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The examination of polysaccharides as potential antioxidative compounds for topical administration using a lipid model system

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Abstract

Aim of this study was the detection of polysaccharides with antioxidative properties as potential lipid protectors for topical administration. The effects of eight different polysaccharides on UV irradiation induced lipid peroxidation were investigated in a concentration dependent manner. An aqueous linolenic acid dispersion was used as an in vitro test system to examine the influences of acacia gum, agar agar, alginic acid, guar gum, novelose 330 and xanthan gum on the lipid peroxidation level after UV exposure. Four different samples of pectin and locust bean gum resulting from a swing mill grinding series were tested as well. Iron ions were added as transition metal catalysts. A UV irradiation device was used to create high level radiation. The amount of lipid peroxidation secondary products was quantified by the thiobarbituric acid assay detecting malondialdehyde. All of the tested polysaccharides showed antioxidative effects at least at one concentration. For acacia and xanthan gum, a concentration dependency of the protective effects was measured. The samples of agar agar, guar gum and novelose 330 acted antioxidatively without showing any concentration dependency. For alginic acid, prooxidative effects were determined. A correlation between grinding time and the effects of pectin and locust bean gum on the model lipid was not observed. The administration of lipid protective polysaccharides in cosmetic formulations or sunscreens could be helpful for the protection of the human skin against UV induced damage. In vivo experiments with the lipid protective polysaccharides found in this study should follow.

Keywords: Polysaccharides; Oxidative stress; Topical application; Antioxidants; Thiobarbituric acid assay

1. Introduction

The human skin is constantly exposed to ultraviolet radiation, oxygen and other noxious environmental influences (Thiele, 2001). These factors can lead to premature skin ageing, mutation and skin cancer (Cunningham, 2000; Sander et al., 2002; Ichibashi et al., 2003). The outermost layer of the human skin,

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the stratum corneum, determines its barrier function (Wertz, 2000). Peroxidation of the intercellular lipid matrix of this layer caused by UV radiation is harmful and causes loss of function (Shibimato, 1994).

Therefore, active protection is needed for the largest organ of the human body and new powerful substances for topical administration are required for this. One opportunity to find novel sunscreening agents for skin protection is the isolation of antioxidants occurring in marine UV extremophilic organisms and their test as chemopreventives in vivo (Dunlap et al., 1999). Polysaccharides could be advantageous for this task as well because they are also naturally occurring substances with no or minor adverse effects (Tombs and Harding, 1998). Recently, lipid protective effects of the glycosaminoglycan hyaluronic acid were determined by our group and the polysaccharide and its fragments from enzymatic degradation were suggested to be used in semisolid formulations as skin protective agents (Trommer et al., 2003b). The hyaluronic acid fragments with lower molecular weights showed lipid protective properties as well and the incorporation of them into creams or ointments should be facilitated.

Continuing our investigations on polysaccharides in this study five different concentrations of the polysaccharides acacia gum, agar agar, alginic acid, guar gum, novelose 330, xanthan gum, locust bean gum and pectin were examined for the revelation of the antioxidative behaviour of these carbohydrates.

Since polyunsaturated fatty acids are the main components found in the cell membranes of biological tissues (Reis et al., 2003) and a main part of the epidermal stratum corneum lipid matrix (Wertz and van den Bergh, 1998) we used an aqueous linolenic acid dispersion as a simplified stratum corneum in vitro model for the monitoring of the peroxidative changes (Trommer et al., 2001).

An irradiation chamber, allowing selective well-defined UV exposure, was used for ultraviolet irradiation (Trommer et al., 2002).

For the evaluation of the amount of the oxidative damage the thiobarbituric acid (TBA) assay detecting malondialdehyde (MDA) as a classic lipid peroxidation secondary product was employed (Trommer et al., 2003a,b). It is the most widely used method for the quantification of peroxidative lipid damage (Janero, 1990).

Transition metal catalysts play a key role in UV induced lipid peroxidation and skin damage. To guarantee the comparability to the in vivo situation, iron ions were added to each sample before irradiation. UV radiation is able to increase the skin pool of non-heme iron (Fe^{II}) in dermis and epidermis significantly (Guy et al., 1999). This is an indirect pathway leading to oxygen radicals in addition to a direct biomolecule attack by UV light. The involvement of iron ions in UV irradiation induced free radical formation in the skin was confirmed by Buettner and Jurkiewicz (1996). The treatment of skin samples with an iron chelator resulted in a significant reduction of a spin adduct after irradiation which supports the key role of iron in UV mediated free radical formation.

Eight different polysaccharides were tested in this study for their properties to prevent a linolenic acid dispersion acting as a stratum corneum lipid model from UV induced oxidative damage. For an exact evaluation, these experiments were carried out in a concentration dependent manner in the presence of iron ions as transition metal catalysts.

2. Material and methods

2.1. Reagents

α-Linolenic acid (LLA), ferrous sulfate, malondialdehyde-bis-(dimethylacetal), 2-thiobarbituric acid and trichloroacetic acid for the thiobarbituric acid assay (all of analytical grade) as well as guar gum were obtained from Sigma (Deisenhofen, Germany). Acacia gum was provided by Colloides Naturels International (Frankfurt/Main, Germany). Agar agar was supplied by Setexam (Kenitra, Maroc). Xanthan gum was purchased from Jungbunzlauer (Vienna, Austria). Alginic acid was obtained from Serva (Heidelberg, Germany). Novelose 330 came from National Starch & Chemical (Hamburg, Germany). The swing milled samples of pectin and locust bean gum were provided by the Department of Food Chemistry and Preventive Nutrition (German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany). An overview providing further information about source and composition of the polysaccharides used for the experiments is given in Table 1. The E numbers

Table 1
The polysaccharides used for the experiments

Polysaccharide	Source	Composition	E-Number
1. Acacia gum	Exudate from stems and branches of *Acacia senegal* and *Acacia seyal*	Mixture of arabinogalactan oligosaccharides, polysaccharides and glycoproteins	E 414
2. Agar agar	Obtained from red seaweed species (*Gelidium* and *Gracilariae*)	Mixture of agarose and agaropectin	E 406
3. Alginic acid	Product of brown seaweeds *Phaeophyceae* (mainly *Laminaria* species)	Unbranched polymers containing β-(1-4)-linked D-mannuronic acid and α-(1-4)-linked L-guluronic acid residues	E 400
4. Guar gum	Obtained from the seed of *Cyamopsis tetragonoloba*	Galactomannan consisting of α-(1-4)-linked β-D-mannopyranose backbone with branchpoints from their 6-positions linked to α-D-galactose	E 412
5. Novelose 330	Resistant starch used in the food industry		
6. Xanthan gum	Microbial polymer prepared commercially by aerobic submerged fermentation from *Xanthomonas campestris*	α,β-(1–4)-D-glucopyranose glucan backbone with side chains of -(3-1)-α-linked D-mannopyranose-(2-1)-β-D-glucuronic acid-(4-1)-β-D-mannopyranose on alternating residues	E 415
7. Pectin	Acidic structural polysaccharide in fruit and vegetables, prepared from citrus peel and apple pomace	Polymers of galacturonic acid linked by α-(1-4)-linked bonds where the carboxyl groups are methylated to varying degrees and rhamnose residues are present in the linear chain	E 440
8. Locust bean gum	Extracted from the seed of the carob tree (*Ceratonia siliqua*)	Galactomannan consisting of α-(1-4)-linked β-D-mannopyranose backbone with branchpoints from their 6-positions linked to α-D-galactose (i.e. 1-6-linked α-D-galactopyranose)	E 410

as the code numbers of the European commission for food additives are listed in the table as well.

Methanol of gradient grade and chloroform (LiChrosolv®) were purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

The lipid model system used for the experiments was an oil in water dispersion of LLA. The samples were obtained after shaking the dispersion for 120 min using the laboratory flask shaker GFL 3006 (Gesellschaft für Labortechnik, Burgwedel, Germany).

The concentration of the polysaccharide stock solutions was 1%. The irradiation of the 5 ml samples was started after 15 min incubation time.

The final concentrations used in this study for the test substances are given in figures of Section 3.

Ferrous sulfate (10 μM) was added to the samples as an electron donor and catalyst of the Haber–Weiss reaction to initiate reactive oxygen species (ROS) generation via a Fenton type reaction. Previous investigations have shown that there is no effect when irradiating lipid model systems without transition metal catalysts (Trommer et al., 2001).

The fatty acid dispersions and test substance solutions were freshly prepared just before use.

2.3. UV irradiation

UV-B irradiation was carried out using a UV irradiation chamber (Dr. Gröbel UV-Elektronik, Ettlingen, Germany) enabling a selective exposure to UV-B because of the special lamp F15/T8 15 W with a main emission range of 290–320 nm (Sankyo Co., Tokyo, Japan). Prior to irradiation, 5.0 ml sample (polysaccharide concentrations as given in figures) were transferred to 55 mm open glass dishes. The optical path length was 2.1 mm and a homogeneous exposure was assumed in spite of light scattering.

The samples were treated with an UV-B dose of 0.25 J/cm^2 which corresponds approximately

with the 10-fold of the minimal erythema dose (MED) of normal pigmented (type II in the skin type classification) people (Kindl and Raab, 1998). This high dosage was required to provoke stress conditions.

2.4. Thiobarbituric acid assay

The thiobarbituric acid test is a quantitative assay for the detection of MDA, and is the most widely used technique to determine lipid peroxidation products (Trommer et al., 2003b; Schaffazick et al., 2005). In this study, the Buege–Aust method of the TBA assay was applied (Buege and Aust, 1978). Briefly, 2.0 ml of a stock TBA reagent containing 15% (w/v) trichloroacetic acid in 0.25 M HCl and 0.37% (w/v) thiobarbituric acid in 0.25 M HCl were added to 1.0 ml of the UV-B treated sample. After heating at 90 °C for 15 min and cooling down, the red TBA:MDA complex (2:1) appears allowing fluorescence measurement. A HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an auto sampler AS-4000A, interface D-6000A, pump L-6200A, UV–vis-Detector L-4250, fluorescence detector F-1080 was used to quantify the pigment. A reversed phase column (LiChrospher® 100, RP 8, particle size 5 μm) was used with a mobile phase methanol/water 30:70 for HPLC procedure. The excitation wavelength was 515 nm and the emission measurement was performed at 555 nm.

A calibration curve was generated using MDA which was formed from malondialdehyde-bis-(dimethylacetal) under acidic conditions.

2.5. Statistical analysis

All data shown represent the mean values ± S.D. of sextuple measurements ($n = 6$). Statistical analysis of the effects of the different polysaccharides on the TBA-RP concentration after UV-B irradiation was performed using a one-way ANOVA.

In all cases, post hoc comparisons of the means of individual groups were performed using Dunnett's multiple comparison test. A significance level of $P < 0.05$ between groups was accepted as being statistically significant. All calculations were performed using GraphPad Prism 2.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Acacia gum

Fig. 1A shows the results of the thiobarbituric acid assay when adding acacia gum in different concentrations. The effects of 0.002, 0.004, 0.04, 0.08 and 0.4% acacia gum on the LLA dispersion after UV exposure are shown. Secondary lipid peroxidation products (measured as malondialdehyde units) were significantly decreased after the addition of acacia gum. This abatement shows concentration dependency in the investigated range. The highest protection capacity was measured for the samples with the highest amount of acacia gum. In the samples containing 0.4% acacia gum, the TBA level after irradiation was decreased from 130 ng/ml for the samples without any additives to 40 ng/ml for the polysaccharide containing samples.

Biochemical effects of pharmaceutically used adjuvants have already been shown by Bachmann et al. (1978), where several substances including arabic gum caused uncoupling of oxidative phosphorylation in liver and heart mitochondria and partial inhibition of mixed function oxidases. After feeding the hydrocolloids guar gum, locust bean gum and acacia gum to weanling rats an increase in caecal microbial enzyme activity was measured (Mallett et al., 1984). There are several examples in the literature where the connection between arabic gum application and the following reduction of oxidative stress was observed. Arabic gum has been shown to protect mice from acetaminophen induced hepatotoxicity (Gamal el-din et al., 2003). Former investigations of this research group resulted in protective effects after oral arabic gum administration on gentamicin induced nephrotoxicity in rats. It was concluded that the gum protected the rats at least in part by inhibition of the production of oxygen free radicals that cause lipid peroxidation (Al-Majed et al., 2002). Furthermore, the effect of arabic gum on doxorubicin induced cardiotoxicity in mice has been examined showing cardio protection by the polysaccharide as well (Abd-Allah et al., 2002). This protective effect was accompanied by a decrease of cardiac lipid peroxides and the confirmation that arabic gum was able to scavenge both enzymatic and non-enzymatic generated superoxide anion radicals. This oxygen radical scavenging properties were explained to be responsible, at least in part, for the

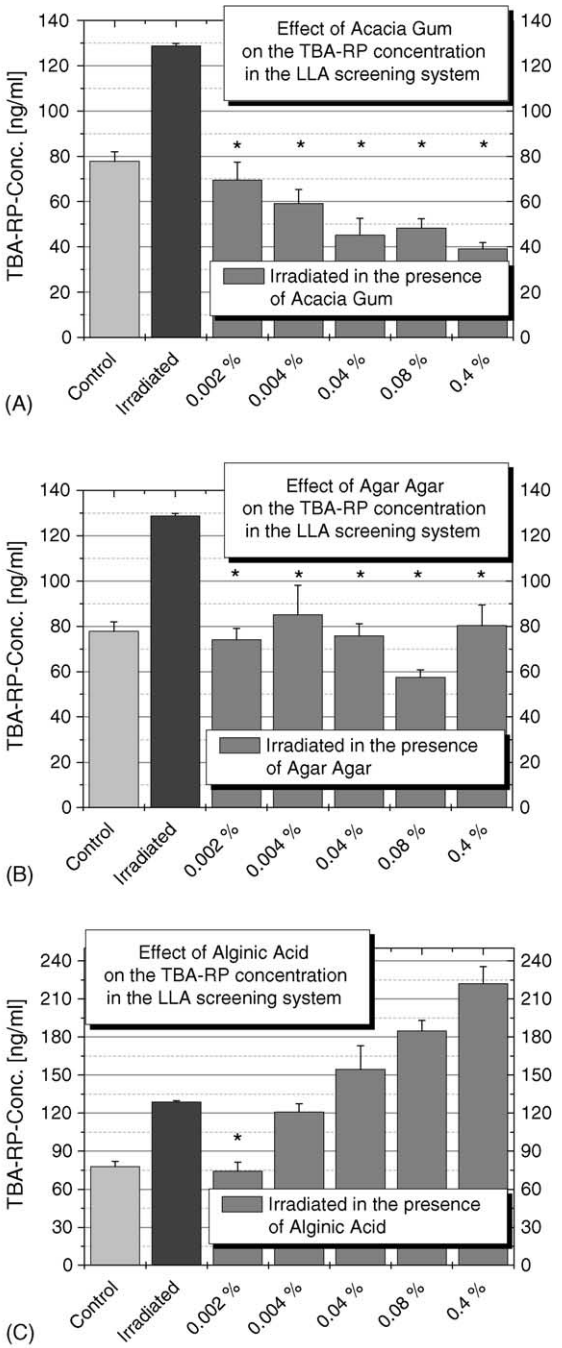

Fig. 1. Concentration of the thiobarbituric acid reaction products (TBA-RP conc.) and influence of ultraviolet irradiation and polysaccharides in the LLA system: (A) effects of acacia gum; (B) effects of agar agar; (C) effects of alginic acid.

protection from doxorubicin induced cardiotoxicity by arabic gum. Our results confirm this explanation.

3.2. Agar agar

In Fig. 1B, the TBA results for the experiments with agar agar are visualized. Agar is along with carageenans and furcellaran a red seaweed polysaccharide and consists of a mixture of agarose and agaropectin. Agar is the least soluble member of this group and forms the strongest gel (Tombs and Harding, 1998). Agar agar was shown to exhibit antioxidative effects after irradiation treatment of the LLA system as well. In contrast to the acacia gum experiments, a linear concentration dependency of this effect was not observed for agar agar in the amounts tested in this study. The most effective protection was determined when using an agar agar concentration of 0.08%. The lipid peroxidation level was reduced in this case from 130 to 58 ng/ml.

3.3. Alginic acid

The effects of alginic acid on the TBA level after UV irradiation are illustrated in Fig. 1C. The lowest concentration of the marine polysaccharide alginic acid used in this study (0.002%) was able to decrease the amount of lipid peroxidation products significantly. The TBA level was decreased by the seaweed polymer from 130 ng/ml for irradiation without alginic acid to 75 ng/ml for irradiation in the presence of 0.002% polymannuronic acid. 0.004% alginic acid did not affect the TBA level of the simplified stratum corneum lipid model after irradiation. For all the other concentrations of alginic acid tested in this study (0.04, 0.08 and 0.4%) prooxidative effects were measured. The lipid damage correlated with the concentration of the polysaccharide in a linear manner. A therapeutic potential of alginates for the treatment of various inflammatory disorders associated with an increase of endothelial leukocyte adhesion molecules was deduced by Son et al. (2001). They demonstrated that highly mannuronic acid containing alginates were able to inhibit the gamma irradiation induced expression of various adhesion molecules and NO production in human endothelial cells. In a following study, the inhibition of TNF-alpha induced ICAM-1 expression and of NO and H_2O_2 production by alginate has been

shown as well (Mo et al., 2003). These effects were explained to be due to a modulation of the generation of reactive oxygen or reactive nitrogen species.

In the present study, only the samples containing 0.002% alginic acid showed a lipid protective potential, and therefore, only this concentration is suitable for topical administration to prevent the skin from reactive oxygen species attack.

3.4. Guar gum

In Fig. 2A, the lipid protective effects of guar gum are demonstrated. Guar gum is a product of the guar plant *Cyamopsis tetragonoloba* and is used particularly in the food industry as a thickener and stabiliser. All the concentrations of the galactomannan used in this study were able to protect the unsaturated model lipid from UV induced damage. This protection showed no linear correlation to the applied amount of polysaccharide in the observed concentration range. Galactomannan concentrations of 0.002–0.08% were able to reduce the TBA level down to the malondialdehyde content measured for the unexposed lipid samples. The additionally tested polysaccharide concentration of 0.4% had less antioxidative power. A TBA level decrease from 130 to 105 ng/ml was achieved in this case.

Rat experiments revealed a protective effect of guar gum against stress-induced gastric ulcers in vivo (Harju and Sajanti, 1991). These findings are in correspondency with our results obtained by the in vitro experiments.

3.5. Xanthan gum

The effects of a concentration range of the microbial polysaccharide xanthan gum are presented in Fig. 2B. Figure shows that the lipid protection of LLA from UV induced damage by xanthan gum is more effective when the polysaccharide is added to the samples in higher amounts. A linearity of the effect is visible in the figure.

Xanthan gum is an anionic polysaccharide consisting of glucose, mannose, glucuronic acid, acetate and pyruvate in a defined stoichiometric ratio. *Xanthomonas campestris* cultures excrete the polymer when kept under adequate fermentation conditions. The treatment of bioreactor cultures of *X. campestris* with the reactive oxygen species hypochlorous acid

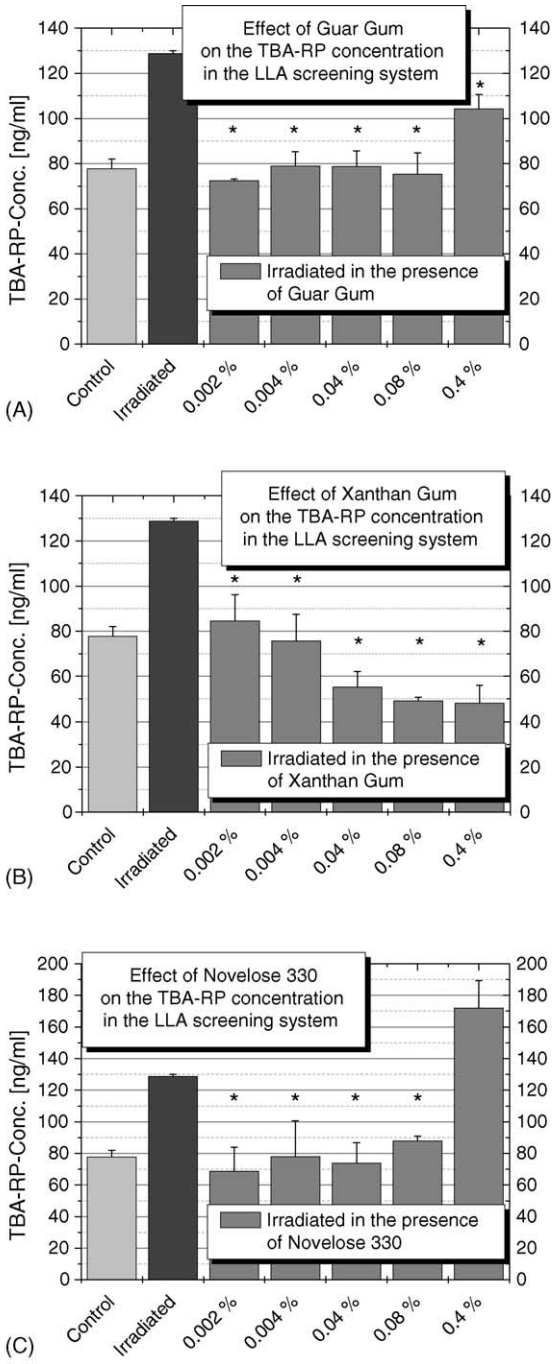

Fig. 2. Concentration of the thiobarbituric acid reaction products (TBA-RP conc.) and influence of ultraviolet irradiation and polysaccharides in the LLA system: (A) effects of guar gum; (B) effects of xanthan gum; (C) effects of novelose 330.

(HOCl) resulted in the improvement of both the bioreactor productivity and the quality of xanthan gum (Rao and Sureshkumar, 2001). Xanthan gum showed no adverse dietary or physiological effects in man (Eastwood et al., 1987). The degradation of the xanthan macromolecule by hydrogen peroxide in the presence of ferrous ions (Fenton system) was examined by Christensen et al. (1996). The degradation resulted in a more than 10-fold reduction of the molecular weight. These fragments are easier to handle and the incorporation into semisolid formulations for topical administration as aimed by the authors should be improved. Thus, future investigations should deal with the testing of these degradation products and also of enzymatically digested xanthan fragments to find out whether they still own the antioxidative properties of their macromolecular counterparts. This was shown for hyaluronan and its fragments by our group recently (Trommer et al., 2003b).

3.6. Novelose 330

Novelose 330 is a commercially developed so-called resistant starch. These kinds of starch are able to escape digestion in the small intestine but are fermented in the large intestine by bacterial microflora (Yue and Waring,

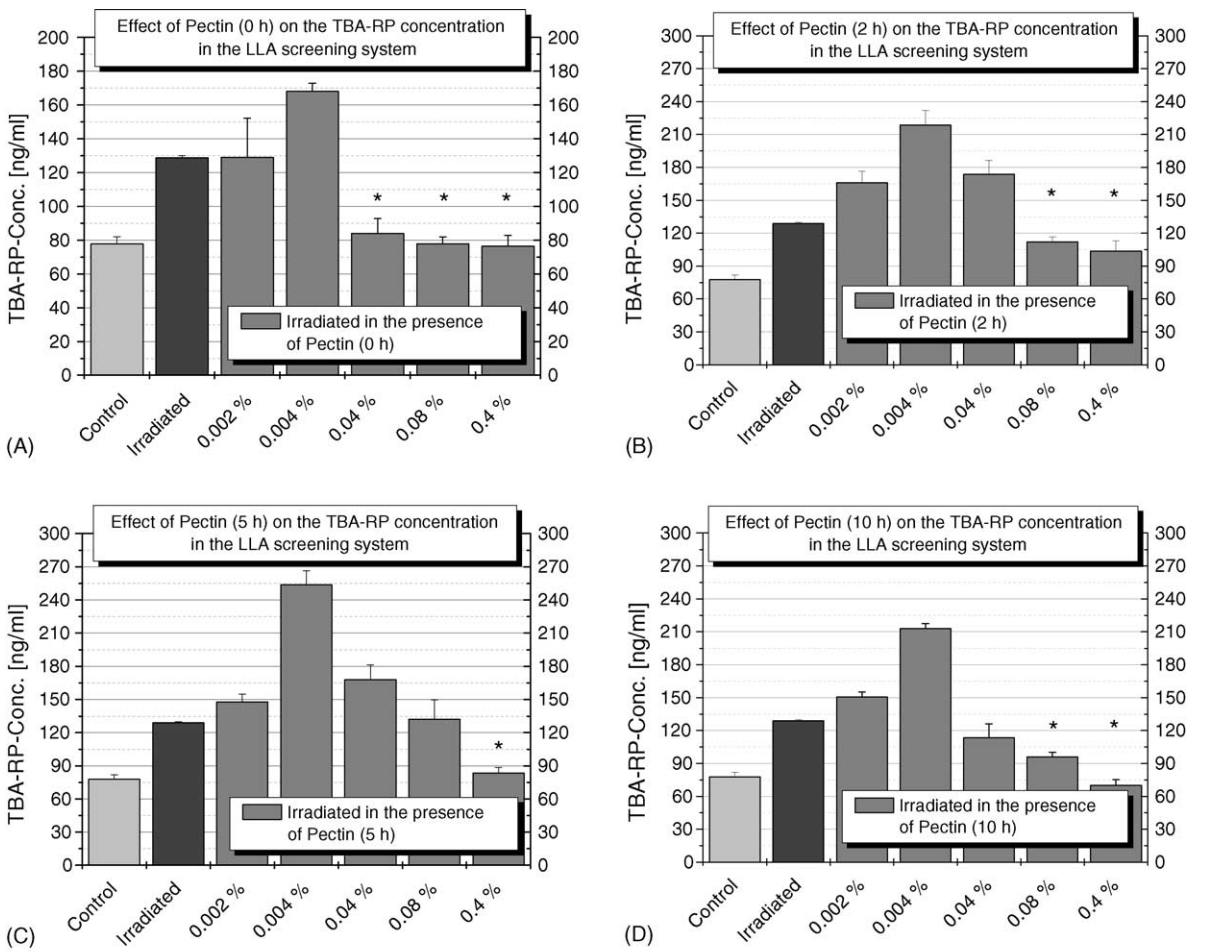

Fig. 3. Concentration of the thiobarbituric acid reaction products (TBA-RP conc.) and influence of ultraviolet irradiation and a swing mill grinding series of pectin in the LLA system: (A) effects of unmilled pectin; (B) effects of pectin milled for 2 h; (C) effects of pectin milled for 5 h; (D) effects of pectin milled for 10 h.

1998). Resistant starches have drawn broad interest in the last few years because they have fiber like properties and show promising physiological benefits in humans which could be important for disease prevention (Richardson et al., 2000).

In the TBA assay (Fig. 2C), lipid protective effects were measured for samples containing 0.002, 0.004, 0.04 and 0.08% of novelose 330. The lipid peroxidation level was decreased by the starch down to the stage of the unirradiated samples.

For the samples with the highest amount of resistant starch (0.4%), the converse result was measured. The malondialdehyde content was increased from 120 up to 170 ng/ml by 0.4% novelose 330.

3.7. Pectin

The protective relation of dietary viscous fiber intake (for example, pectin) and the risk of cardiovascular disease events have been found in several epidemiological studies (Wu et al., 2003). Furthermore, the cell wall polysaccharides have been shown to be prone to the attack of reactive oxygen species. The scission of these polymers by peroxidase-generated

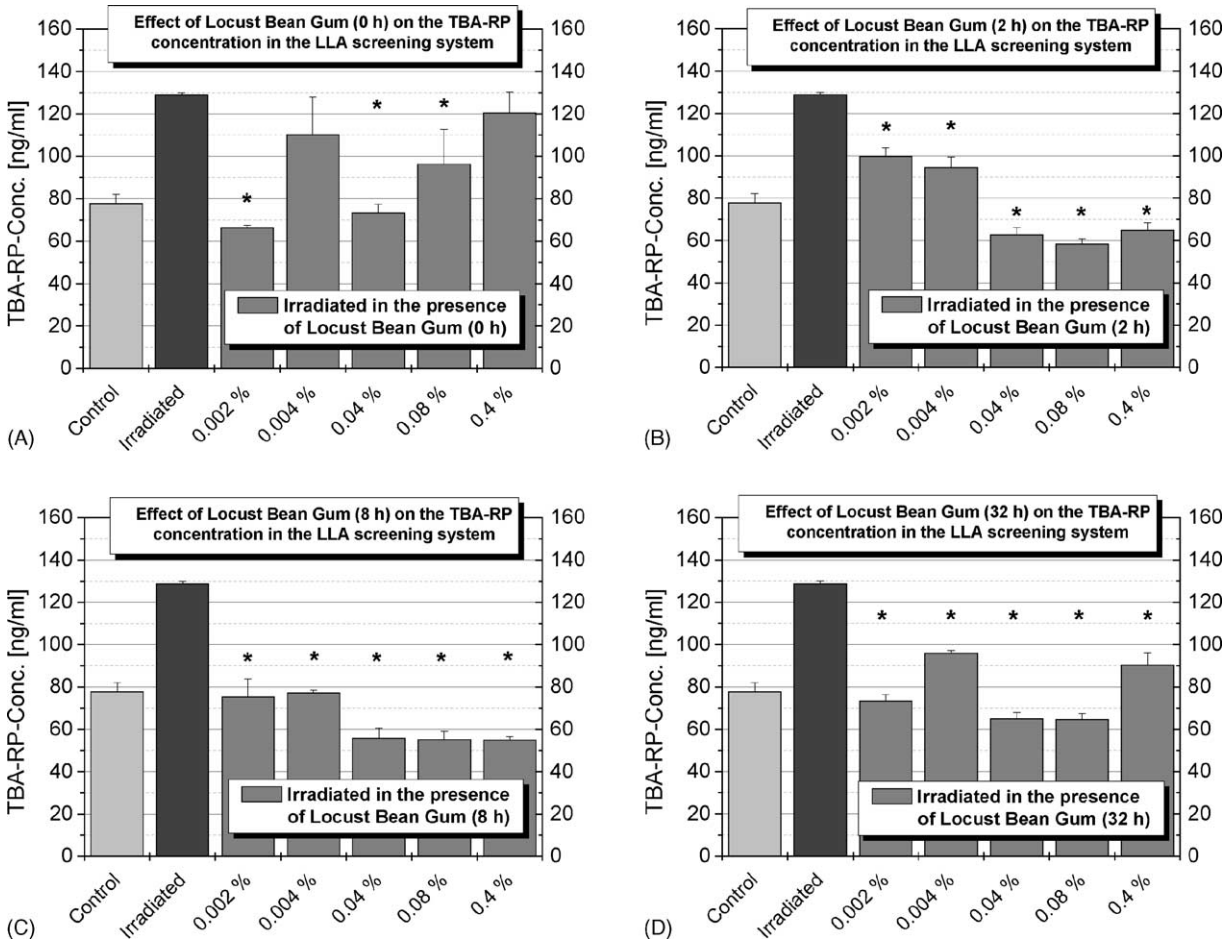

Fig. 4. Concentration of the thiobarbituric acid reaction products (TBA-RP conc.) and influence of ultraviolet irradiation and a swing mill grinding series of locust bean gum in the LLA system: (A) effects of unmilled locust bean gum; (B) effects of locust bean gum milled for 2 h; (C) effects of locust bean gum milled for 8 h; (D) effects of locust bean gum milled for 32 h.

hydroxyl radicals was measured using a viscometric assay (Schweikert et al., 2000). A fingerprinting assay of polysaccharides degraded by hydroxyl radicals gave evidence to the occurence of radical attack during the softening process of pears (Fry et al., 2001).

Fig. 3 shows the results of the TBA assay using pectin as a test polysaccharide for potential skin protection. Four pectins were tested for antioxidative properties in the LLA system. The acidic cell wall polysaccharide pectin was swing milled for different intervals (0, 2, 5, 10 h). Comparing Fig. 3A–D, it is remarkable that the antioxidative properties of the different pectin samples swing milled for different intervals seem to have similar effects on the TBA-RP levels of the lipid system. In all cases the lowest concentration (0.002%) has no (Fig. 3A) or slightly prooxidative effects (Fig. 3B–D) whereas a pectin concentration of 0.004% leads to explicitely enhanced TBA levels after UV exposure in all cases. With increasing pectin concentration, the peroxidation level is decreased ending in antioxidative action of pectin when present in high concentration.

3.8. Locust bean gum

Antioxidant activity of carob tree (*Ceratonia siliqua*) plant material has already been shown (Kumazawa et al., 2002). This group extracted polyphenols from carob pods and evaluated the in vitro antioxidant activity of the crude polyphenol fraction. The isolation and structure elucidation of the major individual polyphenols in the carob fiber was carried out by Owen et al. (2003). The data showed that carob fibre is rich in both amount and variety of phenolic antioxidant substances. It has been concludes that its inclusion in the diet may have chemopreventive effects.

Our investigations dealt with the polysacharidic *Ceratonia siliqua* ingredient. The reserve polysaccharide locust bean gum was tested for its antioxidant power in a lipid model system as well. Four samples grinded for different intervals (0, 2, 8, 32 h) were included in the study. Fig. 4 clarifies the results of these test rows. The unmilled sample of locust bean gum in concentrations of 0.004 and 0.4% (Fig. 4A) had no effects on the TBA-RP concentration. At all other concentrations used locust bean gum was able to reduce the level of lipid peroxidation products significantly. The optimum was measured for the polysaccharide swing milled for 8 h and used in concentrations between 0.04 and 0.4%. Hereby, the malondialdehyde content was reduced from 130 to 55 ng/ml. This is significantly below the measured value of the non-UV irradiated control.

Lipid peroxidation reducing effects of certain polysaccharides are described in the literature (Albertini et al., 2000). The mechanism of these lipid protecting effects seems to be the chelation of transition metal ions. Sipos and co-workers suggested complexes of the kind $L[Fe(OH)_3]_n$, where L is the polysaccharide monomer (Sipos et al., 2003). This chelation can lead to anti- and prooxidative effects and, therefore, has to be tested in every isolated case (Gutteridge et al., 1979).

Further investigations should be carried out to check the compounds found as protective substances in this study for its in vivo influences, for example, on the MED after topical application with test persons. This is of importance for in vivo evaluation of the generated data and its clinical interpretation.

4. Conclusion

Eight different polysaccharidic compounds were tested for antioxidative effects using an in vitro lipid model system. For acacia gum, agar agar, guar gum, xanthan gum and the samples of a swing mill series of locust bean gum lipid protective effects were measured in the thiobarbituric acid assay. The polysaccharides alginic acid, novelose 330 and the swing milled samples of pectin were able to reduce the amount of secondary lipid peroxidation products as well when applied in certain concentrations. Acacia gum, agar agar, xanthan gum and locust bean gum were capable to lower the TBA assay levels of the UV irradiated test samples below the value measured for the non-irradiated control. On the other hand, prooxidative effects were found for alginic acid, novelose 330 and pectin when used in certain concentrations.

These results show new opportunities of skin protection by topical application of polysaccharidic substances with antioxidative potency.

Considering human skin and its constant exposure to UV light and oxygen, combined with an increased iron ion content of the exposed skin, the topical administration of some of the substances found in this study in

cosmetic and pharmaceutical semisolid formulations could be protective for the lipids within the human skin.

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