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Immobilized Proteases for Wound Cleaning

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Abstract—Data on the immobilization of proteolytic enzymes for creation of medicines for the first phase of wound healing are summarized. The most common methods of immobilization, media, and pharmaceutical forms are characterized.

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Healing wounds with a high content of necrotic tissues which are quite difficult to remove by traditional methods is an urgent problem of modern surgery.

The wound process is a complex of physiologic reactions induced by tissue damage [1]. The "Golden Standard" in cleaning septic wounds is still scalpel surgery. However, in certain situations this methods does not work in view of the fact that it can only be applied when the wound is surrounded by sufficient healthy tissues and no heavy ischaemia is observed at the site of mechanical injury. In the case of weak damages, the use of scalpel for wound cleaning is, too, unreasonable [2]. Healing wounds with a high content of necrotic tissues which are impossible to remove by surgery still remains quite an urgent problem.

Necrotic tissues are removed using various pharmaceutics, largely proteolytic enzymes (proteases), which is considered the most promising and biologically soft technique [3]. There is ample published evidence showing that the wound process involves enzymes [4] and, therefore, the use of enzyme-containing agents for wound healing is a pathogenetically justified approach [5].

Even though proteases have already long been use in the therapy of the wound process, in the "Federal Guidance for the Use of Medical Preparations" for 2010 [6] mentions as little as three corresponding drugs (trypsin crystalline, chymotrypsin, chymopsin), and, what is important, these agents are recommended for use exclusively in the case of pyoinflammatory processes in the maxillofacilal area (the Guidance contains no recommendations concerning medicines for the therapy of wounds and skin burns). The enzyme preparations recommended in the Guidance are included in the section "Anti-inflammatory agents of other groups." It should also be noted that the previously popular Iruxol ointment is excluded from the list in [6].

Derimedved' et al. [7] lists 10 medicines recommended for use at the first phase of the wound process, but only one of them contains a proteolytic enzyme capable of acting as an antinecrotic agent [7]. As to the foreign wound-cleaning preparations containing proteolytic enzymes, in the "Encyclopedia of Chemical Technology" they are included in a separate subgroup titled "Topical Enzymes," i.e. enzymes for topical application, which are designated for selective removal of necrotic tissues from wounds and burns. This subgroup contains such medicines as Travase, Santyl (contains collagenase), Elase, Granulex (contains trypsine and papain), Panifil, and Panifil White (contains papain and urea) [8]. In the Mashkovskii's Drug Handbook, a wide range of preparations on the basis of proteolytic enzymes for wound cleaning is presented. Thus, group IV "Enzyme Preparations and Inhibitors" contains the subgroup "Enzyme Preparations Primarily Applied for the Therapy of Purulent Necrotic Processes," including 13 enzyme preparations. Of them 11 are based on proteolytic enzymes: Three contain immobilized proteases, and the others are native proteases (trypsin, chimotrypsin, terrilitin, etc.). These enzymes are applied on necrotic tissues as powders, as well as aqueous solutions prepared *ex tempore* or applied on textile supports [9].

Proteases catalyzing degradation of tissue proteins favor wound cleaning and also exhibit antiinflammatory, fibrinolytic, and antiedemic effects [9–11]. However, native proteases, when directly applied on a wound, undergo fast deactivation, which makes such enzyme therapy inefficient. Thus, according to [12], the necrolytic effect of proteolytic enzymes in this case lasts as little as 15–30 min. In this connection at present increasing application is being found for immobilized protease preparations. Immobilization allows the effect of proteases to be localized in a required region and enhances their stability under the conditions of the wound process [13, 14].

We have analyzed the published information on preparations on the basis of proteolytic enzymes, taking into account sources of proteases, dosage forms, and methods of enzyme immobilization, as well as combinations of proteases with substances from other pharmacological groups, specifically antibiotics.

Wound-cleaning preparations are produced in various dosage forms: suspensions and emulsions, powders, films and membranes, gel bands, fiber materials, ointments, and gels.

Suspensions and emulsions are scarcely used as dosage forms of proteases. To our knowledge, there are only one enzyme preparation in the form of emulsions [15] and two enzyme preparations in the form of suspension [9, 16–18]. Even though suspensions and emulsions relate to one dispersion group, specifically disperse systems with a liquid dispersive medium, but the principles of production of these systems as dosage forms and methods of protease immobilization in them differ from each other. Thus, for emulsions a dissolved proteolytic enzyme is dispersed in a liquid medium, i.e. the inclusion technique is used, and the dispersive medium serves as a carrier matrix. Known medicines, Profezim and Procelan, are produced by a more complicated technology. The enzyme (here protosubtilin) is preliminarily chemically immobilized on aminoethyl cellulose (carrier) [19], and the resulting powder is suspended in an isotonic solution of sodium chloride (dispersive medium). Thus, in the case of suspensions, double immobilization is realized: enzyme is immobilized on a carrier which, in its turn, is introduced into a dispersive medium.

The most common dosage forms for immobilized proteases are powders. This group includes five medicinal preparations differing from each other by carriers, methods of enzyme immobilization, and application. Terridecasa is a proteolytic enzyme territilin (waste product of *Aspergillus terricola* mold fungus) covalently bound with an oxidized derivative of the dextran polyglycine [9, 17]. Before use the enzyme is dissolved in water, and the resulting solution is applied on a surface to be treated. The other immobilized preparations of this group are applied as powders. The carriers for such preparations are swelling polysaccharide polymers: sodium alginate in the Sipralin dusting powder containing protease C [20] or various dextrans in powders containing papain, trypsin, and chymotrypsin [21].

Dusting powders are prepared using various immobilization techniques. Thus, the immobilization of protease C on sodium alginate is performed by adsorption due to weak chemical bonding (hydrogen bonds, coordination, etc.) between the carboxy groups of algic acid and amino and other positively charged groups on the enzyme surface. Covalent bonds of proteases with dextrans are formed after the latter have been treated with activating agents: bromocyan, a solution of 4-nitrophenyl glycidyl ether and sodium hydrazide in toluene, or 2-amino-4,6-dichloro-s-triazine. The chemical bonds that form strongly fix enzyme molecules on the carrier and prevent their desorption during application.

Films and membranes are designated for application on a wound or burn surface, which is made possible by the adhesive properties of matrices. The matrices are copolymers and modified polymers, and, therewith, their composition varies over a wide range: from one carrier polymer (for example, a polyvinyl butyryl membrane [22]) to complex compositions including several components (for example, a composition formed by polyethylene oxide (PEO) 400, copolymer of vinyl-pyrrolidone and vinyl alcohol, hydroxypropyl cellulose, and dextran-40 [23]).

The immobilization of proteases in films and membranes is performed by the inclusion technique (protease is incorporated into the structure during film or membrane formation) and via covalent binding. Two modes of covalent binding are possible: either support is preliminarily activated by special reagents and then treated with an enzyme (protease) solution or matrix is first kept in an enzyme solution and then the surface with adsorbed enzyme is treated with a cross-linking agent. The most common cross-linking agent is glutaraldehyde, but some other substances, for example, 1,1'-carbonyldiimidazole or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesul-

fonate, can be used [17, 22]. Upon treatment with cross-linking agents not only enzyme–carrier, but also enzyme–enzyme bonds can form on the carrier surface. In certain cases pretreatment leads to modification of the enzyme, rather than the carrier. Thus, Kil'deevaet al. [24] bound trypsin with carboxymethyl dextrans containing 2-amino-4-chloro-s-triazine residues, aiming at enhancing stability and preventing desorption from a possible matrix, specifically cellulose triacetate. A complicated composition is characteristic of films with hydrolytin (proteolytic enzyme isolated from the *Streptomyces hygroscopicus* culture), whose matrix consists of polyether resins and vinyl chloride copolymers and is cross-linked with polyvinylpyrrolidone (PVP) gel and copolymers of vinylpyrrolidone with acrylic monomers [25]. This dosage form was peoduced using a unique two-stage immobilization method: The first stage involves chemical activation of the support surface and the second, copolymerization of the activated support with an enzyme-containing PVP gel. Thus, the enzyme proves to be spatially restricted by a carcass of polymer molecules cross-linked with the support.

The best known of medical films are Prodioxilong pellicles containing protosubtilin. In this medicine, the protease is immobilized in a biosoluble composition of cacao oil and a copolymer of acrylamide, *N*-vinylpyrrolidone, and ethyl acrylate. This film slowly dissolves in wounds, gradually releasing the protease incorporated in the matrix, which allows the enzyme to be consumed entirely [26]. Moreover, protosubtilin is introduced into a lyophilized procelan film which forms the dispersive phase of the above suspension [18], thus ensuring stability of the protease in the target site.

A new type of film dosage forms, viz. "film plus carrier," was suggested by Kovalenko [17]. They are prepared by applying a thin film of BF-6 glue on a polyethylene film or a hydrate cellulose membrane, after which the surface in coated with granules of SUMS-1 and SUMS-2 carbon mineral carriers. The resulting materials are dried for the granules to fix tightly on the surface and then immersed into a protosubtilin solution to adsorb the enzyme. The author also considers the variant of subsequent treatment of protosubtilin composite preparations with a cross-linking agent (glutaraldehyde) to enhace their stability.

Prototypes of modern gel bands are ointment bands: a gauzy fabric impregnated by a hydrophilic ointment. Previously ointment bands were prepared before use in bandaging rooms by applying an ointment composition on gauze with a spatula. However, the very process of preparing such bands was time consuming, and underdosage or non-uniform ointment layers were not excluded. Moreover, evidence is available showing that ointment bands prepared in bandaging rooms may not always conform to sterility requirements [27].

Modern gel bands consist of a fibrous base, largely a textile fabric (an unwoven material is also possible) supporting a gel containing a proteolytic enzyme. The technology of production of this form of immobilized proteases includes two stages: (1) preparation of gel (a solution of gel-forming polymer) and immobilization of proteolytic enzyme in it by the inclusion technique and (2) application of gel on support (or impregnation of support with gel). The most common gel-forming agents are polysaccharides and their derivatives: sodium alginate [28–30], carboxymethyl chitosan and carboxymethyl chitin [30], aubasidan, xanthan and polymixin [13], and pectin [14]. Of synthetic polymers, polyvinyl alcohol cross-linked by sodium tetrafluoro-borate is used [31, 32]. The range of textile supports is not very wide and includes cellulose (gauze) and polyether materials. The very term "gel bands," to our knowledge, is not fixed in the existing legal documents containing definitions of drug forms, but it is fairly widespread and has quite an unequivocal interpretation in different sources. However, the lack of a solid definition leads to certain controversy. Thus, Kravchenko and Davidenko [14] have described "medical plasters" with proteolytic enzymes, but their production technology is completely the same as the above-described production technology for gel bands. However, this is the only precedent, and we failed to find other sources in which this dosage form is defined in a way different from "gel band."

The most abundant group of dosage forms of wound-cleaning medicines is formed by fibrous materials. This group includes both surgical fibers (sutural material) and various bandaging materials.

The overwhelming majority of protease-containing fibrous materials are produced by means of covalent immobilization [17, 33–36]. The surface of fibers is activated with a bifunctional cross-linking agents, specifically glutaraldehyde which is suitable both for cellulose and polyamide fibers. Callulose fibers can be activated with oxidants to form dialdehyde cellulose [33, 35]; surface grafting polymerization with acrylic

acid is also possible [36]. In any case, the fiber surface acquires active chemical groups (as a rule, aldehyde) which react with surface functional groups of protease molecules to form strong covalent bonds.

Inclusion is a much rarer immobilization technique. The inclusion process in itself is realized directly during fiber formation, it is relatively simple to implement and requires no chemical modification of the carrier polymer. Fibers to include enzymes during formation can be readily obtained on an equipment used for production of ordinary fibers, and, therewith, a lot of enzyme can be included into the fiber structure. However, fibers are not infrequently produced using substances inactivating proteolytic enzymes (for example, methylene chloride). The inactivation effect can be avoided by means of certain engineering measures (by using spinning solutions in the form of enzyme suspensions in organic polymer solutions), as well as by chemical modification of the enzyme itself. For example, before inclusion into cellulose triacetate, cellulose diacetate, or Fluoroplast-42 fibers protosubtilin is modified by adding it to a styrene–maleic acid copolymer in the presence of Cr^{3+} ions, which gives more active fibers and enhances resistance of the enzyme to unfavorable factors [37].

The paper of Sevast'yanova et al. [38] on the production of carbon fabrics with immobilized proteases deserves special attention. The authors resorted to a technique unusual for developers of fibers with immobilized proteases, i.e. adsorptive immobilization. Moreover, for the carriers the authors took, instead of traditional polymers (cellulose and its derivatives or polyamides, a highly adsorptive Dnepr-MN activated carbon fiber material. However, it was shown that adsorption on carbon materials adversely affects the activity of proteolytic enzymes and, sometimes, the strength of the carbon fiber is also affected. To minimize adhesive interactions and stabilize enzymes, polymer carriers cross-linked with sodium tetraborate were applied, and, therewith, the cross-linking agents here were PEO 400 and PEO 1500 rather than polyvinyl alcohol, a traditional reagent in such cases. To obtain an immobilized dosage form, the enzyme is dissolved in an aqueous solution of PEO 400 or 1500, the solution is applied on the surface of a carbon fabric, modified with a sodium tetraborate solution, and then dried. This technology is similar to the technology for gel bands, but the polymers used in the former case are not gel-forming ones.

Sevast'yanova et al. [38] suggested two possible immobilization mechanisms: (1) adsorption on the carbon surface of micellar aggregates of polyethylene oxides including protease molecules; and (2) cross-linking by sodium tetraborate of terminal hydroxy groups of the polymer and hydroxy and primary amino groups of the enzyme. It is also not excluded that the carbon matrix is involved in some way.

A great number of developed wound-cleaning preparations are based on the immobilization of proteolytic enzymes in soft dosage forms: ointments and gels, among which prevailing are hydrophilic ointments. Thus, Kovalenko [17] made use of silica gels (gelatinized sols of sodium silicate neutralized with HCl) including enzyme molecules. Such gels exhibit a high proteolytic activity. Enzymes immobilized in activated chitosan gels are prepared in a different way. The typical technology of preparation of such medical gels, described in the patent [39], includes several consecutive stages. A preprepared chitosan gel is treated with a glutaraldehyde solution. The polysaccharide is activated by forming aldimine bonds between the chitosan amino group and one aldehyde group of a bifunctional reagent, namely glutaraldehyde. The resulting activated chitosan gel is then doped with an enzyme which is fixed by bonding between the functional group of glutaraldehyde and the amino group of the protease molecule. This technology was used to obtain gels containing covalently bound papain [40, 41], trypsin [42], and a complex of enzymes isolated from the hepatopancreas of *Paralithodes camtschatica* crab [39].

Enzymes are suggested to be immobilized on hydrophilic gels prepared on the basis of synthetic polymers, largely polyethylene oxides. For example, this is a PEO gel with collase (partially purified enzyme preparation from *Paralithodes camtschatica* crab hepatopancreas, containing, along with collagenase, a number of other proteolytic enzymes) [43, 44], a PEG-155 gel cross-linked with protosubtilin under the action of accelerated electrons (Immosimase preparation) and a hydrogel formed by PEO-5000 and glycerol, containing immobilized protosubtilin (Profesimum preparation) [19]. In the latter case we can speak about consecutive combined immobilization: the enzyme was preliminarily immobilized on a polymer carrier, after which the carrier with immobilized enzyme is introduced into an ointment matrix. The same technology was used to produce one more immobilized protosubtilin form, namely Procelan. One difference was that the enzyme was included into a

PEO 400 + PEO 1500 polyalloy, rather than a hydrogel [18].

Hydrophylic matrices can have a complicated composition. For example, protease C is suggested to immobilize on a polyvinylpyrrolidone, glycerol, PEO 1500, and 1,2-propylene glycol polyalloy [45]. For inclusion of crab collagenase [46] a composition containing vinyl glutarate, vinyl acetate, and vinyl alcohol is suggested. On contact with the wound content the composition swells and forms gel. Ointment compositions are also prepared using lipophilic bases. For example, an ointment containing a bacterial collagenase from *Clostridium histolyticum* is prepared on a mineral oil base [15]. The Iruxol ointment containing clostridiopeptidase A [6] as well as ointments with protease C [47] and moricrase [48] are prepared on lipophilic bases. Moricrase (proteinase isolated from *Paralithodes camtschatica* crab) is also a component of the Moricrol ointment on a lipophilic base, specifically eiconol (a complex of eicosapentaenoic and docosenoic fatty acids and vitamins A, E, D, and F [49]. Inclusion of proteolytic enzymes into a lipophilic base is accomplished by suspending in the latter a native enzyme.

We have analyzed publications on immobilization technologies for preparing wound-cleaning medicines with proteolytic enzymes to estimate the shares of publications concerning one or another technology. Most frequently (42%) proteolylic enzymes were immobilized by the inclusion technology in various versions, depending on the carrier. In the case of high-molecular gels, enzymes are immobilized due to spacial restrictions imposed by the 3D polymer structure. In the case of a lipophilic base, the spacial separation of protease on a carrier base is reached in a mechanical way, i.e. by suspension. The latter version of the inclusion technology is similar to that for preparing polymer fibers with proteolytic enzymes from enzyme suspensions in spinning solutions. It is important that enzymes immobilized by the inclusion technology do not bind to anything, and, therefore, there are no steric hindrances arising on covalent or electrostatic enzyme–polymer binding [50]. Moreover, the method is easy to perform and suitable for virtually any enzymes. However, a rigidly cross-linked polymer matrix may hinder, by diffusion reasons, penetration of the substrate, and is completely inapplicable in the case of high-molecular substrates (proteins) [51].

The second most used technology (in 29% of the publications) is covalent binding. The conjugates obtained by this technology are very strong, making immobilized enzymes stable in external conditions [51]. The main disadvantages are as follows: labor consumption (difficulties in selecting carrier, cross-linking (activating) agent, and reaction conditions) [50]; covalent biding with carrier may entail irreversible structural changes in immobilized enzyme and, as a result, its low residual activity [52]; and the active center should be protected from chemical modification [51].

The absorption technology of immobilization of proteolytic enzymes was used in 11% of cases. This technology is the earliest immobilization method. Adsorption is accomplished by simply mixing enzyme and carrier for a certain period of time and involves no covalent binding. However, immobilized enzymes are formed in a low yield and show a low residual activity. Moreover, thus prepared immobilized enzyme forms are extremely unstable, and enzymes may be desorbed even on minor changes of the medium [50]. The main advantages of this technology include simplicity of implementation and availability of sorbents [51].

The use in wound healing of preparations composed of substances of different pharmacologic groups makes it possible to avoid the disadvantage of traditional preparations, namely, a unidirectional effect [1]. In the first phase of the wound process, drugs should suppress infection in the wound, normalize the local hemostasis, activate sloughing, and adsorb wound secrets [4]. To solve the first of this task, i.e. to suppress wound infection, would-cleaning preparations contain antimicrobial substances, both individual or in mixed with other antimicrobial agents. Some authors [28, 29, 32, 38] reported the use of natural substances, lytic enzymes (lysozyme) and their complexes, as antimicrobial substances. However, such combination casts some doubts, since lytic enzymes, while contributing to the necrolytic effect of immobilized proteases, are proteins, i.e. substrates for proteolytic enzymes.

Synthetic antimicrobial agents, too, pose certain problems. Thus, for example, from 80 to 100% of microbial secrets associated with suppurative inflamemation are insensitive to gentamicin. Furacilin exhibits almost no antimicrobial activity toward principal surgical pathogens [1]. Research on the efficiency of synthetic chemotherapeutic preparations (antibiotics and antiseptics) active toward principal representatives of wound microflora [53] revealed the highest activity of chlorhexidine and dioxidine. Semisynthetic antibiotics of the penicillin, cephalosporin, and amino-

glycoside groups were found to be unsuitable for healing large wounds [53]. Chloramfenicol (Laevomycetin), a broad-spectrum antibiotic, a component of the Iruxol, Laevomecol, and Laevosin ointments applied for healing septic wounds in the first phase of the wound process, deserves attention. However, this antibiotic is not very active against acid-resistant bacteria, *Pseudomonas aeruginosa*, and *Clostridium* bacteria [9].

The main advantage of soft dosage forms of immobilized proteases is that they combine a number of factors enhancing the efficiency and convenience in use. First, soft dosage forms can be applied on wounds of any relief, and, therewith, full contact with wound bed is guaranteed. Second, the amount of a soft dosage form and, as a result, the level of the necrolytic effect, can be controlled in view of the current therapeutic situation, irrespective of how much other means, for example, bandage materials, are used. Third, dosage forms on the hyperosmolar base act as dehydratants favoring efflux of necrosis products and toxins from tissues surrounding the wound. Absorption involves the entire base volume and then develops, due to the osmotic gradient, into the band. Fourth, with hydrophilic bases, the softest and efficient im-mobilization technique, i.e. inclusion, is feasible. As the base matrix absorbs the wound secret, and, in doing so, changes consistency, "opening" of the internal structure with gradual release of the entire immobilized enzyme takes place. Moreover, the technology of production of soft dosage forms allows one to relatively easily introduce in them additional active substance or their combinations. However, the described advantages are characteristic of not all soft dosage forms. For example, gels from cross-linked polymers are similar to films and feature all disadvantages of the latter form.

Thus, our analysis of the literature concerning drug dosage forms allows a conclusion that the preferable dosage forms for immobilized proteolytic enzymes are soft dosage forms (ointments and gels) with a hydrophilic hyperosmolar base, which involve no strong (covalent) bonds between base components and enzyme.

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