roqueforti* Thom

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Abstract—The mechanism of action of potassium sorbate, a widely used food preservative on the lipid composition of the *Ascomycete* fungus *Penicillium roqueforti*, the main contaminant of cheese, was investigated. The inhibition of fungal growth by potassium sorbate was found to be associated with a change in the composition of phospholipids (a decrease in phosphatidylcholine content and an increase in phosphatidylethanolamine and phosphatidic acid content) and of neutral lipids (a decrease in the triacylglycerol and sterol content and an increase in the free fatty acid content). The fatty acid composition of fungal lipids also changed. A drastic decrease in the linoleic acid content occurred both in the total lipid fraction and in the triacylglycerol and total phospholipid fractions, whereas the oleic acid content increased correspondingly. This suggests that sorbic acid (SA) affects Δ12 desaturase activity, which controls the adaptive response of mycelial fungi to deleterious environmental factors.

Key words: mycelial fungi, *Penicillium roqueforti*, preservatives, food items, potassium sorbate, phospholipids, neutral lipids, linoleic acid.

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In recent years, much attention in the food industry has been given to measures aimed at eradicating food-spoiling mycelial fungi. Ascomycete fungi are the main contaminants affecting food production, with the dominant role played by *Penicillium* species that account for up to 80% of food-spoiling microorganisms [1]. A promising method of conserving food items such as hard cheese is based on using preservatives. The most widely used preservatives are sorbic acid (SA) and its salts [2].

Sorbic acid ($CH_3CH=CH–CH=CHCOOH$; 2,4-hexadienoic acid) is a natural compound which was originally isolated by A. Hoffman from mountain ash fruit juice over a century ago (in 1859). Subsequently, a complete chemical synthesis of sorbic acid was carried out. Forty years later, its antimicrobial properties were revealed [3]. Presently, sorbic acid is one of the main food preservatives (conservant E200).

Sorbic acid as a preservative possesses important advantages: it has virtually no effect on the taste and the odor of food items and it is nontoxic to humans at concentrations below 0.2% [2]. However, even though SA has long been used on a large scale in the food industry and its fungicide activity has been recently investigated in detail [4, 5] some aspects of its mechanism of action have not yet been elucidated.

A number of suggestions exist concerning the mechanism of action of SA. Since SA belongs to the group of unsaturated fatty acids including crotonic acid, inhibition of the activity of a number of membrane enzymes was suggested, particularly dehydrogenases [6]. Uncoupling of respiration and oxidative phosphorylation, resulting in the disruption of metabolic processes and, ultimately, in suppression of microbial growth is a possible cause of the inhibition of fungal growth [6, 7]. According to another hypothesis, SA, a lipophilic compound, affects cell membranes, disrupting the transfer of a number of compounds including amino acids [8].

The data presented in the literature suggests the conclusion that membrane structures are the sites of sorbate action on fungal metabolism. This results in a change in the activities of a number of dehydrogenases. In the light of present-day concepts, polar lipids of the bilayer membrane influence the activities of a number of enzymes [8] including desaturases. Therefore, the goal of the present work was to investigate the effect of sorbic acid on the main lipid fractions and their fatty-

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acid composition in ascomycete fungi (exemplified by *Penicillium roqueforti*) that affect food items such as hard and soft cheeses.

MATERIALS AND METHODS

The subject of our studies was the ascomycete fungus *Penicillium roqueforti* Thom VKM FW-3072 isolated from the cheese surface. *P. roqueforti* was grown on milk whey in 250-ml flasks with 50 ml of medium on a rotary shaker (220 rpm) at 27°C for 72 h (unless otherwise specified). Potassium sorbate was added to a 28-h culture at concentrations of 0.1% (8.9 mM) and 0.3% (26.8 mM).

The lipids were extracted according to [10]. The composition of the neutral lipid (NL) and polar lipid (PL) fractions was analyzed by ascending thin-layer chromatography on plates with a fixed silica gel layer (Merck, Germany). To separate the NL, we used the hexane–diethyl ether–acetic acid (85 : 15 : 1) or the petroleum-ether–diethyl ether–acetic acid (80 : 20 : 2) solvent system. To separate the PL, we employed two-dimensional TLC. We consecutively ran the chloroform–methanol–water (65 : 25 : 4) and the chloroform–acetone–methanol–acetic acid–water (50 : 20 : 10 : 10 : 5) system in the opposite directions. The amount of lipids applied to a plate was 70–120 μg.

Fraction identification was carried out using the standard lipid preparations, as well as by specific qualitative tests for the individual functional groups. For this purpose we employed the Vaskovsky reagent, the Dragendorf reagent, ninhydrin solution, α-naphthol solution, and the mixture of sulfuric and acetic acid. Phosphomolybdic acid was used as a general developer.

The quantitative assay of the fractions of individual lipids was performed using the Dens software (Lenkhrom, Russia).

To analyze fatty acids, we obtained their methyl esters [10], which were identified on a Khromateks-Kristall 5000.1 (Russia) gas–liquid chromatographer equipped with an Optima-240 (0.25 mm × 60 m)–0.25 m Macheray-Nagel GmbH & Co capillar column (Germany); the column packing was 33% cyanopropylmethyl–67% dimethylpolysiloxane.

Based on the data on the contents of individual fatty acids, the lipid properties were calculated, including the desaturation degree and the iodine number.

The statistical treatment of the results was carried out using the median (Me) method at $n = 2–3$ [11].

RESULTS

SA effect on *P. roqueforti* growth. Addition of 0.1 and 0.3 % potassium sorbate to a 28-h culture (with a biomass concentration of 1.81 g/l) caused a strong inhibition of *P. roqueforti* growth. After 72 h of cultivation, the biomass concentration in the control sample was over 8 g/l, while in the experimental samples it was lower by 79.2% and 83.4%, respectively.

SA impact on lipid formation in *P. roqueforti*. The addition of the preservative exerted a significant influence on the process of lipid formation in the fungus, which manifested itself in different dynamics of lipid formation in the control and experimental samples. The specific lipid content (percentage of the cells' dry weight) remained almost constant (about 11%) in control samples and tended to increase in SA-supplemented experimental samples. This trend was particularly manifest in a 72-h culture that demonstrated an almost twofold increase in the lipid level relative to the control sample (19.1% and 22.1% of the dry weight with 0.1% and 0.3% potassium sorbate, respectively).

SA effect on the fatty-acid composition and content of the total lipid fraction of *P. roqueforti*. A study of the composition and content of individual fatty acids in the total lipid fraction (Fig. 1) revealed that in the control sample, the cells of a 72-h culture contained predominately unsaturated fatty acids whose content was 62.23% of the total lipid's acid fraction. The monoenoic oleic acid ($C_{18:1}$) and the dienoic linoleic acid ($C_{18:2}$) accounted for almost one third (20.31%) and two thirds (39.28%) of the total unsaturated acid' fraction, respectively. Palmitic acid ($C_{16:0}$) prevailed in the saturated acid's fraction. Its content in the total fatty acid fraction (21.13%) slightly exceeded one half of the whole saturated acid fraction. The desaturation degree of the lipids was 1.03.

In the presence of both potassium sorbate concentrations, the lipid's desaturation degree decreased almost twofold. It was 0.57 and 0.49 at 0.1% and 0.3%, respectively. Hence unsaturated fatty acids tended to prevail in the lipids, and this trend was more pronounced at a higher SA concentration. With 0.3% preservative, the total unsaturated fatty acid content was 62.56% and the palmitic acid content was 33.11%, which almost 1.5-fold exceeded the control value. In addition, the myristic acid ($C_{14:0}$) content increased more than 2.5-fold. In contrast, the unsaturated acid content decreased to 37.01%, and the oleic acid content remained at the control level (23.52%). The drop in the lipid's desaturation degree was due to a drastic (almost 3-fold) decrease in linoleic acid content (to 10.93%). Hence, the results obtained testify to an influence of SA on the formation of linoleic acid.

SA impact on the phospholipid (PL) composition of *P. roqueforti*. The following lipid species were identified: phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PEA), and phosphatidic acid (PA).

The control sample contained mainly PC and PEA (Table 1). The PC/PEA ratio dropped from 1.53 to 1.32 during cultivation, while PC remained the predominant phospholipid species.

In the presence of SA, a different pattern of PC and PEA accumulation in the membrane was detected dur-

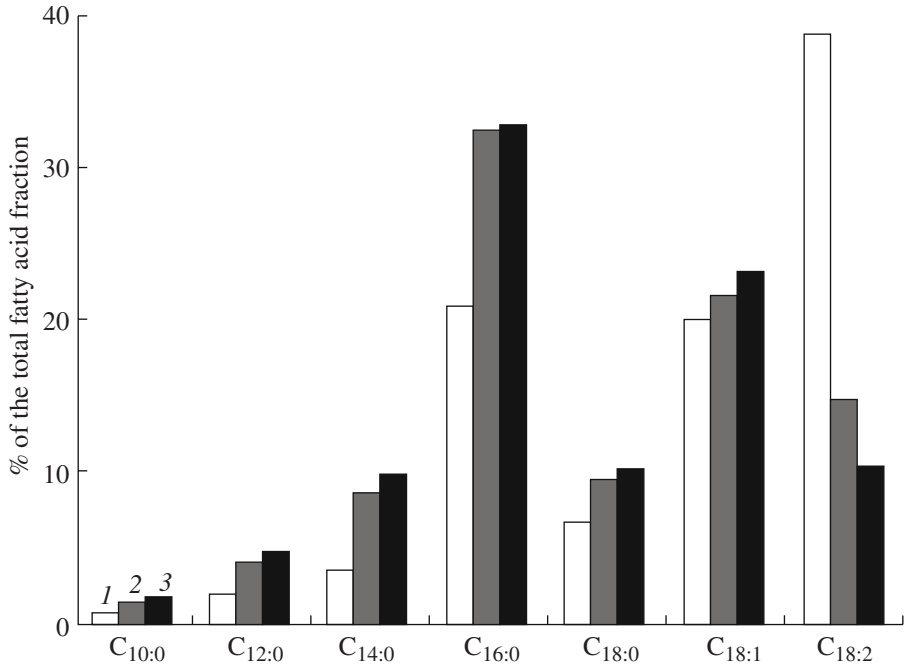

Fig. 1. The composition and content of the main fatty acids in the total lipid fraction of *P. roqueforti* grown on whey for 72 h: control (*1*); 0.1% potassium sorbate (*2*); 0.3% potassium sorbate (*3*).

ing fungal growth (Table 1). The PC content significantly decreased in the experimental sample, and this trend was particularly manifest at higher SA concentrations. The PEA level somewhat decreased after 48 h and increased after 72 h of cultivation with the preservative. For instance, the PC/PEA ratio was 0.69 at an SA concentration of 0.3%, i.e., the PEA content exceeded the PC content in the PL fraction. As for the other phospholipids, the PS content was enhanced in a 72-h culture with SA; the PG level also increased to some extent. Importantly, the PA content increased almost twofold after 72 h of cultivation, compared to the control value.

SA impact on the neutral lipid (NL) composition of *P. roqueforti.* Our research on the SA effect on fungal growth and lipid formation revealed that SA caused changes in the neutral lipid's fraction. Triacylglycerols (TAG) accounted for a half (50.52%) of the control NL fraction. Moreover, we identified the fractions of sterols (11.02%), free fatty acids (9.99%), and sterol esters (7.55%). The content of other individual fractions (of mono- and diglycerides and unidentified compounds) did not exceed 19%. In the presence of potassium sorbate, the NL composition was drastically changed (Table 2), which was more manifest at a higher preservative concentration. In the experimental samples, the

Table 1. The PL composition of *Penicillium roqueforti* grown on whey supplemented with various potassium sorbate concentrations

Type of experiment	Control			Potassium sorbate			
				0.1%		0.3%	
Cultivation time, h	28	48	72	48	72	48	72
PS	2.05	2.64	1.43	2.81	3.49	2.76	4.71
PC	48.99	47.06	44.84	48.94	38.15	31.61	24.99
PG	6.98	2.89	5.99	4.76	4.21	4.19	7.97
PEA	32.06	32.12	34.09	29.51	34.93	26.19	36.39
PA	9.92	15.29	13.66	13.98	19.21	35.26	25.94

Table 2. The NL composition of *Penicillium roqueforti* grown on whey supplemented with various potassium sorbate concentrations

Type of experiment	Control			Potassium sorbate			
				0.1%		0.3%	
Cultivation time, h	28	48	72	48	72	48	72
MAG	8.19	2.57	2.00	3.94	7.07	7.61	4.03
DAG	6.84	6.53	5.26	6.70	5.80	4.75	9.40
Sterols	14.96	12.21	11.02	9.27	9.62	9.93	9.77
X_0	3.01	1.86	1.95	1.82	2.48	2.00	2.78
Free fatty acids	16.20	10.99	9.99	5.35	10.16	10.26	18.75
X_1	1.55	3.43	4.20	2.37	3.94	2.97	2.01
X_2	10.72	5.46	5.26	8.61	10.77	11.09	7.92
TAG	32.18	47.51	50.52	56.86	43.34	43.82	39.80
Sterol esters	4.53	7.29	7.55	4.20	5.73	6.60	4.45
Hydrocarbons	1.82	2.15	2.27	0.89	1.10	0.97	1.09

content of TAG, the main NL fraction, decreased to 43.34% and 39.80% with 0.1% and 0.3% SA, respectively. Against the background of a decrease in the TAG content in the NL of experimental samples, the share of free fatty acids increased. An almost twofold increase was attained with 0.3% potassium sorbate. In addition, a decrease in sterol and sterol ester contents in the NL fraction occurred in the presence of SA.

SA impact on the fatty acid content and composition in the NL and PL fractions of *P. roqueforti.* The SA effect on fungal growth and phospholipid synthesis was more pronounced at an SA concentration of 0.3%. Therefore, our studies on the influence of SA on the fatty-acid composition of TAG, the predominant fraction of neutral lipids, and the total PL fraction were conducted with 0.3% SA in a 72-h culture.

The results obtained (Fig. 2) demonstrated that the total phospholipids fraction was more unsaturated in both the control and experimental samples. In the control samples, the unsaturated fatty acid content in the total PL fraction was 74.07%. The contents of oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) in the total fatty acid's fraction were 14.99% and 57.96%, respectively, and the $C_{18:2}/C_{18:1}$ ratio was 3.87.

In the TAG fatty acids, the content of unsaturated fatty acids reached 60.05%; the $C_{18:1}$ and $C_{18:2}$ contents were 22.67% and 35.12%, respectively, and the $C_{18:2}/C_{18:1}$ ratio was 1.55 (Fig. 3).

In the experimental sample, the fatty acid content in the TAG and the PL fractions was 32.35% and 50.44%, respectively. It follows that the PL fraction was more unsaturated in both the control and experimental samples, even though the unsaturated fatty acid content in the TAG and PL fractions was higher in the potassium sorbate-containing sample than in the control sample. The TAG fraction in the experimental sample (Fig. 3) was characterized by a drastic increase in the saturated fatty acid content (from 39.95% in the control to 67.65% in the experimental sample), which was due to an almost 3- and 1.6-fold increase in the $C_{14:0}$ and $C_{16:0}$ content, respectively. The $C_{18:0}$ content changed insignificantly. The $C_{18:1}$ content remained almost at the control level (22.91%), while the $C_{18:2}$ content decreased nearly fivefold (to 7.10%). The $C_{18:2}/C_{18:1}$ ratio was 0.31.

Research on the SA effect on the fatty acid content of the PL fraction revealed a somewhat different pattern. The increase in the saturated fatty acid content from 25.93% in the control to 49.56% in the experimental sample was due to such fatty acids as $C_{14:0}$ (an almost fourfold increase), $C_{16:0}$ (a 1.5-fold increase), and $C_{18:0}$ (a 3.5-fold increase). This was not observed in the TAG fraction.

In contrast to TAG, the total PL fraction was characterized by an increase in $C_{18:1}$ content from 14.99% in the control to 29.27% in the experimental sample. Similar to TAG, the $C_{18:2}$ content decreased albeit to a lesser extent, from 57.96% to 18.50% (3.2-fold). The $C_{18:2}/C_{18:1}$ ratio was 0.63.

DISCUSSION

It was established for the first time in this work that sorbate exerts a significant influence on (i) the composition of membrane phospholipids including the bulk lipid species PEA and PC and (ii) the sterol content. Changes in the PL composition are accompanied by a modification of their side chains. In particular, a drastic decrease occurs in the content of linoleic acid, which is the main unsaturated fungal fatty acid. It controls adaptive responses, an important aspect of metabolism. In addition, a decrease in $C_{18:2}$ level under the influence of SA is associated with an increase in oleic acid content in the

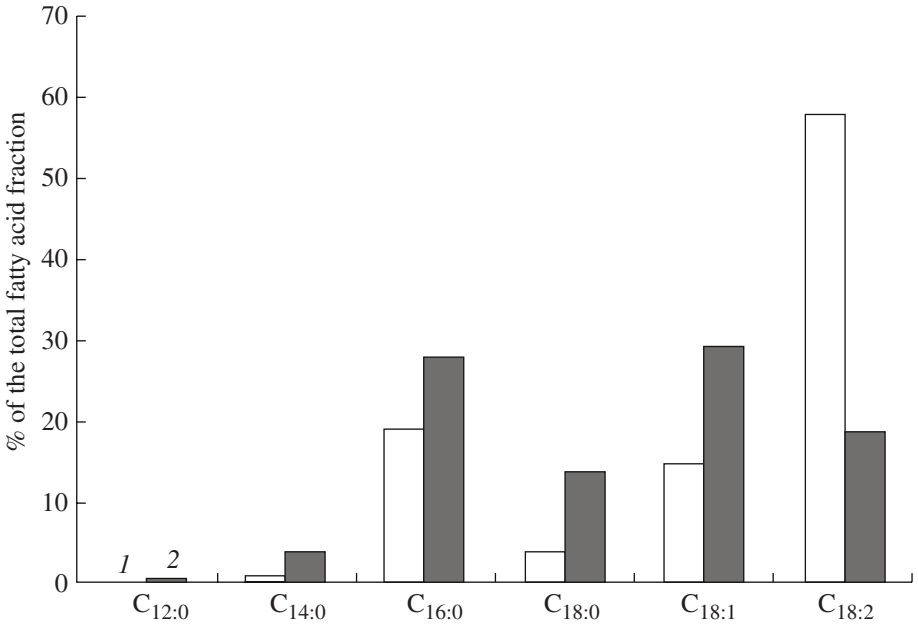

Fig. 2. The composition and content of the main fatty acids in the PL fraction of *P. roqueforti* grown on whey for 72 h: control (*1*); 0.3% potassium sorbate (*2*).

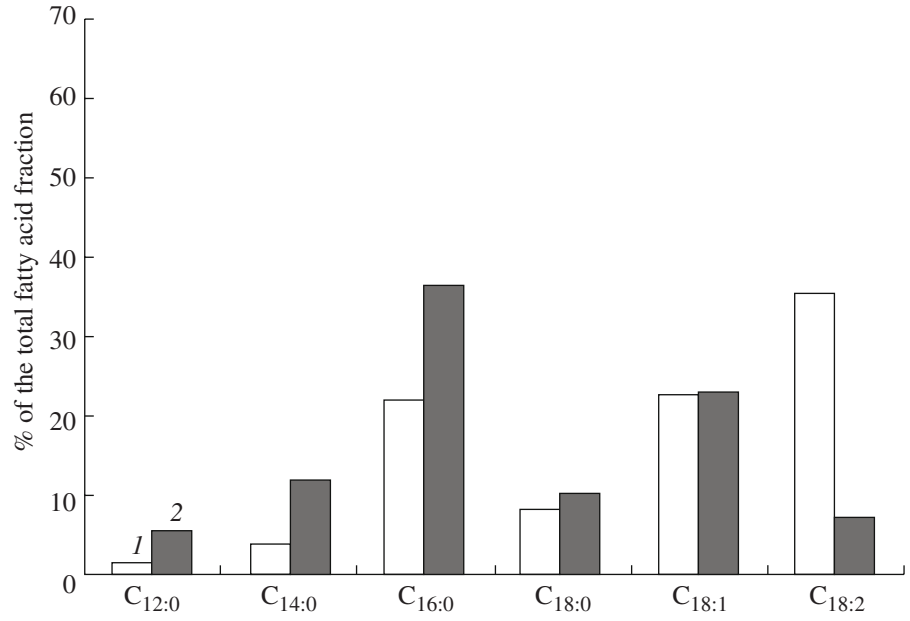

Fig. 3. The composition and content of the main fatty acids in the TAG fraction of *P. roqueforti* grown on whey for 72 h: control (*1*); 0.3% potassium sorbate (*2*).

phospholipids acyl chains. The results obtained suggest that potassium sorbate affects Δ12 desaturase involved in desaturation of oleic acid to linoleic acid [12].

According to the data presented in the literature, PL, and more specifically PC, is the site of action of Δ12 desaturase in plants [13, 14] and fungi [15–18]. Desaturation of PC oleate in the *sn-2* and *sn-1* positions plays a key role in linoleic acid synthesis, although the *sn-2* position is preferable because of its easier accessibility. Accordingly, a possible reason for a decrease in the linoleic acid content in the phospholipid fraction is a decrease in the PC content.

Interestingly, the phospholipid desaturation degree decreases and the content of $C_{16:0}$ and monoenoic fatty

acids (especially $C_{18:1}$) increases under the influence of SA. This results in significant changes in the phase state of the lipid bilayer [19]. For example, if one unsaturated bond appears in the molecule of stearic acid (T_{melt} = 60°C), this lowers its melting point by 10°C, which appreciably influences the phase state of the membrane involved.

It is known that an alteration in an organism's homeostasis caused by deleterious factors such as changes in the growth medium's composition or cultivation temperature is accompanied by changes in the percentage of various fatty acid species in PL acyl chains. Changes in the linoleic acid content occur in ascomycete fungi, particularly during the adaptation process. This enables them to quickly adjust the microviscosity of the lipid bilayer. A relationship between the fatty-acid composition of the membranes, their fluidity, and the stress resistance of yeast organisms has been demonstrated [20].

Thus, the main metabolic processes whose inhibition by SA results in growth suppression include changes in Δ12 desaturase activity. Apparently, account should be taken of the changes in the percentages of membrane bulk PL, their involvement in desaturation processes [21], and the role of the "sterol–sterol ester" adaptive system that is implicated in controlling the lipid bilayer's microviscosity and undergoes changes in the presence of the preservative.

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