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ORIGINAL PAPER

Effect of polar and non-polar carotenoids on *Xanthophylomyces dendrorhous* membranes by EPR

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Abstract The red yeast *Xanthophyllomyces dendrorhous* is one of the microbiological production systems for natural carotenoids. High-performance liquid chromatography (HPLC) and electron paramagnetic resonance spectroscopy (EPR) experiments were performed on *X. dendrorhous* membranes in order to study the effect of incorporation rates of different type of carotenoids. In the case of fluid-phase membranes, it was found that polar carotenoids, such as astaxanthin and *cis*-astaxanthin, increased the EPR order parameter and decreased the motional freedom and phase-transition temperature. In contrast the non-polar carotenoids β-cryptoxanthin and β-carotene decreased the EPR order parameter and increased motional freedom and phase-transition temperature. A noteworthy coherence was observed between the polarities of the strains and the phase-transition temperatures.

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Keywords Plasma membrane · Spin label · Phase-transition temperature · Carotenoids · Astaxanthin · β-Carotene

Introduction

Carotenoids have recently received considerable interest because of their potential for delaying or preventing degenerative diseases such as arteriosclerosis, cancer and aging (Schroeder and Johnson 1993). Epidemiological surveys suggest individuals that with low intakes of antioxidants are at higher risk of developing these diseases (Woodall et al. 1997).

Carotenoids are found in a variety of living organisms (Fraser and Bramley 2004). A great deal of knowledge on the molecular biology and regulation of fungal carotenoid biosynthesis has been obtained from investigations of fungi such as *Neurospora crassa* and *Fusarium fujikuroi* (which produce neurosporaxanthin), *Phycomyces blakesleeanus* and *Mucor circinelloides* (which produce β-carotene), *Rhodotorula mucilaginosa* (which produces torulene), and *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* (which produce astaxanthin) (Visser et al. 2003; Libkind et al. 2003; Palagyi et al. 2006; Higuera-Ciapara et al. 2006). Astaxanthin is formed via the mevalonate pathway, which starts at acetyl-CoA and proceeds via mevalonate to isopentenyl pyrophosphate (IPP), the general precursor of all isoprenoids. Subsequently eight molecules of IPP undergo condensation to form the colourless carotenoid phytoene. Via four dehydrogenations and two cyclisations, phytoene is converted into β-carotene. Finally, β-carotene is oxidised to yield astaxanthin (Visser et al. 2003), for which two alternative pathways have been postulated in *X. dendrorhous*. Both of them imply that, via asymmetric

oxidation at C-4 in one of the rings in β-carotene and in the single ring in torulene, a keto group is formed, first while oxidation to a keto group at the second C-4 in β-carotene is preceded by hydroxylation at C-3 on the same ring, yielding the intermediates 3-OH-echinenone or 3-hydroxy-3′,4′-didehydro-β-ψ-caroten-4-one from β-carotene and torulene, respectively (Andrewes et al. 1976; Johnson and Lewis 1979).

The wild-type strains of *X. dendrorhous* produce astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione). This is a potent antioxidant, the protective effect of which against peroxide radical-mediated phospholipids peroxidation may be stronger than that of β-carotene (Lim et al. 1992). It is well documented that carotenoids react rapidly with oxidising agents and free radicals. Reaction courses have been inferred from the identification of carotenoid products formed in small quantities at the end of an oxidation reaction in which most of the carotenoid has disappeared (Woodall et al. 1997).

Carotenoids protect the cell membrane from oxidation (Subczynski and Wisniewska 2000; Rengel et al. 2000). The incorporated lipid-soluble carotenoids, their localisation and their stability all determine the membrane dynamics (Niewska et al. 2003). They are located in the hydrophobic core of the bilayer of the plasma membrane and other cellular membrane components, such as the microsome and vacuole (Wataru 1991; Wisniewska and Subczynski 1998). Carotenoids' polar groups anchored in the head group region on both sides of the membrane, with their long axis perpendicular to the membrane surface, leading to a decrease in fluidity in biological membranes (Subczynski et al. 1993). The molecular structures of carotenoids are usually considered to have a modifying effect on the biomembranes, allowing them to perform different physiological functions (Goto et al. 2001). The in vitro experiments have shown that the effects of carotenoid pigments on the molecular dynamics of a lipid membrane are based on hydrophobic interactions between rigid pigment molecules and hydrocarbon lipid chains; which are undergoing continuous *gauche-trans* isomerisation, and on hydrogen-bond formation between polar groups of the xanthophylls (e.g. astaxanthin and *cis*-astaxanthin) and lipid heads (Subczynski et al. 1992). In contrast, the non-polar carotenoids, such as β-carotene and β-cryptoxanthin, do not bind with the lipid head group (Jezowska et al. 1994). These various interactions can result in the fluidisation of the well-ordered structure of the lipid membrane or rigidifying effect on a membrane in its fluid state (Subczynski et al. 1993). The fluidisation of a membrane is directly related to the increased motional freedom of lipid molecules, which is well pronounced for β-carotene, while a rigidifying effect has been reported for polar xanthophylls (Gabrielska and Gruszecki 1996).

The aim of our study was to determine the ratio of the carotenoids in the *X. dendrorhous* plasma membrane, and their influence on the plasma membrane dynamics and the phase-transition temperature in vivo. The carotenoid content and ratio in different colour mutant was determined by high-pressure liquid chromatography (HPLC). Spin-labelling method was used to study the structure and dynamic properties of *X. dendrorhous* plasma membranes in relation to temperature.

Materials and methods

Strain, culture conditions and chemicals

Xanthophyllomyces dendrorhous strain CBS 6938 was used to produce seven stable colour mutants by inducted mutagenesis (Palagyi et al. 2006). Four of them (C27, C29, C30 and C31), exhibiting different colours, were selected for further investigation. The strains were cultivated in malt extract-yeast extract broth (YM: 0.5% malt extract, 0.25% yeast extract, 1% glucose, 0.25% peptone and 1% agar, pH: 5.3) or on YM agar plates at 20°C. The sensitivity of the strains to the oxidative stressors H_2O_2 and $CdSO_4$ has been previously determined (Lee et al. 1995).

Chemicals: benzene, cadmium sulphate, 5-(4′,4-dimethyloxazolidine-*N*-oxyl)stearic acid (5-SASL), dimethyl sulphoxide (DMSO), ethanol, ethyl acetate, *Helix promatia* gastric juice (L-1419), hexane, hydrogen peroxide, mercaptoethanol, potassium chloride, sodium phosphate and *Trichoderma* lysing enzyme (L-1390) were obtained from Sigma.

Carotenoid extraction and HPLC analysis

Strains were grown for 6 days on YM agar plates. For the carotenoid extraction, the DMSO-based method was used (Sedmak et al. 1990). Cells were washed twice with deionised water, pelleted and dried at laboratory temperature. After the addition of 15 ml of DMSO preheated to 55°C, the samples were vortex-agitated for 30 s. To separate the carotenoids, 0.1 ml of sodium phosphate (0.01 M, pH 7.0) was added to 1 ml of organic solvent. For compatibility with our HPLC analysis of carotenoids, 1 ml of hexane:ethyl acetate 50:50% (v/v) (HPLC grade) was used. The organic phase was removed and the carotenoid content was determined by spectrophotometry in benzene at 450 nm. A method was developed for the identification of aldehyde, ketone, 5,6-epoxy and 5,8-epoxy groups, and *cis* double bonds with high-intensity UV/vis *cis* peaks. This strategy made use of a simple chemical reaction, such as the furanoid oxide reaction and reduction, combined with an appropriate variation of the detection wavelength. This

method was used earlier for the quantitative and qualitative determination of the individual carotenoids in *X. dendrorhous* (Deli and Molnar 2002).

HPLC: A *Dionex-580* pump, an *HP-1050* detector with *HP* ChemStation software and a *Waters 991* photodiode-array detector, and a *Chromsyl* C_{18} (6 µm end-capped) column (250 × 4.6 mm i.d.); gradient elution (in linear steps) with solvent *A* (H_2O/MeOH 12:78), *B* (MeOH), and *C* (CH_2Cl_2/MeOH 30:70): 0–2 min 100% *A*; 2–10 min to *A/B* 80:20; 10–18 min to *A/B* 50:50; 18–25 min to 100% *B*; 25–27 min 100% *B*; 27–34 min to 100% *C*; 34–41 min 100% *C*, at a flow rate of 1.25 ml/min. UV/VIS: a *Beckman DU 65 spectrophotometer*.

Preparation for electron paramagnetic resonance (EPR) spectroscopy

Six-day-old colonies were transferred into the protoplasting solution containing 0.5% (w/v) *Helix pomatia* lyophilised gastric juice, 0.5% (w/v) *Trichoderma* lysing enzyme and 2‰ mercaptoethanol in 0.7 M KCl solution of and kept at 25°C for 2 h with occasional shaking (Farkas et al. 2003).

Spin-labelling: From a stock solution (3 mg/ml in ethanol), 13 µl of 5-SASL spin label was added to 10^8 protoplast for incorporation into the membrane (Belagyi et al. 1999).

EPR measurements

The EPR spectra were recorded with an ESP 300E spectrometer (Bruker BioSpin, Germany) equipped with a 412 VT temperature regulator. The spectra arising from the fatty acid spin probe 5-SASL incorporated into the membranes were taken in the temperature range from 0 to 30°C. The conventional spectra were obtained at 20 mW microwave power and 100 kHz field modulation, with of 2.0 G; 1024 data points were recorded with of a time constant of 20 ms and the scan rate was 11.4 min. The spectra were scaled to the same peak-to-peak amplitude or normalised to an identical double integral. The spectra were characterised with the hyperfine splitting constant ($2A'_{zz}$), obtained by measuring the distance between the outermost extremes. $2A'_{zz}$ was calculated by a computer algorithm developed earlier. The computer program allows the polynomial fitting of the derivative signal at the low-field and high-field region independently of each other by a least-square procedure, estimates the extreme, and then calculates the distance between them with an accuracy of ±0.25 G.

In biological membranes molecular ordering exists, which characterized with an order parameter (*S*). It shows the mean orientation of the spin labels in the membrane and used usually to describe the effects of different substances on the membrane (Gaffney 1976; Goldman et al. 1972; Marsh 1981). The spectra at lower temperature did not give resolved turning points from which the inner hyperfine splitting could be estimated. Accordingly, the estimation of the order parameter *S* was calculated by the procedure suggested by Isralachvili et al. (1974), which is particularly useful in cases where the inner hyperfine splitting constant is not measurable from the experimental spectra:

$$S = \frac{1}{2}\left(\frac{3(A'_{zz} - A_{\perp})}{A_{zz} - A_{\perp}} - 1\right), \quad (1)$$

where $A'_{zz} \approx A'_{\parallel}$ is the outer hyperfine splitting constant measured in the experimental spectrum, $A_{zz} \approx A_{\parallel}$ and $A_{\perp} = (A_{xx} + A_{yy})/2$, where A_{xx} and A_{yy} are the principal values of the *A* tensor. The order parameters were calculated by using $A_{\parallel} = 33.6$ G and $A_{\perp} = 6.05$ G (Gaffney 1976). The above-mentioned approximation caused a systematic error in the determination of *S*, which was not taken into account during the calculation of EPR results.

In order to study the behaviour of the modified membranes, experiments were performed at different temperatures on the spin-labelled membranes. The spectra were analysed in terms of order parameter *S* against temperature. It turned out that the experimental data could not be satisfactorily fitted by a straight line (Figs. 4, 5, 6). The problem of finding the possible breakpoint of two straight lines requires the applications of statistical methods (Jones and Molitoris 1984). In accepting the existence of two straight lines in our different membrane preparations, a computer-assisted procedure was used. The computer programme compared the residual sum of squared deviations in the case of a signal straight line and two intercepting line segments. The results are included in Table 3. Statistical analysis by *F*-test showed that in all five samples the fitting resulted in a better approximation by two straight lines. The error of break point determination was ±0.2–0.3°C. This supports the suggestion of a phase change in the membrane at rising temperature. The temperature value of the breakpoint depends on the carotenoids incorporated into the membranes.

Results and discussion

Characterisation of strains

The *X. dendrorhous* is capable of producing astaxanthin, an optically active, lipid-soluble antioxidant, to protect itself from the oxidative damage caused by reactive oxygen species (Visser et al. 2003). Several stable colour mutants had been obtained previously by UV-induced mutagenesis from the well-characterised parental strain CBS 6938

(Palagyi et al. 2006, 1995). Four differently coloured mutants were selected for further investigations. Their generation times proved to be almost the same as those of their parental strain, suggesting that hidden mutation events did not influence the basic housekeeping genes. In order to acquire information on the stress behaviour of the mutants, they were grown in the presence of oxidative stressors such as hydrogen peroxide and $CdSO_4$, a glutathione-depleting agent (Halliwell and Gutteridge 1999; Pocsi et al. 2004). In agreement with earlier findings (Woodall et al. 1997; Goto et al. 2001), differences in carotenoid content and carotenoid composition resulted in altered sensitivities of the mutants to the stressors (Table 1).

In the lipid phase, carotenoids have antioxidant effects as scavengers of free radicals and quenchers of singlet oxygen. The quenching ability of carotenoids depends on the length of the conjugated polyene chain. For efficient energy transfer, at least nine conjugated double bonds are necessary (Baltschun et al. 1997). The mechanism and rate of scavenging are strongly dependent on the nature of the oxidizing radical species, but much less dependent on the carotenoid structure (Mortensen et al. 1997). Certain oxygen species also regulate carotenoid levels via the induction of synthesis and oxidative degradation of pre-existing pools of carotenoids, and through the degradation of end-products they relieve feedback inhibition of the astaxanthin pathway (Schroeder and Johnson 1993).

Identification of carotenoids

The carotenoid contents of strain CBS 6938 and its mutants can be seen in (Table 2). Carotenoids were identified by means of their chromatographic properties, using the absorption spectra. We identified polar carotenoids (astaxanthin and *cis*-astaxanthin) and non-polar carotenoids (*β*-cryptoxanthin and *β*-carotene) and small amounts of unidentified polar and non-polar carotenoids were also detected (Fig. 1).

Strains CBS 6938 and mutant C31 contained higher amounts of polar carotenoids, such as astaxanthin and

Table 1 Growth of *X. dendrorhous strain* CBS 6938 (wild type) and its mutants (C27, C29, C30 and C31) on oxidative stressors

Strain	Colour	Generation time (h)	Growth on	
			Cd^{2+} (80 μM) (%)	H_2O_2 (5 μM) (%)
CBS 6938	Red	3.69	100	100
C27	White	3.89	75	100
C29	Orange	3.73	75	100
C30	Pink	3.90	125	50
C31	Claret	3.75	75	75

Table 2 Carotenoid content, order parameter (at 18°C) and phase-transition temperature of *X. dendrorhous* parental strain CBS 6938 and its colour mutants C27, C29, C30 and C31

Strain	Weight percentage of total carotenoid content	Order parameter (*S*)	Phase-transition temperature (°C)
CBS 6938	40.5% astaxanthin	0.67	13.3
	22.7% *cis*-astaxanthin		
	12.0% unknown polar carotenoids		
	16.6% *β*-cryptoxanthin		
	3.1% *β*-carotene		
	5.0% unknown non-polar carotenoids		
C27	Not detected	0.72	15.0
C29	1.0% astaxanthin	0.54	17.7
	1.0% *cis*-astaxanthin		
	5.0% *β*-cryptoxanthin		
	85.0% *β*-carotene		
	8.0% unknown non-polar carotenoids		
C30	0.7% astaxanthin	0.56	16.0
	9.2% *cis*-astaxanthin		
	47.9% *β*-cryptoxanthin		
	0.4% *β*-carotene		
	41.6% unknown non-polar carotenoids		
C31	40.0% astaxanthin	0.67	13.7
	15.0% *cis*-astaxanthin		
	15.5% unknown polar carotenoids		
	17.0% *β*-cryptoxanthin		
	3.3% *β*-carotene		
	8.2% unknown non-polar carotenoids		

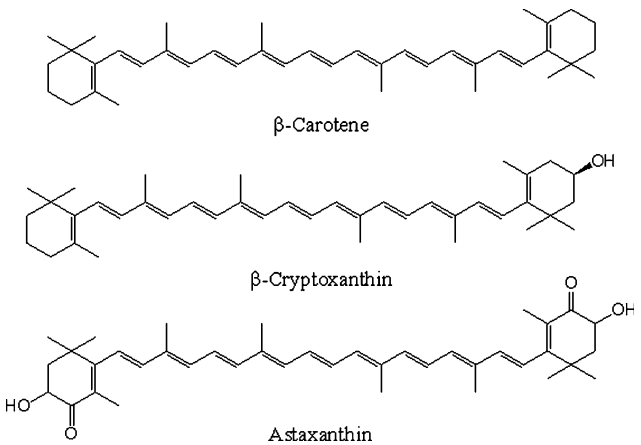

Fig. 1 Chemical structures of astaxanthin, *β*-cryptoxanthin and *β*-carotene

cis-astaxanthin, and trace amounts of unidentified polar carotenoids (Table 2). The predominant carotenoids of CBS 6938 are astaxanthin (40.5% w/v) and *cis*-astaxanthin (22.7% w/v): this is in agreement with earlier findings (Girard et al. 1994). Mutants C29 and C30 proved to contain significantly more non-polar carotenoids: β-cryptoxanthin, β-carotene and unidentified non-polar carotenoids. Carotenoids were not found in detectable amounts in the white mutant C27. In this type of mutant has previously described (Girard et al. 1994), mutations in the phytoene synthesis result in the lack of any carotenoid intermediates (Table 3).

EPR experiments

The spin-label EPR technique may be utilised to show that the various carotenoids can produce significant perturbations in the structure and function of the biological membranes: these effects depend on their chemical structure and their solubility in the membrane. The order parameter S and hyperfine splitting constant $2A'_{zz}$ are the parameters most commonly used to evaluate changes in the membranes; they can be derived from the EPR spectra (see EPR measurements). They both characterise the rotational dynamics of the incorporated probe molecules and thereby reflect the "fluidity" of the lipid regions. The term "fluidity" describes the different aspects of the molecular motions in the membrane.

The plasma membranes of protoplasts of the *X. dendrorhous* strains were labelled with 5-SASL. The probe molecules incorporated into the lipid region of the plasma membrane exhibited limited motional freedom depending on the polarity property of different carotenoids. The probe molecules incorporated in plasma membrane containing polar carotenoids (CBS 6938) have only limited motional freedom in contrast to those without carotenoid-containing plasma membrane (C27). Moreover, the spin labels in non-polar carotenoid-containing plasma membranes (C29) display larger mobility. Representative EPR spectra of our experiments can be seen in Fig. 2. The probe mobility decreased in CBS 6938 in comparison with C29, which contains mostly non-polar carotenoids (98%). This is due to the position of astaxanthin within the plasma membrane, which is located parallel to the membrane lipid chain giving the membrane well-ordered, and reduces the spin label motional freedom. In contrast, non-polar β-carotene is lying perpendicular to the membrane lipid and becomes incorporated into C5 of membrane lipid carbon. Subsequently, this particular order does not inhibit spin label motional freedom or change of order (Fig. 3).

The plot of hyperfine splitting against temperature exhibited a non-linear dependence; accordingly, a well-defined phase-transition temperature could be deduced (Figs. 4, 5, 6). Up to 30°C the distance between hyperfine splitting extreme could be estimated without any difficulty and no hysteresis was observed in the hyperfine splitting

Table 3 Computer-assisted analysis of spectroscopic data

Sample	Breakpoint, (°C)	RSS_{min} broken line	RSS_{min} single straight line	F-test[a] ($P = 0.05$)
CBS6938	13.3	0.678	1.074	$F_{[2,7]} = 51.882$
C27	15.0	1.535	9.319	$F_{[2,10]} = 25.347$
C29	17.7	4.368	18.757	$F_{[2,9]} = 14.821$
C30	16.0	0.951	8.270	$F_{[2,9]} = 34.615$
C31	13.7	0.487	7.099	$F_{[2,8]} = 54.253$

Different types of carotenoids were incorporated into the membrane of *X. dendrorhous*. After preparation the membranes were spin-labelled with fatty acid spin probes and EPR spectra were taken at different temperatures in the range from 0 to 30°C. From the spectra the relevant parameters (hyperfine splitting constant $2A'_{zz}$ and order parameter S) were calculated and plotted as a function of temperature

[a] F-test was calculated to compare the fit of a broken line or a single straight line by the residual sum of squares (RSS). The broken lines gave significantly better fit to measured spectroscopic data ($2A'_{zz}$ or S against T) than the single straight lines (Jones and Molitoris 1984)

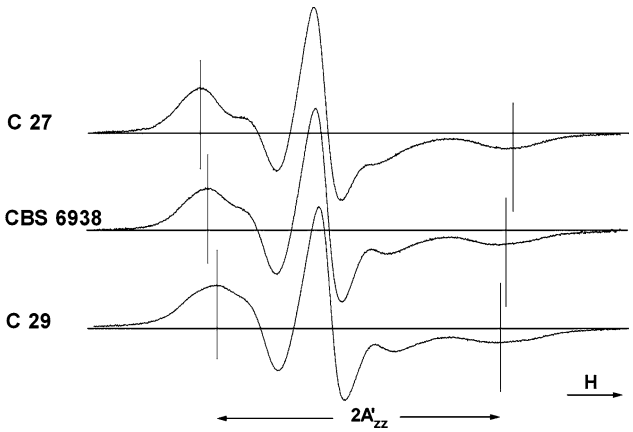

Fig. 2 EPR spectra of plasma membranes (labelled with 5-SASL) of the parental strain CBS 6938 and its carotenoid mutants (C27 and C29) at 10°C. The field scan was 100 G

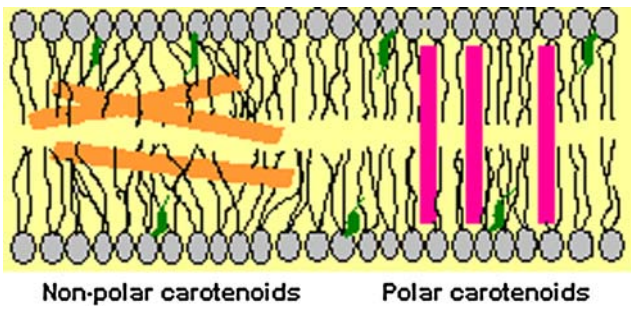

Fig. 3 Localization and orientation of polar and non-polar carotenoids within the biological membrane

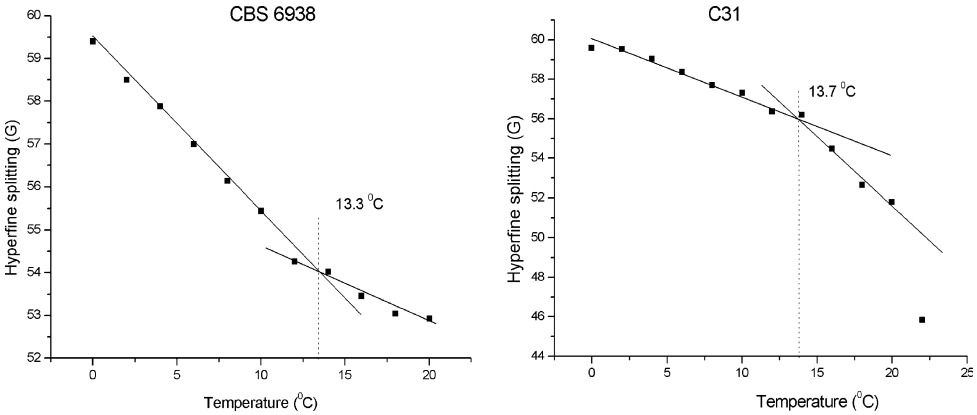

Fig. 4 Plot of hyperfine splitting (G) for the parental strain CBS 6938 and its C31 colour mutant of *X. dendrorhous* (labelled with 5-SASL) as a function of temperature

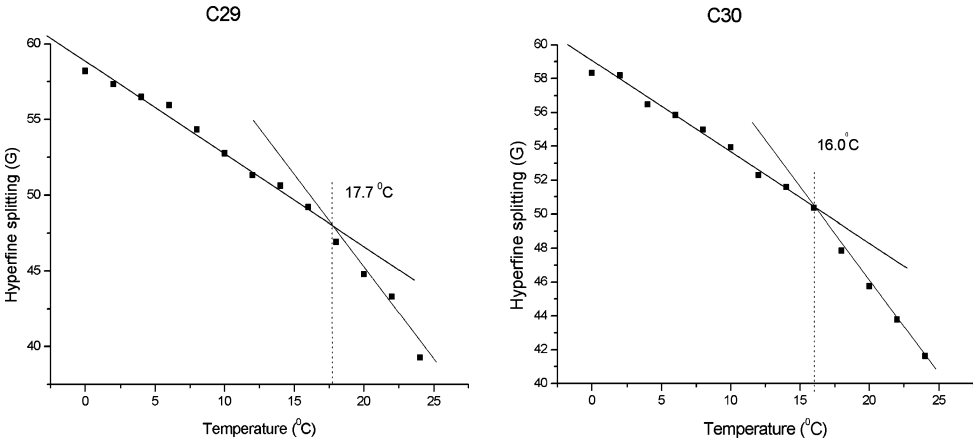

Fig. 5 Plot of hyperfine splitting (G) for mutants C29 and C30 of *X. dendrorhous* CBS 6938 (labelled with 5-SASL) as a function of temperature

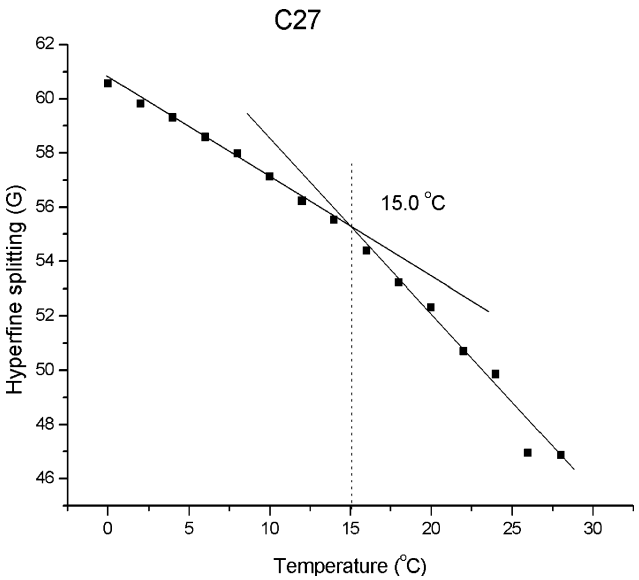

Fig. 6 Plot of hyperfine splitting (G) for the carotenoid non-producing mutant (C27) of *X. dendrorhous* (labelled with 5-SASL) as a function of temperature

constants. At higher temperatures, carotenoids have much less effect and above 30°C almost no outermost components are detected, which is a sign of the increased rotational mobility of the probe molecules in the membrane. The mobility of the probe molecules within the plasma membrane is highly dependent on the temperature and the type of carotenoid.

A noteworthy coherence was observed between the polarities of the mutants and the phase-transition temperatures. Since the mutants contained various carotenoids, their phase-transition temperatures did not match exactly on the linear line. The strain CBS 6938, containing the largest amount of polar carotenoids (75.2%), had the lowest phase-transition temperature (13.3°C), while the highest temperature was measured in the case of mutant C29, which had a high non-polar carotenoids (98%) concentration (17.7°C). The mutant C31 contained slightly more non-polar carotenoid than the parental strain, which explains its higher phase-transition temperature (13.7°C). Due to its structure, the *cis*-astaxanthin interaction with the lipid hydrocarbon chain is slightly disturbed; consequently, it increases the order less than that for astaxanthin, and as a result decreases the phase-transition temperature even less than that for astaxanthin. The mutant C30 with β-cryptoxanthin has a higher polarity than that of β-carotene and presumably produces a stronger van der Waals interaction with the head group: It therefore has less effect on the phase-transition temperature (16.0°C) than in the case of β-carotene (Fig. 7).

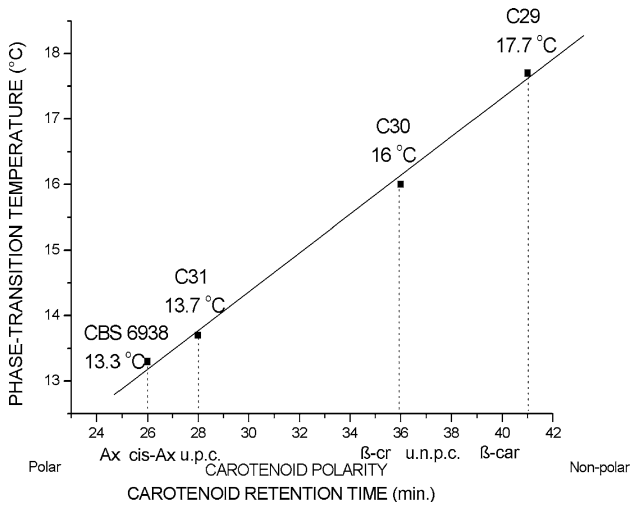

Fig. 7 Phase-transition temperature dependence of carotenoid retention time (min) of *X. dendrorhous* parental strain CBS 6938 and its colour mutants C27, C29, C30 and C31. The polarity of the carotenoids changes from polar to non-polar. (*Ax* astaxanthin, *cis-Ax cis*-astaxanthin, *u.p.c.* unknown polar carotenoids, *β-cr β*-cryptoxanthin, *u.n.p.c.* unknown non-polar carotenoids, *β-car β*-carotene)

The phase-transition temperature correlates with the polarity of the plasma membrane carotenoids. Non-polar carotenoids increase the phase-transition temperature, while polar carotenoids decrease it. The results of the present study prove that strains containing the highest levels of astaxanthin had the lowest phase-transition temperatures, and the highest temperatures were measured for mutants with the highest *β*-carotene contents.

According to our measurements the HPLC retention time was determined by the polarity of carotenoids, and it was coherent to the phase-transition temperature. The polar carotenoids of CBS 6938 strain (containing most polar carotenoids) eluted on HPLC column up to 26 min and showed lowest phase-transition temperature (13.3°C). On the other hand non-polar carotenoids of C29 strain (containing most non-polar carotenoids) eluted completely in 38 min on the HPLC column with highest phase-transition temperature (17.7°C) (Fig. 7).

The degree of tumbling motion of probe molecules between the membrane lipid hydrocarbon chains and the binding of the surrounding carotenoids in the plasma membrane is reduced as the temperature decreases. Consequently, the mobility of the incorporated probe molecules is reduced in parallel with decreasing temperature. The astaxanthin-containing membrane is therefore rigid, having stable structure while the *β*-carotene-containing plasma membrane proved to be fluid due to its less stable structure. This indicates that astaxanthin and *cis*-astaxanthin have a rigidifying effect on the plasma membrane, whereas *β*-carotene and *β*-cryptoxanthin exert a fluidising effect; this is not observed in mutant C27 where the carotenoid synthesis is inhibited.

In the eukaryote cell, the polarity of the carotenoids regulates the membrane fluidity by generating a hydrophobic barrier in the membrane to polar molecules and ions, and also a barrier to small non-polar molecules (Schroeder and Johnson 1993; Woodall et al. 1997; Schroeder and Johnson 1995). Astaxanthin and *cis*-astaxanthin are localised perpendicularly in the plasma membrane, protruding into the head groups of both the inner and outer lipids. Consequently, this allows the protection of the alkyl chains from the adverse effects of the oxidants. The *β*-carotenes and *β*-cryptoxanthin have a weaker ability to protect the membrane as they bond at an oblique angle in the plasma membrane and do not anchor to either head group (Jezowska et al. 1994) (Fig. 3). The lack of polar groups in the *β*-carotene molecule prevents it from interacting with the polar regions of the bilayer in order to bind with the long axis of the pigment perpendicular to the membrane plane. As a consequence of the repelling forces between the lipid head groups and the pigments in the polar region of the membrane, the oblique localisation of non-polar *β*-carotene and *β*-cryptoxanthin is highly likely. This effect leads to a considerable mismatch in the well-ordered lipid bilayer structure, which is directly related to the presence of the hydrocarbon carotenoid.

The effects of *β*-carotene and *β*-cryptoxanthin pigment are more marked in the hydrophobic core where all the CH_2 groups are located, as proved by the higher mobility of the spin-labelled molecules (C29). The high mobility of the CH_2 groups of fatty acid indicates that the same free space has been created in the network of well-ordered lipid molecules due to a *gauche-trans* isomerisation (temperature effect) or mismatch (*β*-carotene effect). Astaxanthin and *cis*-astaxanthin increase the order in lipid bilayer membranes and result in decreased motional freedom of the spin label (CBS 6938). Thus, the localisation and orientation of *β*-carotene, *β*-cryptoxanthin and other carotenoid species in a membrane should take into account when determining a membrane's dynamic properties (Jezowska et al. 1994; Strazalka and Gruszecki 1994).

References

Andrewes AG, Phaff HJ, Starr MP (1976) Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry 15:1003–1007

Baltschun D, Beutner S, Biriviba K, Stenhorst F (1997) Singlet oxygen quenching abilities of carotenoids. Liebigs Ann/Recueil 1887–1893

Belagyi J, Pas M, Raspor P, Pesti M, Pali T (1999) Effect of hexavalent chromium on eukaryotic plasma membrane studied by EPR spectroscopy. Biochim Biophys Acta 1421:175–182

Deli J, Molnar P (2002) Paprika carotenoids: analysis, isolation, structure elucidation. Curr Org Chem 6:1197–1219

Farkas N, Pesti M, Belagyi J (2003) Effect of hexavalent chromium on the plasma membranes of sensitive and tolerant mutants of *Schizosaccharomyces pombe*. An EPR study. Biochim Biophys Acta 1611:217–222

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265

Gabrielska J, Gruszecki WI (1996) Zeaxanthin (dihydroxy-β-carotene) but not β-carotene rigidifies lipid membranes: a ^{1}H-NMR study of carotenoids-egg phosphatidylcholine liposomes. Biochim Biophys Acta 1285:167–174

Gaffney BJ (1976) Practical consideration for calculation of order parameters for fatty acid or phospholipids spin labels in membranes. In: Berliner LJ (ed) Spin labeling: theory and applications, vol 1. Academic Press, New York, pp 567–571

Girard J, Falconnier B, Bricout J, Vladescu B (1994) β-Carotene producing mutants of *Phaffia rhodozyma*. Microbiol Biotechnol 41:183–191

Goldman SA, Bruno GV, Freed JH (1972) Estimating slow-motional rotational correlation times for nitroxides by electron spin resonance. J Phys Chem 76:1858–1869

Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E, Terada H (2001), Efficient radical trapping at the surface and inside the phospholipids membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. Biochim Biophys Acta 1512:251–258

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. Oxford University Press, Oxford

Higuera-Ciapara I, Felix-Valenzuela L, Goycoolea FM (2006) Astaxanthin: a review of its chemistry and applications. Crit Rev Food Sci Nutr 46:185–196

Isralachvili J, Sjönsten J, Ericsson LEG, Mhrström M, Graslund A, Ehrenberg A (1974) Theoretical analysis of the molecular motion of spin labels in membranes. ESR spectra of labeled *Bacillus subtilis* membranes. Biochim Biophys Acta 339:164–172

Jezowska I, Wolak A, Gruszecki WI, Strzalka K (1994) Effect of β-carotene on structural and dynamic properties of model phosphatidylcholine membranes. II. A ^{31}P-NMR and ^{13}C-NMR study. Biochim Biophys Acta 1194:143–148

Johnson EA, Lewis MJ (1979) Astaxanthin formation by the yeast *Phaffia rhodozyma*. J Gen Microbiol 115:173–183

Jones RH, Molitoris BA (1984) A statistical method for determining the breakpoint of two lines. Anal Biochem 141:287–290

Lee J, Dawes I, Roe JH (1995) Adaptive response of Schizosaccharomyces pombe to hydrogen peroxide and menadion. Microbiology 141:3127–3132

Libkind D, Brizzio S, Broock M (2003) *Rhodotorula mucilaginosa*, a carotenoid producing yeast strain from a Patagonian high-altitude lake. Folia Microbiol 49:19–25

Lim BP, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K (1992) Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipids peroxidation. Biochim Biophys Acta 1126:178–184

Marsh D (1981) Electron spin resonance: spin labels. In: Grell E (ed) Membrane spectroscopy, Springer, Berlin, pp 52–198

Mortensen A, Skibsted LH, Sampson J, Rice-Evans C, Everett SA (1997) Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. FEBS Lett 418:91–97

Niewska AW, Draus J, Subczynski WK (2003) Is a fluid-mosaic model of biological membranes fully relevant? Studies on lipid organization in model and biological membranes. Cell Mol Biol Lett 8:147–159

Palagyi Zs, Nagy Á, Vágvölgyi Cs, Ferenczy L (1995) A new mutation protocol for obtaining mutants of the yeast *Phaffia rhodozyma*. Biotechnol Technol 9:401–402

Palagyi Zs, Linka TB, Papp T, Vágvölgyi Cs (2006) Isolation and characterization of *Xanthophyllomyces dendrorhous* mutants with altered carotenoid content. Acta Aliment Hung 35:223–228

Pocsi I, Prade RA, Penninclex MJ (2004) Glutathione, a metabolite in fungi. Adv Microb Physiol 49:1–76

Rengel D, Díez-Navajas A, Serna-Rico A, Veiga P, Muga A, Milicua JCG (2000) Exogenously incorporated ketocarotenoids in large unilamellar vesicles. Protective activity against peroxidation. Biochim Biophys Acta 1463:179–187

Schroeder WA, Johnson EA (1993) Antioxidant role of carotenoids in *Phaffia rhodozyma*. J Gen Microbiol 139:907–912

Schroeder WA, Johnson EA (1995) Single oxygen and peroxyl radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. J Biol Chem 270:18374–18379

Sedmak JJ, Weerasinghe DK, Jolly SO (1990) Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. Biotechnol Technol 4:107–112

Strazalka K, Gruszecki WI (1994) Effect of β-carotene on structural and dynamic properties of model phosphatidylcholine membranes. I. An EPR spin label study. Biochim Biophys Acta 1194:138–142

Subczynski WK, Wisniewska A (2000) Physical properties of lipid bilayer membranes: relevance to membrane biological functions. Cell Mol Biol Lett 47:613–623

Subczynski WK, Markowska E, Gruszecki WI (1992) Effect of polar carotenoids on dimyristoylphosphatidylcholine membranes: a spin-label study. Biochim Biophys Acta 1105:97–108

Subczynski WK, Markowska E, Sielewiesiuk J (1993) Spin-label studies on phosphatidylcholine-polar carotenoids membranes: effect of alkyl-chain length and unsaturation. Biochim Biophys Acta 1150:173–181

Visser H, Ooyen AJJ, Verdoes JC (2003) Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*. FEMS Yeast Res 4:221–231

Wataru M (1991) Biological functions and activities of animal carotenoids. Pure Appl Chem 63:141–146

Wisniewska A, Subczynski WK (1998) Effect of polar carotenoids on the shape of the hydrophobic barrier of phospholipids bilayers. Biochim Biophys Acta 1368:235–246

Woodall AA, Britton G, Jackson MJ (1997) Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. Biochim Biophys Acta 1336:575–586