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Topical formulations with superoxide dismutase: influence of formulation composition on physical stability and enzymatic activity

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Abstract

Three different topical formulations were supplemented with superoxide dismutase (SOD) and evaluated concerning physical and chemical stabilities in order to determine the most stable formulation that would maintain SOD activity. Physical stability was evaluated by storing the formulation at room temperature, and at 37 and 45 °C for 28 days. Samples were collected at 7-day intervals for assessment of rheological behavior. Chemical stability was evaluated by the measurement of enzymatic activity in formulations stored at room temperature and at 45 °C for 75 days. The formulations showed a pseudoplastic behavior, with a flow index of less than 1. There was no significant difference in the initial values of flow index, hysteresis loop or minimum apparent viscosity. The simple emulsion and the one stabilized with hydroxyethylcellulose showed decreased viscosity by the 21st day and with higher temperature, but no significant changes concerning the presence of SOD. Although there were no significant changes concerning storage time or temperature, the formulation stabilized with hydroxyethylcellulose showed a marked loss of SOD activity. The addition of SOD to the formulations studied did not affect their physical stability. Simple emulsions or emulsions stabilized with carboxypolymethylene seem to be better bases for enzyme addition than emulsion stabilized with hydroxyethylcellulose.

Keywords: Superoxide dismutase; Physical stability; Rheology; Chemical stability; Enzymatic activity; Emulsions with proteins

1. Introduction

The discovery of superoxide dismutase (SOD) has attracted great attention in the scientific community because it catalyzes the dismutation of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [1]. The use of this enzyme as an anti-inflammatory agent has

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been studied by several authors [2,3]. SOD is predominantly an intracellular enzyme and its activity has been shown to be regulated by UVB irradiation [4]. Only small amounts can be found in extracellular fluids [5], and the level of SOD in human epidermis is low, approximately 23 μg/g of fresh tissue, only about 10% of that reported for human liver [6]. Moreover, several authors have found SOD activity to be decreased after exposure to UV irradiation [7–11]. Inal et al. [12] showed that SOD activity decreases with aging, as well.

Topical application of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system and thus may be useful against photodamage and inflammation [13–15]. Treatment with exogenous SOD can reduce the loss of activity and prevent the UV-induced formation of sunburn cells in skin [16]. Mizushima et al. [17] and Niwa [18] showed that a topically applied SOD cream was effective against several skin diseases associated with increased reactive oxygen species' levels in human.

Nevertheless, one of most challenging tasks in the development of formulations with proteins is to deal with the physical and chemical instabilities of proteins [19]. 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 [20]. Proteins in formulations are highly susceptible to both chemical and physical instabilities when compared with traditional drugs. Chemical instability results in the generation of a new chemical entity by bond formation or cleavage. Physical instability involves changes in the secondary, tertiary, or quaternary structure of the molecule, which can be manifested as denaturation, adsorption, aggregation, and precipitation. Both chemical and physical changes in proteins can result in a loss of biological activity [21].

Due to the importance of this enzyme in topical formulations and its chemical and physical instabilities, in the present study we developed three different formulations (a simple emulsion, an emulsion stabilized with an anionic polymer, and an emulsion stabilized with a non-ionic polymer) 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. Materials and methods

2.1. Test formulations

Dismutin®-BT (SOD) was purchased from Pentapharma (São Paulo), and the remaining raw materials for the formulations were purchased from Galena (Campinas, São Paulo). Three different emulsions were developed based on a commercially available self-emulsifying wax, macadamia nut oil and squalane were added as emollients, and propylene glycol was added as a moisturizer (Table 1). The preservative used was a mixture of phenoxyethanol and parabens. Two emulsions were stabilized with polymers, an anionic hydrophilic colloid (carboxypolymethylene, Carbopol® 940) and a non-ionic polymer (hydroxyethylcellulose, Natrosol® 250 HHR). Distilled water was used in the preparations of all formulations. Dismutin®-BT (0.4%) was incorporated at room temperature (Table 1). All formulations were allowed to equilibrate for 24 h before the studies.

2.2. Physical stability evaluation

Physical stability was evaluated by submitting the formulation to storage at room temperature, and at 37 and 45 ± 2 °C for 28 days [22]. Samples were collected for the evaluation of rheological behavior and pH measurements at the initial time and then at 7-day intervals. The rheological measurements were made using a rotational rheometer with a cone-plate configuration (Brookfield RV-III) with a CP52 spindle, and 0.5 g of the sample. Measurements were made at progressively higher rotation speeds and shear rates (0.5–10 rpm and 1–20 s^{-1}, respectively) to obtain the ascending curve, and the procedure was repeated in reverse with progressively slower rates (10–0.5 rpm and 20–1 s^{-1}) to obtain the descending curve. 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. All

Table 1
Composition in w/w percentages of the emulsions added or not with SOD

Component	Simple emulsion	Emulsion with a carbomer	Emulsion with hydroxyethylcellulose
Self-emulsifying wax	10.0	2.0	2.0
Macadamia nut oil	2.5	2.5	2.5
Squalane	1.0	1.0	1.0
Propylene glycol	5.0	6.0	6.0
Phenoxyethanol and parabens	0.4	0.4	0.4
Carboxypolymethylene	–	6.0	–
Hydroxyethylcellulose	–	–	1.5
Distilled water qs	100	100	100

measurements were made at room temperature in triplicate for each analyzed sample.

2.3. Chemical stability evaluation

Chemical stability was assayed by the method of McCord and Fridovich [23] using the xanthine–xanthine oxydase system. All chemicals (xanthine, xanthine oxidase, and cytochrome *c*) were purchased from Sigma. The formulations were stored at room temperature and at 45 ± 2 °C for 75 days. For the assay of enzymatic activity, the formulations containing or not SOD were diluted 1:5 in phosphate buffer (pH 7.8, 0.05 M) with 0.5 M NaCl and mixed at 4 °C for 120 min. The enzymatic activity of the homogenate of diluted formulations was determined at the initial time, after 24 h, 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 setup 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 or not SOD were diluted 1:3 in phosphate buffer (pH 7.8, 0.05 M) with 0.5 M NaCl, mixed at 4 °C for 120 min, and centrifuged at 15 000 rpm for 40 min at 4 °C. 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. [24] using bovine serum albumin as a standard. All measurements were made at room temperature in triplicate for each emulsion.

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. The Tukey test was used to analyze the changes in viscosity in terms of period of study, storage temperature, and presence of SOD. The level of significance was set at $P < 0.05$. The same test was used to analyze the enzymatic activity in terms of period of study, storage temperature, and activity.

3. Results

3.1. Evaluation of rheological behavior

The average pH was about 5.6, 7.2, and 6.9 for the simple emulsion, and for the emulsions containing carbomer and hydroxyethylcellulose, respectively. The flow index of all formulations was less than 1, indicating a pseudoplastic behavior. The non-Newtonian behavior can be mathematically determined by applying the model of Oswald–De Waele:

$$T = K\gamma^n,$$

where T is the shear stress, γ the shear rate, K the consistency index, and n the flow index [25].

The simple emulsion showed an initial flow index of 0.43 with a range 0.46–0.35. The emulsion stabilized with the carboxypolymethylene polymer was the most pseudoplastic formulation, with an initial value of about 0.33 and a range 0.39–0.26. The initial value for the emulsion stabilized with hydroxyethylcellulose was 0.40, with a range 0.38–0.48. By the 28th day, at 45 °C there was an increase in values of about 0.53, showing a reduction of the pseudoplastic behavior. The addition of a macromolecule did not affect the flow index.

There were no significant differences in the initial minimum apparent viscosity among formulations supplemented or not with SOD. 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 simple emulsion showed a greater variation of viscosity values. The initial values were 4135.0 and 4322.0 cP for the formulation containing or not SOD, respectively, and the ranges were 5577.0–2133.0 cP and 5963.7–2106.7 cP for the formulation containing or not the enzyme. The highest values were observed when the formulation was stored at 37 °C and the lowest when it was stored at 45 °C, but there was no significant difference in terms of storage temperature. Although there was also no significant difference regarding SOD presence, the formulation showed a reduced viscosity by the 21st day. The variations in viscosity of the simple emulsion regarding period of study and presence of enzyme at room temperature, and at 37 and 45 °C are shown in Fig. 1.

The emulsion stabilized with carbomer showed initial values of 5799.7 and 5308.3 cP, and the ranges 6396.0–4436.7 cP and 5705.0–4046.7 cP for the base and the base plus SOD, respectively. There was a significant decrease at 45 °C, but no significant changes regarding the presence of SOD. The changes in viscosity of the emulsion stabilized with the carbomer polymer regarding the period of study and presence of enzyme at room temperature, and at 37 and 45 °C are shown in Fig. 2.

The initial value of the minimum apparent viscosity was 6556.7 cP and the values ranged from 6173.3 to 4649.5 cP for the emulsion stabilized with hydroxyethylcellulose. The formulation containing SOD showed an initial value of 6753.3 cP and a range from 6088.0 to 4802.0 cP. Again, the apparent viscosity decreased at higher temperature, but no significant changes were observed regarding temperature or presence of SOD. There was a significant decrease after the 21st day. The changes in viscosity of the emulsion stabilized with hydroxyethylcellulose in terms of the period of study and the presence of the enzyme at room temperature, and at 37 and 45 °C are shown in Fig. 3. 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.

All formulations were found to be thixotropic. The hysteresis loop was calculated by the area under the curve for 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).

3.2. Assessment of chemical stability and free-protein measurement

The enzymatic activity found in 1 g of each formulation is shown in Table 2. The simple emulsion and the emulsion containing carbomer polymer were the formulations that maintained a more stable SOD activity when stored at room temperature, although the activity found initially in 1 g of each formulation was about 70%. When the formulations were stored at 45 °C, the simple emulsion showed a decrease after the 7th day, and it lost the activity by the 75th day. The emulsion stabilized with carbomer lost its activity after the 45th day. However, there were no significant differences in enzymatic activity in terms of control activity (blank + SOD), or storage time and temperature for either formulation. The protein percentage recovered after extraction in the aqueous phase, concerning the mass of protein added, was about 18% for both formulations.

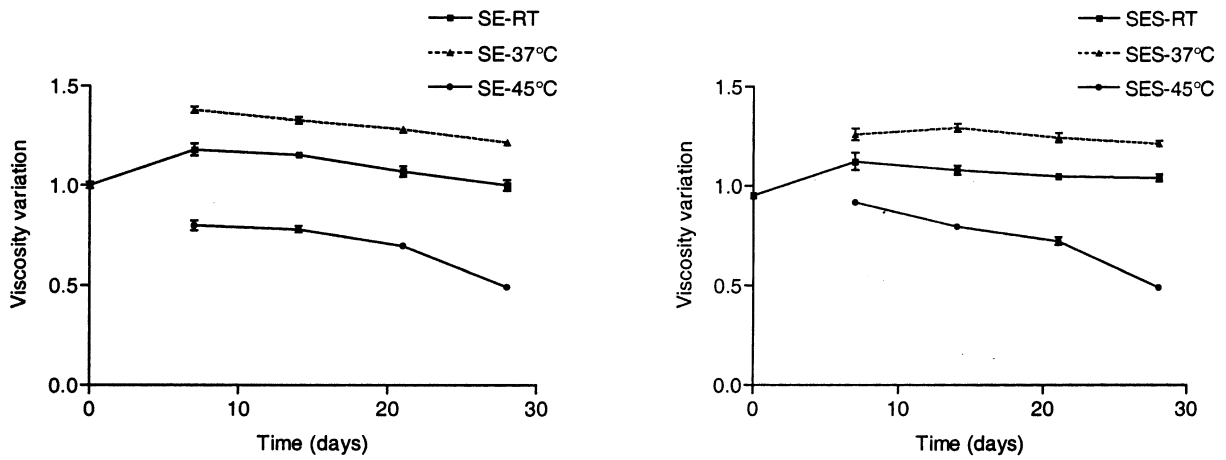

Fig. 1. Viscosity variation of a simple emulsion in terms of period of study and SOD presence at room temperature, and at 37 and 45 °C. SE, simple emulsion; SES, simple emulsion supplemented with SOD.

Initially, the enzymatic activity of the emulsion stabilized with hydroxyethylcellulose was similar to that found in the other formulations, but after 24 h it decreased and after 7 days SOD lost its activity, with a small recovery after 30 days. SOD activity was slightly higher when the formulation was stored at 45 °C. There were no significant differences in enzymatic activity in terms of control activity (blank+SOD) or temperature, but after 45 days of storage there was a significant decrease in SOD activity. The protein percentage recovered after extraction, in the aqueous phase, was about 48%, more than that found for the simple emulsion and the emulsion stabilized with carbomer.

4. Discussion

The use of enzymes, especially scavengers of free radicals in topical formulations, has been increasing. Thus, it is necessary to use appropriate vehicles and excipients in order to obtain good formulation stability and enzymatic activity.

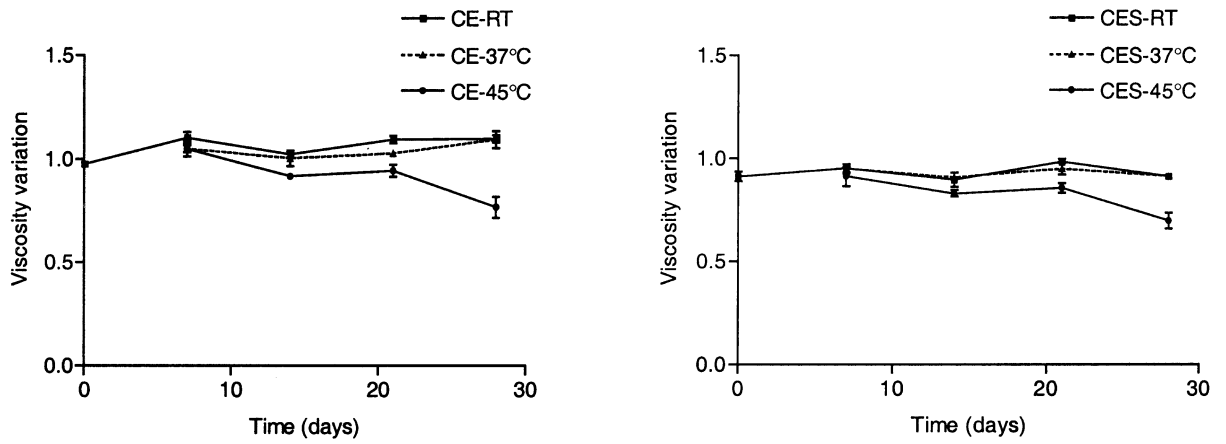

Fig. 2. Viscosity variation of an emulsion with a carbomer in terms of period of study and SOD presence at room temperature, and at 37 and 45 °C. CE, emulsion with a carbomer; CES, emulsion with a carbomer supplemented with SOD.

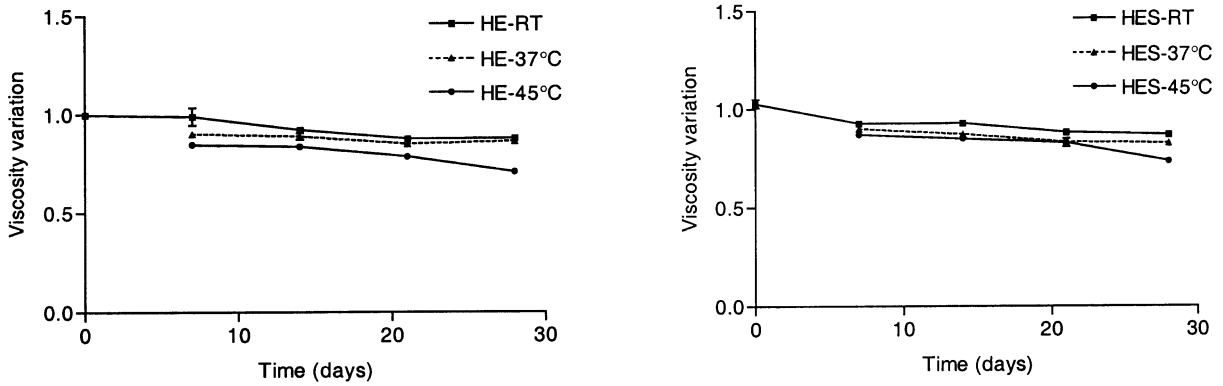

Fig. 3. Viscosity variation of an emulsion with hydroxyethylcellulose in terms of period of study and SOD presence at room temperature, and at 37 and 45 °C. HE, emulsion with hydroxyethylcellulose; HES, emulsion with hydroxyethylcellulose supplemented with SOD.

Three different formulations were prepared and studied for stability when SOD was incorporated. The formulations were stored at room temperature, and at 37 and 45 °C for 28 days. Temperature is one of the most important factors for the rheology of a system [26]. It can affect formulations through changes in viscosity and solubility, partitioning of molecules between the two phases, melting of waxes, and hydration of polymers, and can facilitate creaming and coalescence [27]. By studying the rheological behavior of formulations, one can verify signs of instability. The range of shear rate was 1.0–20 s^{-1} due to the fact that torque was adjusted between 10 and 100% to optimize the assay's conditions. All the rheograms showed a pseudoplastic flow, a desirable rheological behavior in these preparations. In this case, the shear stress decreases as the shear rate increases. This kind of behavior leads to a decrease in apparent viscosity. The addition of a macromolecule did not affect this flow behavior, and the most pseudoplastic formulation was the one stabilized with carbomer polymer.

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. The simple emulsion showed the greatest variation of viscosity values, although there was no qualitative change in the type of pseudoplastic flow behavior. The increase in viscosity at 37 °C can be explained by loss of the water present in the formulation or may be due to

Table 2
SOD units found in 1 g of each formulation during the assessment of chemical stability

Time	Simple emulsion		Emulsion with a carbomer		Emulsion with hydroxyethylcellulose	
	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 h)	62.20	–	67.85	–	66.67	–
48 h	75.70	79.50	50.00	42.87	33.33	46.55
7 days	69.00	77.80	38.30	85.71	0	31.50
15 days	51.10	53.33	88.00	50.00	0	10.00
30 days	53.45	0	58.00	64.00	13.40	0
45 days	59.60	42.87	44.47	55.15	13.30	31.93
60 days	43.43	36.35	45.75	0	17.17	28.20
75 days	61.55	0	60.30	0	17.00	29.83

RT, room temperature.

the cetostearyl alcohol present in the self-emulsifying wax, which shows a tendency to increase the viscosity with preparation aging. The opposite occurred at 45 °C, with viscosity showing a decrease. This could be related to a possible internal structural disarrangement due to the higher temperature, with this disarrangement subsiding upon the water loss or the cetostearyl alcohol effect, which is the reason why the viscosity decreased.

Storage time as well as temperature can affect the enzyme stability by changing the secondary, tertiary, and quaternary structures leading to protein unfolding and/or aggregation [19]. Thus, stability studies under accelerated storage conditions are a useful tool for evaluating formulations with proteins [28].

The chemical stability of these formulations was determined by the method of McCord and Fridovich [23], which allows a wide variation because two reactions must run sequentially, i.e. the liberation of superoxide ion followed by its scavenging by SOD. Thus, the method involving the measurement of variation of enzymatic activity was validated by the observation of 13% within-assay precision and 21% between-day repeatability. During the chemical stability study, we could observe absence of enzymatic activity in 1 g samples of the analyzed emulsions (Table 2). These results may be explained by the analytical method variation and by the small volume of SOD added to the formulations due to its high activity.

The SOD activity in the simple emulsion and in the emulsion stabilized with carbomer was reduced at the beginning of the chemical stability test, regarding the quantity of SOD added (92.10 U/g). However, after this loss, the activity remained constant during the study period at room temperature. The reduced activity shown by both formulations may be first explained by the method variation, and second by the high interference of the formulation components with the analytical method used or to interactions between SOD and formulation components, leading to a lower detection of SOD activity. 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. Only 18% of the protein added was recovered in the aqueous phase, and therefore 82% of the protein could be interacting with the formulation components present in the fatty phase. These results show that the activity of SOD present in the fatty phase could be determined by the analytical method used.

The emulsion stabilized with hydroxyethylcellulose showed a loss of activity after 24 h of storage similar to the other formulations, while the protein recovered in the aqueous phase was about 48%. This protein recovery could be explained by a greater interaction between the enzyme and the non-ionic polymer hydroxyethylcellulose. This interaction could prevent the enzyme from binding to the fatty components, a fact that did not occur in the other formulations (simple and stabilized with carbomer).

The interactions between SOD and the fatty phase components could be due to the nature of the emollient added to the formulations, the macadamia nut oil, which has a chemical structure similar to that of the cell membrane because it is rich in palmitoleic and oleic acids [29]. SOD is an enzyme which binds to cell surfaces and collagen [13], so it may show affinity for the macadamia nut oil components.

Moreover, SOD (Dismutin®-BT) utilized in these formulations is conjugated with polyethyleneglycol (PEG). SOD subjected to covalent modifications with PEG showed remarkable preservation of activity and exhibited much longer plasma lifetimes in mice. It appears that attachment of 2–4 PEG chains per glycoprotein causes minimal loss of enzymatic activity [30]. The conjugation of SOD with PEG can increase the solubility of the enzyme in hydrophobic media [31]. This may explain the low percentage of protein extracted from the simple emulsion and the emulsion stabilized with carbomer, and reinforces the hypothesis of SOD binding to hydroxyethylcellulose.

The emulsion stabilized with hydroxyethylcellulose proved not to be a good base for adding the enzyme, since a marked decrease in enzymatic activity occurred during the storage period at room temperature. After 48 h, at room temperature, a loss about 50% of the initial activity (24 h)

occurred. This may be explained by an increase of the SOD–PEG conjugate and hydroxyethylcellulose interaction during the storage period. This interaction may prevent the access of the substrate to the active site of SOD. Moreover, the interaction with hydroxyethylcellulose may expose the hydrophobic groups of the molecule, and with formulation aging, there is a greater exposure of these groups leading to conformational changes that may cause a loss of enzymatic activity. The decrease was more marked when the formulation was stored at room temperature than at 45 °C. This may be probably due to an internal structural disarrangement with the high temperature that led to a decrease in viscosity. This decrease was observed during the rheological behavior studies and hence, the enzyme present in the fatty phase should be more available for measurement.

5. Conclusions

- All formulations showed pseudoplastic behavior, with a flow index of less than 1. The emulsion stabilized with the anionic polymer, carbomer, was the most pseudoplastic formulation;
- the presence of SOD did not affect the physical stability of the formulations studied; and
- both the simple emulsion and the emulsion stabilized with the anionic polymer showed a good stability of enzymatic activity, although the protein recovery was low. In contrast, the emulsion stabilized with hydroxyethylcellulose was found not to be a good base for the addition of SOD. Although the protein recovery was higher than the other formulations, SOD lost its activity after 24 h of storage at room temperature.

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