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Radiation Technology in the Preparation of Polyethylene Oxide Hydrophilic Gels and Immobilization of Proteases for Use in Medical Practice

E. I. Vereschagin[1], Do-Hung Han[3], A. W. Troitsky[1], O. V. Grishin[1], S. E. Petrov[2], E.P . Gulyaeva[1], L. A. Bogdanova[1], M. V. Korobeinikov[2], and V. L. Auslender[2]

[1]Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Ac., Lavrentyev pr., 10, Novosibirsk 630090, Russia, [2]Budker Institute of Nuclear Physics, Siberian Branch of Russian Academy of Sciences, Ac., Lavrentyev pr., 11, Novosibirsk 630090, Russia, [3]School of Chemical Engineering, Yeungnam University, Gyonsan 713-712, Korea

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This paper deals with the development of a technology for making a hydrophilic gel of polyethylene oxide reception in which radiating ability is employed to cause cross-linking of polymers in a water solution. The gel of polyethylene oxide was shown to be non-toxic, contain 5-50% of polymer and be useful in composite medicinal forms along with biologically active substances including *Bac. subtilis* proteases. Proteases immobilized in the gel possess high thermal stability and proteolytic activity and are readily applied in medicine. The effect of immobilized proteolytic and glucolytic enzymes of *Bac. subtillis* (Immozimase) on the warm ischemia-reperfusion (I/R) which can cause hepatic and jejunum injury was also studied. These enzymes were immobilized on water-soluble polymer polyethylene glycol by means of an electron beam. The number of degranulated mast cells as well as serum ALT after I/R in the group with Immozimase was decreased to almost half as compared with the control group. Pretreatment with Immozimase resulted in significant reduction of hepatic and gut neutrophil accumulation as compared with control animals. It was concluded that Immozimase has a protective effect for hepatic and gut ischemia/reperfusion, and this effect seems to be associated with prevention of leukocyte accumulation.

Key words: Electron beam, Hydrophilic gel, Immobilized enzyme

INTRODUCTION

Composite means for external application are a large part of the world's pharmaceutical technology. Hydrophobic bases, such as Vaseline, lanolin and vegetable oils are traditionally produced by these means. Essential disadvantages of hydrophobic ointment bases include low gas-penetrability that propagates anaerobic microorganisms which in turn cause infectious inflammation, and incompatibility with most biologically active substances. In particular, the presence of a hydrophobic basis in a composition can cause irreversible denaturation and loss of specific activity in various enzymes. Nevertheless, interest in development of ointment forms containing enzymes such as bacterial proteinases is growing constantly. Such a tendency is connected first of all with the enhancement of purulent inflammation treatments. Until now medical approaches mainly based on 4th and 5th generations antibiotics have only allowed the problem of purulent inflammation pathology to be solved partially. The most impressive results have been achieved by a combination of antibacterial therapy with immobilized enzymes. Necrotic tissue proteins which provide a nutritious environment for propagating pathogenic microorganisms were effectively eradicated by proteolytic enzymes. Thus, hydrophilic gels of natural and synthetic polymers are the most acceptable composite bases for creation of ointments containing enzymes. Gels based on agar, chytosan, carboxymethylcellulose, *etc.*, have high hydrophilic properties and gas permeability, and influence the activity of dissolved enzyes very little. The

Correspondence to: Do-Hung Han, School of Chemical Engineering, Yeungnam University, Gyonsan 713-749, Korea
E-mail: dhhan@yu.ac.kr

main drawback of gels based on biopolymers is their low resistance to microorganism cleavage. Organic polymers, especially polyethylene oxides, are most perspective for manufacturing of hydrophilic gels for medical purposes. Polyethylene oxide is known to be extremely inert chemically, resistant to biodegeneration, and non-harmful to humans. The main problem in manufacturing hydrophilic gels of polyethylene oxide is associated with the lack of simple and accessible technologies for cross-linking polymer molecules in a three-dimensional structure. Chemical methods based on the use of bifunctional reagents are difficult and require tedious purification procedure for removal of toxic impurities in the gel.

It seemed likely that a physico-chemical method of manufacturing polyethylene oxide gel from its aqueous solutions using radiation-induced cross-linking technology would most perspective. In our previous work (Gonchar, *et al.*, 1996), radiation technology was employed for the immobilization of *Bac. subtilis* proteases on polyethylene oxide 1500. Further investigations have shown that the radiation-chemical cross-linking of a polyethylene oxide in an aqueous solution can be applicable not only to the immobilization of proteases, but also to the manufacture of hydrophilic gels.

In the present work we discuss the radiation technology for preparation of polyethylene oxide-based hydrophilic gels, as well as the physico-chemical and toxicological properties of the gels, and properties of medicinal preparations based on both the gel and immobilized *Bac. Subtilis* proteases on polyethylene oxide.

MATERIALS AND METHODS

The following chemicals were used in the study:

- Polyethylene oxide -1500 (molecular weight 1300 - 1600), pharm.
- Polyethylene oxide - 4000 (molecular weight 3800 - 4100), pharm
- Proteases obtained from highly productive industrially producing *Bac. subtilis* (strain CH-15).

Aqueous solutions of polyethylene oxide 1500 and 4000 (2-14%) were used. The solutions were placed in polyethylene test tubes with a capacity of 1 mL, then irradiated by bremsstrahlung radiation (braking radiation) from an the electron accelerator (ILU-6, manufactured by BINP, Russia) with an electron energy of 2.5 MeV and an absorbed dose between 2 and 10 kGy at a constant dose rate of 1.65 kGy/hour.

Carbonyl-group contents in the solutions of polyethylene oxides were analyzed by the method of Lappin and Klark (Gonchar, *et al.*, 1998) after irradiation using. *o*-Phthalaldehyde (Sigma, USA) for calibration.

Biological effects of obtained water-soluble immobilized enzymes were investigated on a model of warm liver and jejunum ischemia-reperfusion. Hepatic Ischemia-reperfusion (I/R) and circulatory hypoxia disorders associated with jejunum in adult male Wistar rats (body weight 200-250 g) were induced by cross-clamping the portal triad for 20 min. Animals were divided into three groups: sham-operated (group I), I/R without Immozimase (Group II), and animals pretreated with Immozimase before I/R (Group III). Immozimase was injected intraperitoneally at a dose of 1000 U 30 min. before I/R.

Samples of the liver and of hepatic lymph nodes were studied at the light optical level. After sections of the liver and nodes 4-6 mm thick were dyed with Meier hematoxylin and eosine, morphometric studies were performed using commonly accepted stereological methods. The areas of both the cytoplasm and the hepatocyte nuclei, nuclei-cytoplasm ratios, the total area of sinusoidal cells and the total content of hepatocytes and sinusoidal cells were determined by the point counting technique at standard magnification.

Statistical analysis was done using Student's t-test. The differences between groups of data were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Technology of manufacturing polyethylene oxide gel using bremsstrahlung radiation (braking radiation)

The majority of organic compounds including the organic polymers experience oxidation in solutions containing oxygen (Siggia and Hanna, 1983, Picaev, 1987). The mechanism of radiation-chemical oxidation has been investigated in a number of works (Siggia and Hanna, 1983, Picaev, 1987, Picaev, 1986, Bensasson, *et al.*, 1987). Oxidation of organic substances occurs basically due to the action of a hydroxyl radical generated in the course of radiolysis of the solvent. The oxidative products of organic substances are peroxide, carbonyl, hydroxyl and carboxyl substances and similar functional groups in the irradiated substances. Among the functional groups formed in polyethylene oxide by radiolysis of its aqueous solutions, the carbonyl groups, which have high reactivity to nucleophilic groups of enzymes, aroused the greatest interest.

Our research has shown that carbonyl groups are formed by radiation-chemical oxidation of aqueous solutions of polyethylene oxide. The yield of carbonyl groups was dependent on dosage from 2 to 10 kGy (Fig. 1).

It is generally accepted that only terminal hydroxyl groups take part in oxidation during irradiation of polyethylene oxide and that the mechanism of carbonyl group-formation is similar to the oxidative destruction of aliphatic alcohols (Bensasson, *et al.*, 1987). The yields of carbonyl groups from polyethylene oxides, even with

identical concentrations of solutions and stationary doses, are somewhat dependent on the molecular weights of polyethylene oxides. However, our investigation for radiation-chemical oxidation has not revealed significant differences in the behavior of polyethylene oxides with various molecular weights with either stationary dose and varied concentrations of solutions, or stationary concentrations of solutions and various doses (Fig. 1 and Fig. 2). Such independence of the polyethylene oxide molecular weight in a carbonyl group-yield may be due to radiolysis at the ether-binding sites in polyethylene oxide.

It is known that irradiant gel-formation is typical only for those polymers in which the cross-linking processes prevail over destruction under irradiation. Radiolytic destruction in aqueous polyethylene oxide usually prevailed in the presence of oxygen, while gel formation prevailed in its absence.

As stated above, the technology of polyethylene oxide gel production represents a special interest associated with creation of prospective medicinal ointments with immobilized proteases. It was hypothesized that enhancement of absorbed dose and increased dose rate could lead to gel formation even in oxygen-containing solutions of polyethylene oxide, due to a more than critical concentration of free radicals and reactive groups for gelation.

To test this hypothesis, we investigated the influence fo an accelerated electron beam at 2 MeV with a high dose rate on aqueous polyethylene oxide (molecular weight of 1500). An ILU-6 industrial electron accelerator was used as the source of radiation. As has been shown, the irradiation of polyethylene oxide 1500 solutions at a dose rate of 5 kGv/s or higher results in formation of the gel even without removal of dissolved oxygen. The dose for gel-formation depended on the polyethylene oxide concentration, ranging from 40 to 420 kGy for concentration from 1 to 50% (Fig. 3). Gels obtained with this technology were colorless and glassy. After homogenization, the dynamic viscosity of the plastic gels ranged from 3 to 6 Pa/s, depending on the concentration of polyethylene oxide.

A gel of polyethylene oxide 1500 with 10% of basic substance was used for a composite preparation of Stomatozim, with proteases of *Bac. subtilis* immobilized on polyethylene oxide in the gel. As has been shown, the *Bac. subtilis* proteases have high thermal stability even in the absence of substrate. Stomatozim kept 37% of activity even after heating at 50°C during 24 h while nonimmobilized proteases lost more than 80% 1 h under the same conditions. The stabilizing effect of the polyethylene oxide gel on the proteases seems to be related to the "double immobilization" effect, in which proteases con-

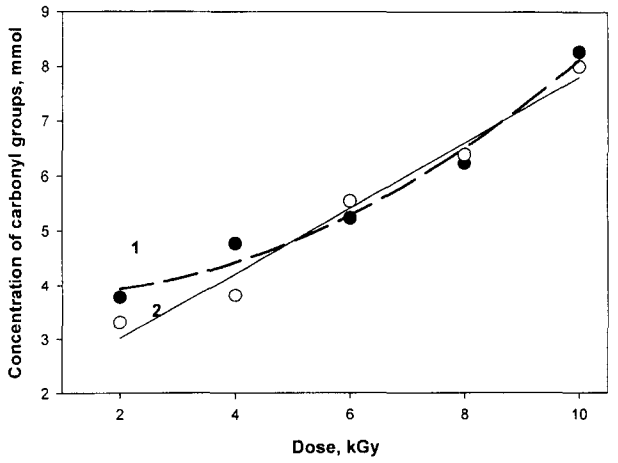

Fig. 1. The yield of carbonyl groups depending on absorbed dose: 1 - black circles and dashed line for PEO-4000; 2 - white circles and solid for PEO-1500.

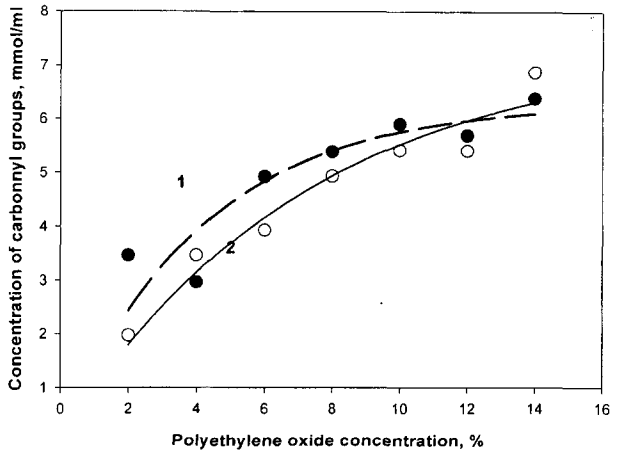

Fig. 2. The yield of carbonyl groups depending on initial polyethylene oxide concentration: 1-black circles and dashed line for PEO-4000; 2- white circles and solid line for PEO-1500.

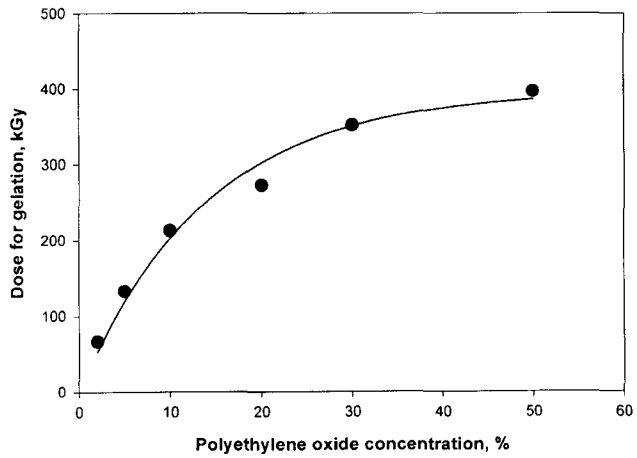

Fig. 3. Dose of polyethylene oxide (PEO-1500) gelation depending on initial concentration.

nected with both polyethylene oxide and a gel matrix.

The ointment-like structure of the compound in combination with its high protease activity allowed it to be applied successfully in stomatology as "Stomatozim" (Siggia and Hanna, 1983). Clinical tests proved that such a preparation effectively removed non-vital sites of dentine and mucus and also provided anti-inflammatory and anti-edematic action. Therefore "Stomatozim" provied to be an effective drug for treatment of caries and parodontosis.

Biological effects of proteases immobilized on polyethylene glycol (PEG)

Proteases of Bac. subtillis immobilized on PEG using braking radiation, but without gel formation, possessed somewhat significant pharmacological effects per se. Investigation of biological effect of proteinases immobilized on PEG in liver and jejunum ischemia/reperfusion(I/R) in rats showed the following results. The study of liver samples with cross-clamping of the portal triad indicated considerable change in the organ's structure: no characteristic lobulation was present, but sharply pronounced intratissue and perivascular edemata were observed. Most of the hepatocytes had ares of discomplementation and sites of necrosis. The hepatocytes cytoplasm had a pronounced basophilic color and the nuclei were round, with the central position inside the cell. Isolated basophilically-colored sinusoidal cells were observable in the apertures of poorly defined sinusoids. The parenchyma of the liver absorbed all leukocytes, mainly neutrophiles and macrophages. The morphological changes in liver were primarily determined by the reaction of its microcirculatory stream and showed themselves in various changes of diameter in sinusoids and central veins. Areas having spasmized capillaries alternated with areas having enlarged edematous full-blooded hepatic sinusoids. The portal vessels were commonly enlarged and had sites of hemorrhage. The parenchyma showed an increase in cell density a considerable decrease in volume density of hepatocytes cytoplasm, considerabley, and a nuclei/cytoplasm ratio that was 4.7 times greater. An increase of the density of cells and sinusoid cells, especially of the Kupffer's cells, was also noted. Well-defined degranulated mast cells were observable around the vessels. Histological study of samples of the liver under correction by the Immozimase preparation, however showed the characteristic lobular structure. The nuclei/cytoplasm ratio increased by 38% as compared with the control group. The sinusoids were enlarged for the most part, there were minor sites of hemmorrhage, and leukocyte infiltration was found only around the portal vessels. There was a 28% increase in the volume density of the parenchymatous cells.

Serum ALT 6 h after ischemia in Group II was significantly greater, compared with Group III (1223 and 811 U/l respectively). Hepatic lymph osmolality in Group III completely recovered in 6 h after ischemia, while it decreased significantly (by 30%) in Group II 6 h after I/R, as compared with Group I.

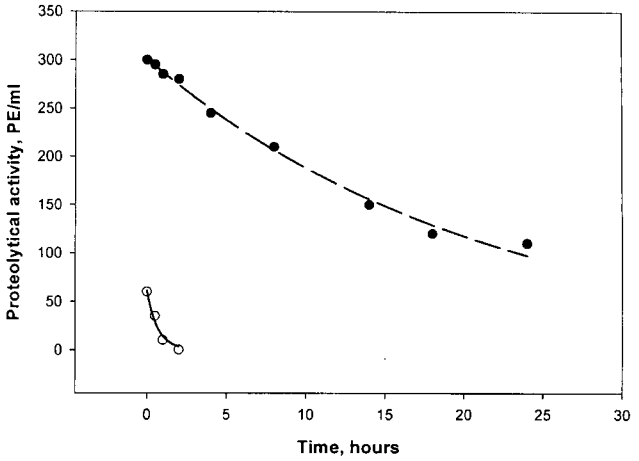

Fig. 4. Proteolytical activity of immobilized proteses depending on time passed after immobilization black circles and dashed line for proteases immobilized on PEO-gel; white circles and solid line for proteases immobilized on polyethylene oxide aqueous solution

In both experimental groups, reactive jejunum hyperemia was noted within 3 h after GCH. Meantime density of functional capillaries in Group III increased by 20% as compared the Group II, but a 25% decrease in functional capillaries along with sings of venous spasm in jejunum was noted in Group II by the 3 h. The number of degranulated mast cells after GCH in the Immozimase-treated animals decreased twice as much as in Group II.

The data obtained makes clear that pretreatment with immobilized enzymes of Bac. subtillis, i.e. enzymes with both proteolytical (see Fig. 4) and glycolitical activities, has a potent protective effect on warm ischemia/reperfusion of liver and jejunum. Moreover, enhancement of functional capillary density as well as rapid restoration of lymph osmolality shows that microcirculatory disorders in the target organs disappeared within 6 h after I/R. Most significant differences between control and Immozimase-treated animals were determined by the degree of leukocyte infiltration of the target organs. The protective effect of Immozimase was shown to correlate with a significant reduction of leukocyte accumulation in liver and jejunum. The probable mechanism for prevention is the deactivation of cell adhesion molecules by Immozimase.

CONCLUSION

Our study has shown that the technology of radiation-induced cross-linking can be adapted for reception of

polyethylene oxide gel. Polyethylene oxide-based gel has high hydrophilic properties and is practically nontoxic in various routes of introduction. This technology can probably be applied to bacterial proteases. However, for higher molecular weight enzymes this technique may be problematical. These larger enzymes can probably be inactivated more readily by oxidizing byproducts of water radiolysis. Considering these aspects, this techique can potentially be applied only for bacterial proteases which are stable for irradiation.

Immobilized on a water-soluble base enzymes of *Bac. Subtillis* (Immozimase) effectively protected liver and jejunum against ischemia/reperfusion. The protective effect was related to a reduction of neutrophil accumulation in the target organs. Immobilized proteolytical enzymes may be recommended for prevention of ischemic and reperfusion damage in trasplants and urgent cardiology. We consider that the protective pharmacological effect of Immozimase is not connected only with its prolonged activity. On the contrary, this effect relates to the ability of immobilized protease to remain active after injection. We suppose that its stability in the body is connceted with the absence of specific inhibitors, and results from the protective effect of the PEO matrix. The ointment-like structure of the compound in combination with its high protease activity allows "Stomatozim" to be applied successfully to stomatology. Therefore Stomatozim should be accepted as an effective drug for treatment of caries and parodontosis.

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