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Physical and chemical stability of different formulations with superoxide dismutase

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Topical formulations with superoxide dismutase (SOD), a scavenger of superoxide radicals, have proved to be effective against some skin diseases. Nevertheless, formulations with proteins are susceptible to both chemical and physical instability. Three different formulations (anionic and non-ionic gel and emulsion) were developed and supplemented with SOD in order to determine the most stable formulation that would maintain SOD activity. Physical stability was evaluated by assessing the rheological behavior of the formulations stored at room temperature, 37 and 45 °C. Chemical stability was evaluated by the measurement of enzymatic activity in the formulations stored at room temperature and at 45 °C. Formulations showed a flow index less than one, characterizing pseudoplastic behavior. There was no significant difference in initial values of flow index, tixotropy or minimum apparent viscosity. Neither gel showed significant changes in minimum apparent viscosity concerning storage time or temperature, as well, SOD presence and its activity. The emulsion showed decreased viscosity by the 28th day, but no significant changes concerning storage temperature or SOD presence, although it showed a decreased activity. The addition of SOD to the formulations studied did not affect their physical stability but gel formulations seem to be better bases for enzyme addition.

1. Introduction

Among the antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase), superoxide dismutase (SOD), which plays an important role by catalyzing the dismutation of superoxide radicals ($O_2^{-\bullet}$) to oxygen (O_2) and hydrogen peroxide (Isoherranen et al. 1997), has been suggested to be of major importance in the cellular defense system against reactive oxygen species (ROS) (Punnonen et al. 1995). However, UV-generated ROS at high levels may substantially compromise the antioxidant defense of the skin (Poswing et al. 1999). When the generation of ROS is beyond the capacity of available SOD, photooxidative damage occurs (Miyachi et al. 1987).

The antioxidant defense system can be restored with topical or systemic preparations. Topical application of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system and thus may be useful against photodamage and inflammation (Darr and Fridovich, 1994; Saija et al. 1998; Fox 2000). Treatment with exogenous SOD can reduce the loss in activity and prevent the UV-induced formation of sunburn cells in skin (Steenvoorden and Beijersbergen van Henegouwen 1997). Moreover, the topical application of creams containing SOD has proved to be effective against the symptoms and injuries caused by progressive systemic sclerosis, systemic lupus erythematosus, simple herpes and burns (Darr and Fridovich 1994), and other skin diseases associated with increased ROS levels in humans (Niwa 1989; Mizushima et al. 1991).

Proteins perform molecular tasks with unparalleled speed and specificity, which makes them useful as pharmaceutical drugs, but their applications are often hampered by their low stability (Sanchez-Ruiz and Makhatadze 2001). Formulations with proteins are highly susceptible to both physical and chemical instability when compared with traditional drugs. Both chemical and physical changes in proteins can result in a loss of biological activity (Burgess 1993). Also, the activity loss due to low stability can occur gradually during storage, a fact that limits the shelf life of formulations with proteins (Sanchez-Ruiz and Makhatadze 2001).

Due to the importance of this enzyme in topical formulations and its chemical and physical instability, in the present study we developed three different formulations (anionic gel, non-ionic gel and emulsion) supplemented or not with SOD in order to determine the most stable formulation, and the one that would maintain a more stable enzymatic activity.

2. Investigations and results

2.1. Rheological behavior

The average pH was about 7.4, 6.4 and 5.8 for the anionic gel, non-ionic and emulsion, respectively. When stored at 45 °C, the pH of emulsion showed a decrease with an

average of 4,3. The flow index of all formulations was less than 1, indicating pseudoplastic behavior. The non-Newtonian behaviour can be mathematically determined applying the model of Oswald-De Waele:

$$T = K \cdot \gamma^n \quad (1)$$

where T is the shear stress, γ is the shear rate, K is the consistency index and n is the flow index.

The anionic gel was the most pseudoplastic formulation, with an initial flow index of about 0.27 and a range of 0.39–0.26. The initial value for non-ionic gel was about 0.41. There were no changes at room temperature, but a decrease in pseudoplastic behavior occurred as the temperature was increased. The value range was 0.40–0.48. The emulsion showed a flow index of about 0.44. There was an increase at 45 °C by the 28th day, with values about 0.55. The addition of the macromolecule did not affect the flow index.

There were no significant differences in the initial minimum apparent viscosity among the formulations containing SOD or not. In addition to the statistical analyses of the initial values, the minimum apparent viscosity was analyzed in terms of storage time and temperature, as well as SOD presence. The anionic gel showed initial values of 8870 and 8018 cP and a range of 9119–7982 cP and 8526–7569 cP for the gel alone and the gel with SOD, respectively. Viscosity slightly decreased at higher temperatures. However, there were no significant changes regarding storage time, temperature or presence of SOD. The variations in viscosity of the anionic gel regarding period of study and presence of enzyme at room temperature, 37 and 45 °C are shown in Fig. 1.

The initial values of the minimum apparent viscosity for the non-ionic gel were 5311 and 5170 cP and range values were 5446–4699 and 5607–4476 for the non-ionic gel alone and the gel containing SOD, respectively. Again, the apparent viscosity showed a small decrease

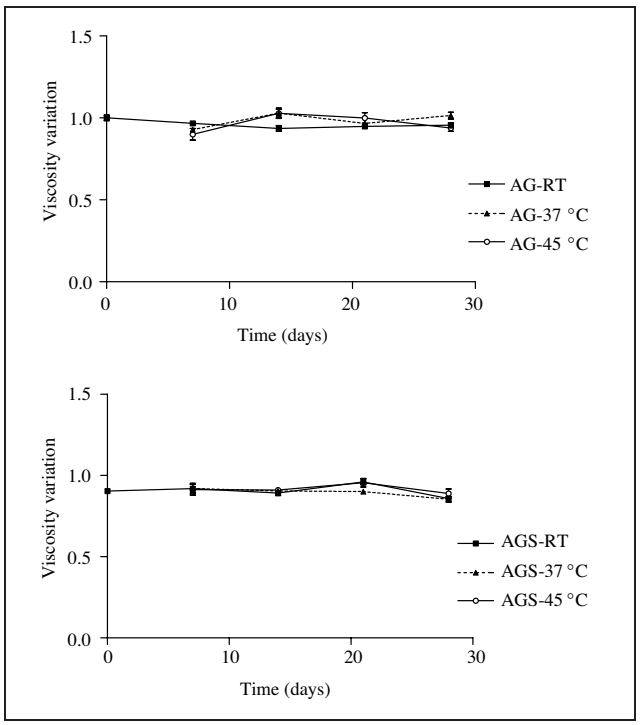

Fig. 1: Viscosity variation of the anionic gel concerning the period of study and SOD presence at room temperature (RT), 37 °C and 45 °C. Results are means ± S.D. of three measurements. AG: anionic gel, AGS: anionic gel supplemented with SOD.

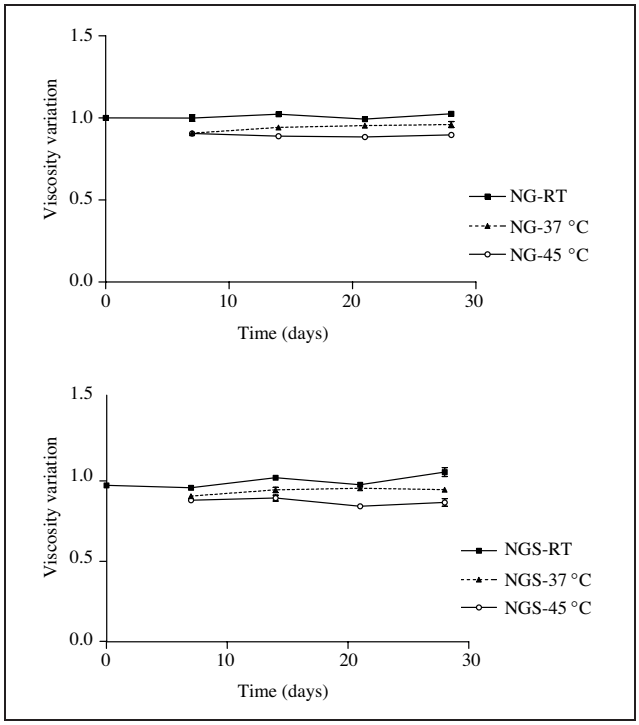

Fig. 2: Viscosity variation of the non-ionic gel concerning the period of study and SOD presence at room temperature (RT), 37 °C and 45 °C. Results are means ± S.D. of three measurements. NG: non-ionic gel, NGS: non-ionic gel supplemented with SOD.

at higher temperature, but no significant changes were observed regarding storage time and temperature or SOD presence. The changes in viscosity of the non-ionic gel concerning the period of study and the enzyme presence at room temperature, 37 and 45 °C are shown in Fig. 2.

The emulsion showed a greater variation of viscosity values. The initial values were 3727 and 3873 cP for the formulation containing SOD or not, respectively, and the range was 4930–2998 and 5302–2680 for the formulation containing the enzyme or not. The highest values were observed when the emulsion was stored at 37 °C and the lowest when it was stored at 45 °C. Although there was no significant difference concerning storage temperature or SOD presence, the emulsion showed a reduced viscosity by the 28th day. The variations in viscosity concerning period of study and SOD presence at room temperature, 37 and 45 °C are shown in Fig. 3.

All formulations were found to be thixotropic. The hysteresis loop was calculated by the area under the curve from the rheograms of each formulation. There were no significant differences in the initial values of the hysteresis loop between the formulations containing or not SOD (data not shown).

Table 1: Composition of formulations added or not with SOD

Component	Anionic gel	Non-ionic gel	Emulsion
Self-emulsifying wax	–	–	9.00 g
Macadamia nut oil	–	–	2.50 g
Squalane	–	–	1.00 g
Propylene glycol	6.00 g	6.00 g	5.00 g
Phenoxyethanol and parabens	0.40 g	0.40 g	0.40 g
Carbomer	0.27 g	–	–
Hydroxyethylcellulose	–	1.50 g	–
Distilled water	93.33 g	92.10 g	82.10 g

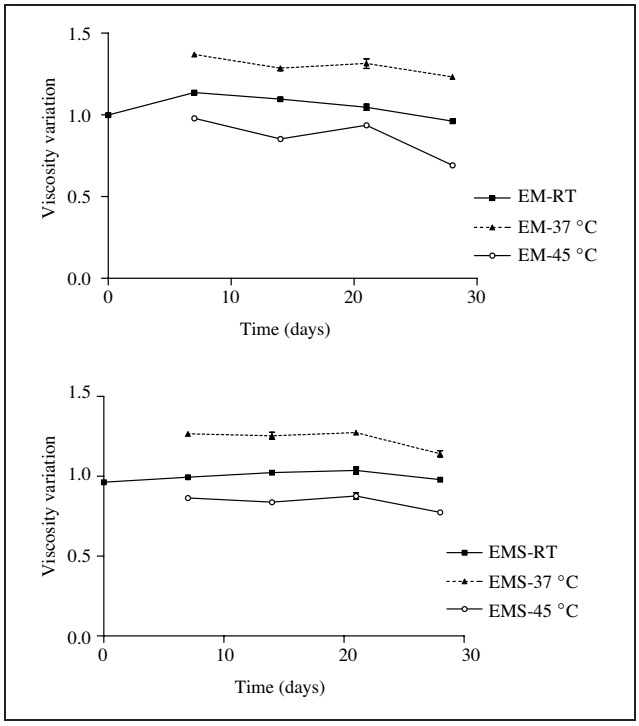

Fig. 3: Viscosity variation of the emulsion concerning the period of study and SOD presence at room temperature (RT), 37 °C and 45 °C. Results are means ± S.D. of three measurements. EM: emulsion, EMS: emulsion supplemented with SOD

2.2. Chemical stability and free protein

The enzymatic activity found in 1 g of each formulation is shown in Table 2. The gels were the formulations that maintained SOD activity more stable. The enzymatic activity of the anionic gel decreased after the 45th day of storage at 45 °C. However, there were no significant differences in enzymatic activity in terms of control activity (blank + SOD), or storage time and temperature. The free protein percentage concerning the mass of protein added was 57%.

The non-ionic gel showed decreased activity after the 30th day at 45 °C. The activity at room temperature was lower than that observed in the anionic gel after the 30th day. There were no significant differences in enzymatic activity in terms of control activity (blank + SOD) or temperature, but after 30 days of storage there was a significant decrease in SOD activity. The free protein percentage was 34%.

Initially, the enzymatic activity of the emulsion did not differ from the other formulations or the control (blank + SOD), but when the activity during the period of study was analyzed statistically, a significant difference was found between the homogenate of formulation containing SOD and the control. Although the emulsions showed a lower activity in terms of the control, it remained constant for the rest of the period, with no significant difference in terms of storage time and temperature. The percentage of free protein recovered after extraction in the aqueous phase, concerning the mass of protein added was 20%.

3. Discussion

Studying the rheological behavior of formulations, one can verify instability signs. In this test the torque was adjusted to be between 10 and 100% to optimize the assays conditions, and this was the reason why the range of shear rate was from 1 to 20 s^{-1}. All rheograms showed a non-Newtonian behavior with pseudoplastic flow, a desirable rheological behavior in these preparations. In this case, the shear stress decreases as the shear rates increases. This kind of behavior leads to a reduction of apparent viscosity. The anionic gel was the most pseudoplastic formulation. The addition of the enzyme did not affect the flow behavior of the formulations.

In addition to the statistical analyses of initial values, the minimum apparent viscosity was analyzed in terms of storage time and temperature, as well as SOD presence. Although the apparent viscosity showed a steady gradual reduction with increasing temperature, there was no qualitative change in the type of pseudoplastic flow behavior with temperature. The emulsion showed increased viscosity at 37 °C. Korhonen et al. (2001) found an increase of the viscosity when their formulation was stored at 25 °C. Most creams have a specific maximum temperature (T_{max}) until which the consistency increases (Barry and Eccleston 1973). In this case, the T_{max} of the emulsion was probably between 37 °C and 45 °C. Also, the cetostearyl alcohol present in the self-emulsifying wax, shows a tendency to increase the viscosity with the preparation aging, hence it could be the cause of the increase in consistency of the emulsion. At 45 °C, the opposite occurred, the viscosity showed a decrease. This could be related to a possible internal structural disarrangement due to the higher temperature. This disarrangement may subside upon the cetostearyl alcohol effect.

Several methods are available for SOD determination and the spectrophotometric ones are among the most widely used techniques. They are indirect methods based on two elements, a superoxide generator and a detector. If SOD is present, it competes with the detector for superoxide radicals (Girotti et al. 2000). The chemical stability of these

Table 2: Units of SOD found in 1 g of each formulation during the chemical stability evaluation

Time	Gel anionic		Gel non ionic		Emulsion	
	RT	45 °C	RT	45 °C	RT	45 °C
SOD added	92.10	92.10	92.10	92.10	92.10	92.10
Initial (24 hours)	62.73	–	94.63	–	68.40	–
48 hours	65.07	56.87	69.33	62.87	42.30	38.90
7 days	83.67	53.33	98.90	72.59	48.30	48.30
15 days	98.77	63.40	85.37	70.83	60.30	72.70
30 days	76.47	47.63	56.90	70.30	54.35	52.00
45 days	82.43	52.63	58.53	29.53	72.50	59.25
60 days	85.30	28.55	47.40	32.10	34.40	54.97
75 days	71.33	25.77	65.47	11.90	45.47	47.85

RT: room temperature

formulations was determined by the method of McCord and Fridovich (1969), which allows variations because two reactions must run sequentially, i.e. the liberation of superoxide ion followed by its scavenging by SOD.

With this analytical method we observed that gel formulations maintained the activity while the emulsion showed reduced activity after 24 hours, although SOD activity did not change during the rest of the study period. The reduced activity shown by the emulsion could be due to methodological variations or to interactions between SOD and formulation components.

In order to verify if this loss of activity was due to a possible interaction of the enzyme with formulation components, the proteins present in the formulations were determined. The anionic and non-ionic gels were not submitted to the extraction process because the components of these formulations do not interfere with the method for protein determination. On the other hand, after homogenization in buffer with 0.5 M NaCl, the emulsion was submitted to centrifugation at 15000 rpm for protein extraction in the aqueous phase, since the components of this formulation interfere with protein measurement.

The anionic gel was formulated with carbomer, a polymer of acrylic acid. This polymer does not interact or interacts very little with proteins. This might explain why this formulation showed a high free protein percentage leading to a good enzymatic activity determination, since the components did not interfere with the measurement. On the other hand, in the non-ionic gel, formulated with hydroxyethyl cellulose, a neuter polysaccharide, 62% of the protein present in formulation could be interacting with the polymer since only 38% of proteins were detected by the method used for protein determination. The results of enzymatic activity measurement showed that, although the enzyme could be attached to the polysaccharide, this did not interfere with the method for activity determination. Both gel formulations showed good stability of enzymatic activity during the study period.

SOD (Dismutin®-BT) used in these formulations is conjugated with polyethyleneglycol (PEG). SOD subjected to covalent modifications with PEG showed a remarkable preservation of activity and exhibited much longer plasma lifetimes in mice. It appears that the attachment of two to four PEG chains per glycoprotein causes minimal loss of enzymatic activity (Zalipsky 1995).

The SOD activity in the emulsion was markedly reduced just at the beginning of the chemical stability test, but after this loss, the activity remained constant during the study period. This result may be explained by a lower detection of SOD activity due to higher interference of the formulation components with the analytical method used or by the conjugation of SOD with PEG. Modification with PEG is useful for making various enzymes soluble and active in hydrophobic media. This increased solubility results from modification of the enzyme's surface characteristics with amphipathic PEG molecules (Inada et al. 1995). Moreover, proteins spontaneously adsorb to oil-water interfaces, essentially due to the hydrophobic properties of these interfaces (Pezennec et al. 2000). Surfactant-protein interactions are complex, especially in emulsions, because adsorption leads to conformational changes, a reduction in protein flexibility, and a decrease in exposed surface area. An adsorbed protein tends to orientate itself so that the hydrophobic segments protrude into the oil phase, whilst the hydrophilic segments protrude into the aqueous phase, because this minimizes the unfavorable contact between polar and non-polar regions (Demetriades and McClements 2000). These facts together may explain the low percentage of protein extracted from the emulsion. SOD may be partially soluble in the fat phase of the formulation and may not be totally extracted.

Also, the Macadamia nut oil, the emollient added to the emulsion, has a chemical structure similar to that of the cell membrane because it is rich in palmitoleic and oleic acid (Cummings et al. 1999). SOD is an enzyme which binds to cell surfaces and collagen (Darr and Fridovich 1994), so it may show affinity for the Macadamia nut oil components and not be completely extracted from the formulation during the extraction process. Thus, the interaction of surfactant or Macadamia nut oil with SOD may cause conformational changes in the enzyme or an impediment of the enzyme active site, preventing the interaction of the enzyme active site and the substrate, superoxide ion, generated in the reaction mixture, and leading to lower enzymatic activity. Although the free protein extracted from emulsion was low, the enzymatic activity could be measured. These results show that the activity of SOD present in the fatty phase could be determined by the analytical method used.

The gel formulations showed a higher availability of SOD, this could be interesting for topical application, since the enzyme needs not to be attached to the formulation components to be effective when applied on the skin.

SOD detection is made difficult by the nature of its substrate, which needs to be generated in the reaction mixture. Although it was possible to measure the enzymatic activity in the formulations prepared, other analytical methods are now being tested in order to obtain a good quality control of formulations supplemented with enzymatic antioxidants.

4. Experimental

4.1. Test formulations

Dismutin®-BT (SOD) was purchased from Pentapharm (Basel, Switzerland). The remaining raw materials for the formulations were obtained from Galena (Campinas, SP, Brazil). The anionic gel (AG) was prepared with carbomer polymer (Carbopol® 940) after the neutralization with triethanolamine. The non-ionic gel (NG) was prepared with hydroxyethylcellulose (Natrosol® 250 HHR). The emulsion (EM) was based on a self-emulsifying wax; Macadamia nut oil and squalane were added as emollients. Propylene glycol was added as a moisturizer to all formulations. The preservative used was a mixture of phenoxyethanol and parabens (Table 1). Distilled water was used in the preparations of all formulations. Dismutin®-BT 0.4% was incorporated at room temperature. All formulations were allowed to equilibrate for 24 hours before the studies.

4.2. Physical stability evaluation

Physical stability was evaluated by submitting the formulation to storage at room temperature and 37 and 45 ± 2 °C for 28 days. Samples were collected the initial time and then at 7 day intervals for the evaluation of rheological behavior and pH measurements. The rheological measurements were made as described previously (Di Mambro et al. 2003) using a rotational rheometer with a cone-plate configuration (Brookfield RV – III, Middleboro, MA, U.S.A), with a CP52 spindle and 0.5 g of sample. The rate was kept constant for 10 s at each shear rate before a measurement was made. The pH of formulations diluted 1 : 10 in distilled water was measured using a Digmed pHmeter (São Paulo, SP, Brazil). All measurements were made at room temperature in triplicate for each analyzed sample.

4.3. Chemical stability evaluation

Chemical stability was assayed by the method described by McCord and Fridovich (1969) using the xanthine-xanthine oxydase system. All chemicals (xanthine, xanthine oxidase and cytochrome c) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The formulations were stored at room temperature and at 45 ± 2 °C for 75 days. For the assay enzymatic activity, formulations containing SOD or not were diluted 1 : 5 in phosphate buffer (pH 7.8; 0.05 M) containing 0.5 M NaCl and mixed

at 4 °C for 120 min. The enzymatic activity of the diluted formulations homogenates was determined at the initial time, after 24 hours, after 7 days and then at 15 day intervals. Two controls were used for this test: the homogenate of the formulation without SOD (blank) and the blank supplemented with SOD at the time of enzymatic activity determination (blank + SOD). This second control was set up to reproduce 100% of free SOD in the reaction mixture. All measurements were made at room temperature in triplicate for each analyzed sample. One SOD unit is defined as the amount that reduces the cytochrome c reduction rate by 50%.

2.4. Assays of free proteins

Formulations containing SOD or not were diluted 1 : 3 in 0.05 M phosphate buffer (pH 7.8) with 0.5 M NaCl and mixed at 4 °C for 120 min. The emulsion was submitted to centrifugation at 15 000 rpm for 40 min at 4 °C and the aqueous phase was used for the assay. A control was prepared with SOD diluted in the same buffer. The free protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. All measurements were made at room temperature in triplicate for each formulation.

2.5. Statistical analysis

Data were analyzed statistically by the non-parametric Mann Whitney U test for initial values of rheological parameters and enzymatic activity. To analyze the changes in viscosity in terms of the period of study, storage temperature and presence of SOD, the Tukey test was used. The level of significance was set at $p < 0.05$. The same test was used to analyze the enzymatic activity in terms of the period of study, storage temperature and activity.

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References

Barry BW, Eccleston GM (1973) Influence of gel networks in controlling consistency of o/w emulsions stabilized by mixed emulsifiers. J Texture Stud 4: 53–81.

Burgess DJ (1993) Drug delivery aspects of biotechnology products. In: Pezzuto ME, Johnson ME, Manasse HR (eds.) Biotechnology and Pharmacy, Chapman & Hall, New York, p. 116–151.

Cummings M, Arquette J, Reinhardt J (1999) Macadamia nut oil. Cosmet Toil 114: 75 –78.

Darr D, Fridovich I (1994) Free radicals in cutaneous biology. J Invest Dermatol 102: 671–675.

Demetriades K, McClements DJ (2000) Influence of sodium dodecyl sulfate on the physicochemical properties of whey protein-stabilized emulsions. Colloids and Surfaces A: Physicochem Eng Aspects 161: 391–400

Di Mambro VM, Borin, MF, Fonseca MJV (2003) Topical formulations with superoxide dismutase: influence of formulation composition on physical stability and enzimatic activity. J Pharm Biomed Anal 32: 97–105.

Fox C (2000) Skin care review 1995–1999. Cosmet Toil 115: 55–77.

Girotti S, Fini F, Ferri E, Budini R, Piazzi S, Cantagalli D (2000) Determination of superoxide dismutase in erythrocytes by chemiluminescence assay. Talanta 51: 685–692.

Inada Y, Furukawa M, Sasaki M, Kodera Y, Hiroto M, Nishimura H, Matsushima A (1995) Biomedical and biotechnological applications of PEG- and PM-modified proteins, TIBTECH 13: 86–91.

Isoherranen K, Peltola V, Laurikainen L, Punnonen J, Laihia J, Ahotupa M, Punnonen K (1997) Regulation of copper/zinc and manganese superoxide dismutase by UVB irradiation, oxidative stress and cytokines. J Photochem Photobiol B: Biol 40: 288–293.

Korhonen M, Hellen l, Hirvonen J, Yliruusi (2001) Rheological properties of creams with four different surfactant combinations – effect of storage time and conditions. Int J Pharm 221: 187–196.

Lowry OH, Rosebrough NJ, Farr AL (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–275.

McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J Biol Chem 244: 6049–6055.

Miyachi Y, Imamura S, Niwa Y (1987) Decreased skin superoxide dismutase activity by a single exposure of ultraviolet radiation is reduced by liposomal superoxide dismutase pretreatment. J Invest Dermatol 89: 111–112.

Mizushima Y, Hoshi K, Yamagawa A, Takana K (1991) Topical application of superoxide dismutase cream. Drugs Exp Clin Res 17: 127–131.

Niwa Y (1989) Lipid peroxides and superoxide dismutase (SOD) induction in skin inflammatory diseases and treatment with SOD preparations. Dermatologica (Suppl) 179: 101–106.

Pezennec S, Gauthier, F, Alonso C, Graner F, Croguennec T, Brulé G, Renault A (2000) The protein net electric charge determines the surface rheological properties of ovalbumin adsorbed at the air-water interface. Food Hydrocolloids 14 : 463–472.

Poswing A, Wenk J, Brenneisen P, Wlaschek M, Hommel C, Quel G, Faisst K, Dissemond J, Briviba K, Krieg T, Scharffetter-Kochanek K (1999) Adaptive antioxidant response of manganese-superoxide dismutase following repetitive UVA irradiation. J Invest Dermatol 112: 13–18.

Punnonen K, Lehtola K, Autio P, Kiistala U, Ahotupa M (1995) Chronic UVB irradiation induces superoxide dismutase activity in human epidermis *in vivo*. J Photochem Photobiol B: Biol 30: 43–48.

Saija A, Tomaino A, Trombetta D, Giacchi M, De Pasquale A, Bonina F (1998) Influence of different penetration on in vitro skin permeation and in vivo photoprotective effect of flavonoids. Int J Pharm 175: 85–94.

Sanchez-Ruiz JM, Makhatadze GI (2001) To charge or not to charge? Trends Biotechnol 19: 132–135.

Steenvoorden DPT, Beijersbergen van Henegouwen GMJ (1997) The use of endogenous antioxidants to improve photoprotection. J Photochem Photobiol B: Biol 41: 1–10.

Zalipsky S (1995) Chemistry of polyethylene glycol conjugates with biologically active molecules. Adv Drug Delivery Rev 16: 157–182.