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SCAVENGE RADICAL ABILITY AND INHIBITION OF LIPID AUTOOXIDATION OF EXTRACTS FROM ANTARCTIC YEAST STRAIN SPOROBOLOMYCES SALMONICOLOR AL1 BIOMASS

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ABSTRACT
In this study three analytical tests were applied for proving the antioxidant activity of the hexane and acetone extracts from biomass of Antarctic yeast strain *Sporobolomyces salmonicolor* AL1. Radical absorbance activity is established by Oxygen Radical Absorbance Capacity method. The acetone extract is fractioned using thin layer chromatography and torularhodin was isolated which displays activity of 2183.4 µmol TE/g, which activity is 98 times higher than that of the acetone extract and 8.5 times higher than the hexane extract's one. Other two analytical approaches, corresponding to different degrees of autoxidation of lipids, where used - formation of conjugated double bonds and reaction with thiobarbituric acid - indicator of byproducts formation such as aldehydes and ketones. At the highest peak of lipid oxidation (at the process' second day) which corresponds to the maximum formation of the primary and secondary products, torularhodin inhibits 32.9±0.1% of the chain reactions as well as the formation of secondary products by 33.0±0.1 %. With the increase of the time for lipoperoxidation (day 5) turolarhodin inhibition of the chain oxidizing reactions and formation of secondary products are increased to 52.0±0.1% and 46.0±0.1% respectively. The inhibitory effect of the hexane and acetone extracts is significantly less than that of torularhodin in both stages of lipid autooxidation.

KEYWORDS: *S. salmonicolor* AL1, Antioxidant activity , L-ORAC, conjugated diene formation, TBARS.

INTRODUCTION
Antarctic red colored yeast belonging to the genera Sporobolomyces and Rhodotorula owe their coloring to carotenoids. The biosynthesis of carotenoids that have the ability to eliminate oxygen radicals generated due to the UV-initiated photochemical reactions[1], is important mechanism for protection against damage to biological macromolecules which mechanism has been built by microorganisms living in places with high UV radiation.[2,3,4] The survival of the psychrophilic yeast at extremely low temperatures is associated also with the accumulation of lipids and carbohydrates at the expense of inhibiting protein synthesis[5], as well as with the regulation of the lipid composition of the cell membrane, in which long-chain polyunsaturated fatty acids prevail.[6,7] The increase in the torularhodin biosynthesis at these conditions is a defense reaction against singlet oxygen, peroxyl radicals and possible chain reaction of lipo peroxidation.[8] Torularhodin shows a considerable antioxidant activity (AOA) that helps the stabilization of membranes under stress conditions[9] and is a more effective antioxidant (AO) in relation to singlet oxygen than β-carotene.[10] Antarctic yeast strain S. salmonicolor AL1 has the potential to synthesize carotenoids as a barrier against UV radiation.[11] These metabolites in the biomass of the strain are natural AO and determination of their activity by in vitro tests expands the options for their use in various products. Particularly appropriate methods for the study of the antioxidant capacity of the lipophilic components contained in extracts of biomass of S. salmonicolor AL1 are ORAC and inhibition of linoleic acid auto oxidation. The main products from the oxidation of unsaturated fatty acids are peroxides and hydroperoxides, precursors of secondary products (aldehydes, ketones, acids). Given that lipid oxidation is multistep process, it is appropriate to necessary to analyze the effects of antioxidants in various stages of the oxidation process.[12]

The purpose of the present study is to prove the antioxidant capacity of the extracts of the biomass from Antarctic strain Sporobolomyces salmonicolor AL1 using their scavenge radical ability and inhibition of lipid autooxidizing.

MATERIALS AND METHODS

Microorganism

The yeast strain S. salmonicolor AL1 was isolated from soil lichen from the region of the Bulgarian base on Livingston Island, Antarctica. It was identified according to the yeast identification criteria of Kurtzman and Fell[13] cited by Pavlova[14] and registered in the National Bank for Industrial Microorganisms and Cell Cultures, entry N 8290.

Extraction

Extraction of well ground biomass of the strain was carried out with n-hexane or acetone on Soksle apparatus for 8h. The solvent is evaporated at 40 ° C under vacuum and the dry residue is weighed. Preparative thin layer chromatography is used for separation of carotenoids β-carotene, torulen and torularodin on plates with Silicagel G60 and mobile phase petroleum ether: diethyl ether (1:1).

Lipophilic ORAC assay

The lipophilic Oxygen Radical Absorbance Capacity (L-ORAC) assay measures the antioxidant scavenging function of lipophilic antioxidants against peroxyl radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C. Fluorescein is used as a fluorescent probe. The loss of fluorescence of fluorescein is an indication of the extent of damage from its reaction with the peroxyl radical. The detailed procedure of the assay is described by Ou et. al.[15]

Antioxidant activity in linoleic acid model system

Linoleic acid emulsions were prepared by mixing 0.285 g of linoleic acid, 0.289 g of Tween 20 as emulsifier and 50 ml phosphate buffer (pH 7.2). The mixture was homogenized for 5 min according to Yen et al.[16] The antioxidant was added at the final concentrations of 0.01% or 0.02 % w/v of oil, butilhydroxytoluene (BHT) 0.01 % was used as control. The mixture was incubated in an oven at 37 °C for 10 d. The course of oxidation was monitored by measuring the conjugated diene formation (CD) and thiobarbituric acid reactive substances (TBARS). The antioxidant activity at the end of the assay time was expressed as reduction percentage of peroxidation (RP %) for each indicator. The control containing no antioxidant was 0 %.

$$RP\% = \frac{peroxidation\ indicator\ value\ without\ AO - peroxidation\ indicator\ value\ with\ AO}{peroxidation\ indicator\ value\ without\ AO} \times 100$$

A higher percentage indicates a higher antioxidant activity.

Determination of conjugated diene formation

Aliquots of 20 µl were taken at different intervals during incubation. After incubation, 2 ml of methanol in deionised water (60 %) were added, and the absorbance of the mixture was measured at 232 nm.[17]

Determination of thiobarbituric acid reactive substances

A modified TBARS method was used to measure the AOA of oil in terms of inhibition on lipid peroxidation. 0.1 ml of sample was taken from the emulsion every day, and the following were sequentially added: the thiobarbituric acid (TBA) - trichloroacetic acid (TCA) solution (20 mM TBA in 15 % TCA). The mixture was heated in a 100 °C water bath for 15 min and cooled at room temperature. After 2 ml of chloroform were added, the mixture was mixed and centrifuged at 2000 rpm for 15 min. The chloroform layer was separated and the absorbance of the supernatant was measured at 532 nm against a blank containing TBA-TCA solution.[18]

Statistical analysis

The statistical differences between the absorbances of the emulsion of linoleic acid and linoleic acid emulsion in the presence of extracts or fractions of torularxodin (at 232 and 532 nm) were analyzed by Student's t-test. Differences showing $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Since there is no universal method for the determination of AOA several methods are used simultaneously to more fully characterize the antioxidant properties of a substance.[19] In this study three analytical tests were used. ORAC assay is widely used for the determination of the antiradical activity, while TBARS method is used for proof of lipid peroxidation by-products. However malondialdechyde (MDA) is not the only product of the decomposition of some primary and secondary products of lipid peroxidation and TBA test is not specific for MDA, as other peroxide producsts are also TBA positive.[20] TBARS method alone is not sufficient for quantification of lipid peroxidation, this is why we apply the method for detection of the formation of a conjugated 1,3 diene product from a 1,4 diene substrate. This method allows the direct detection of hydroperoxides of fatty acids, using their increased absorption at 232 nm and is the basis for most tests for lipoxygenase activity.[21]

Peroxides and alkoxide radicals are very reactive oxidants. They detach hydrogen atom from biomolecules, and this is the initial step in lipid peroxidation. The ability of an antioxidant to neutralize such radicals is most often measured by the L-ORAC method.[15] The hexane and acetone extracts of the biomass of S. salmonicolor AL1 prepared by us are tested for AOA using this method. The carotenoid components in each of the extracts are divided using thin layer chromatography and these components are identified in accordance with their UV spectra.[22] The hexane extract contains β-carotene, torulen, torularodin

in a ratio of 5: 2.5: 1, and the acetone - torularodin, torulen, β-carotene 5: 1: 1. The first extract exhibited 11.5 times the activity against peroxide radicals, compared to the second (Table 1).

Table 1. Scavenge radical ability of extracts from strain S. salmonicolor AL1biomass

Sample	ORAC, µmol TE/g
Torularhodin fraction	2183.4±54.3
Hexane extract	257.9±10.8
Acetone extract	22.3±0.9

Sakaki et al.[10] have proven the torularhodin to be effective against this type of radicals, which determined its protective role against damage from oxidative stress in Rhodotorula glutinis. This fact turned our interest towards investigating the AOA and red-colored torularhodin fraction obtained after the extracts' thin layer separation. The results showed significant activity of this fraction (2183.4 µmol/g), which is 8.5 times higher than the one of hexane extract and 98 times higher than the one of the acetone extract. In the hexane extract the β-carotene is in the highest concentrations compared to other carotenoids, and in that the ratio of the components, the extract's AOA is higher compared to one of the acetone extract. Extracts' low AOA compared with the torularhodin's one indicates the absence of synergism among the components against peroxide radicals. It is possible that other unexplored for the moment factors influence the effectiveness of antiradikal effect of the studied extracts. Dimitrova et al.[23] determine the AOA of an extract from biomass with a mixture hexane:acetone (1:1) using voltammetry method. They found that the efficiency of the total extract does not exceed that of the individual components. The research of Biacs and Daood[24] also suggest that co-administration of several AOs do not lead to improved antioxidant effect. The authors reported that the combined use of the antioxidants vitamin E and vitamin C in certain conditions does not lead to synergistic interactions against lipid oxidation. The separate introduction of these antioxidants in the system has a better effect.[24,25] These studies confirm that the AOA is a complex parameter which is not a mechanical sum of the individual activities of AOs in the studied sample.

Polyunsaturated fatty acids such as linoleic acid are easily oxidized by atmospheric oxygen. This auto-oxidation leads to chain reactions with formation of conjugated double bonds and by-products such as aldehydes, ketones and alcohols. The unoxidized linoleic acid molecules have two unconjugated double bonds and no absorbance at 232 nm. During the oxidation of lipid molecules, conjugated double bonds are formed, whereby lipid peroxides and hydroperoxides are produced, their absorbance at 232 nm increasing in relation to their concentration. Linoleic acid peroxidation caused by the formation of conjugated double bonds showed three absorbance maximums at 232nm: on the 2th, 5th and the 7th day of incubation (Fig. 1 A, B).

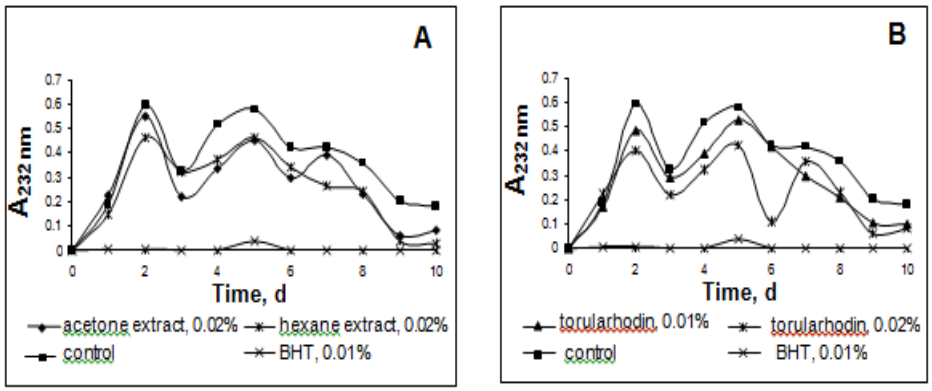

Figure 1. Inhibition of conjugated diene formation in a linoleic acid/water emulsion system by: A) acetone and hexane extracrs; B) torularhodin

It is known that AOA depends on the concentration of the AO in the sample. To establish this relationship, tested hexane and acetone extracts are added to the emulsion of linoleic acid in two concentrations, 0.01 and 0.02% (w/v). The resulting values of absorbance of primary products of lipid autooxidation in the presence of the extracts at a concentration of 0.02% were statistically distinguishable from those of the control. The extracts in concentration 0.01% showed no statistically significant inhibition of lipid autooxidizing. The hexane extract inhibits oxidative chain reactions with the formation of conjugated double bonds of the 2nd, 5th and 7th day, respectively, with 22.61%, 20.52% and 19.15%. Considerably less is the inhibitory action of the acetone extract, which on the 2nd, 5th and 7th day was 7.87, 22.41 and 7.56% (Fig. 1 A). Accordingly, inhibitory activity of the extracts manifested in the interruption of oxidative chain reactions depends on their concentration. Their AOA manifest itself only at a concentration of 0.02%. The fraction containing torularhodin at both concentrations - 0.01% and 0.02% exhibit a statistically significant inhibition of the chain reactions of lipid peroxidation (Fig. 1 B). AOA of torularhodin at concentration of 0.01% inhibited by 30.02% lipid oxidation on day 7, and at concentration of 0.02% even more effective - on the 2nd, 5th and 7th day -

inhibition is respectively 32.99, 26.72 and 52.00 %. Torularhodin, unlike the extracts, may interrupt oxidative chain reactions even at the lowest tested concentration. This confirms the available data from the scientific literature that strong antioxidants exhibit their function also at low concentrations.

The use of thiobarbituric acid as reagent shows the presence of malondialdehyde: a secondary product of the linoleic acid peroxidation. MDA is one of several products formed during the radical induced decomposition of polyunsaturated fatty acids. MDA yielded a pink colour with thiobarbituric acid, with absorption maximum at 532 nm. The control in this study shows one peak in the formation of lipid peroxidation by-products: on the 2nd day. From the day 5 increased formation of secondary products of peroxidation of linoleic acid was observed (Fig. 2 A, B, C).

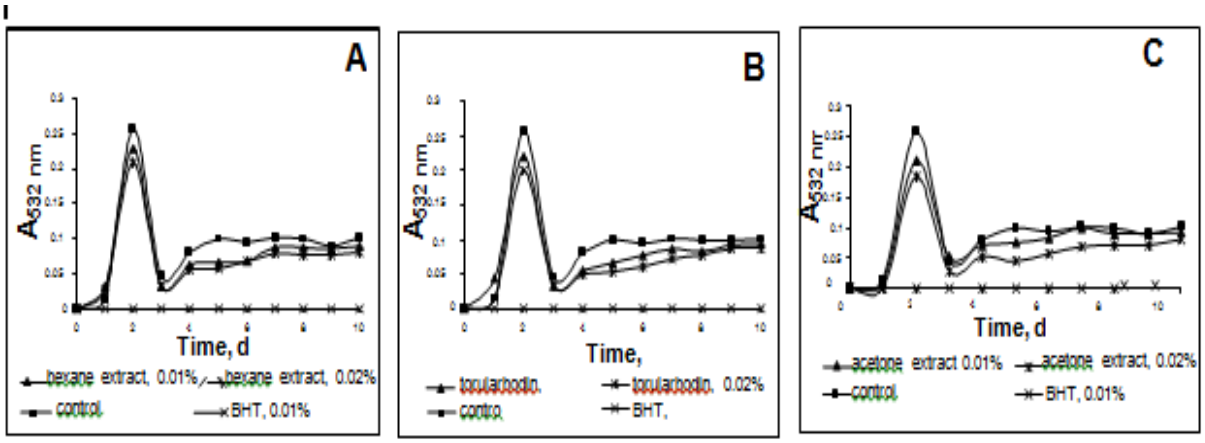

Figure 2. Inhibition of formation and production of by-products of linoleic acid by: A) hexane extract; B) torularhodin; C) acetone extract

The hexane extract inhibits the production of MDA at both concentrations used (Fig. 2 A). The obtained values of absorbance of MDA in the presence of the extract were statistically distinguishable from those of the control. Antioxidant activity of the 0.01% extract causes a decrease in the production of MDA on the 2nd (10.89%) and the 5th day (34.00%). Significantly greater antioxidant activity is exhibited by 0.02% extract. On the 2nd day of the process the inhibition of lipid peroxidation reached 18.67%, and on 5th day - 43.00%.

The presence of torularhodin in the model emulsion in concentrations of 0.01% and 0.02% inhibits the decomposition of linoleic acid related to MDA (Fig. 2 B). At the lower concentration of torularhodin the inhibition of the process of the 2nd and 5th day, respectively, reaches 33.00 and 14.39%, while at the higher concentration it is 33.00% and 46.00%.

The acetone extract exhibits antioxidant activity at both tested concentrations manifested in reduced formation of MDA by lipid peroxidation. Inhibition by acetone extract is as follows: in a concentration of 0.01% on the 2nd day - 18.28%, on the 5th day - 25.00%; at a concentration of 0.02% on the 2nd day - 28.01%, on the 5th day - 55.20% (Fig. 2 C). Synthetic AO BHT, used as a positive control in these studies, performed almost 100% inhibition of lipid peroxidation both in the production of lipid peroxides and hydroperoxides, and in production of secondary products of decomposition of linoleic acid.

Torularhodin reduces the production of MDA in the highest peak of the oxidation of linoleic acid (on the 2nd day) in a higher percentage compared to the extracts, at the same concentrations (0.02%). Inhibition of the production of MDA on the 5th day is most efficiently carried out by the acetone extract at concentration of 0.02%. Probably its effect increases in a lengthier process of lipid peroxidation. Studies on lipid peroxidation and its inhibition by a fraction with torularhodin and extracts are in accordance with the results of the L-ORAC test.

CONCLUSION

The use of various methods in research - ORAC and inhibition of lipid peroxidation prove the antioxidant activity of hexane and acetone extract of the biomass of S. Salmonicolor AL1, as well as the one of the torularhodin fraction. The torularhodin fraction displays the highest radical scavenging activity, exceeding in times that of the extracts. The components of the extracts do not exhibit synergism against peroxide radicals, which may be one of the reasons for their low AOA. The establishment of these reasons could be a topic for future research. Oxidation- prevention activity of the fraction with torularhodin occurs at a lower concentration unlike extracts. Therefore torularhodin may find application as AO in the food, pharmaceutical, cosmetic industries.

CONFLICT OF INTEREST

The authors do not have any conflict of interest related to this research.

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