REVIEW ARTICLE

Results and Achievements in the Engineering of Pharmacological Enzymes for Clinical Application

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Abstract

Research into modified enzyme derivatives and their development have expanded considerably modern arsenal of biopharmaceuticals. The effectiveness of enzyme drugs *in vivo* and their prospectiveness in clinical practice were confirmed by biomedical studies. Several directions have formed in the biocatalyst research, such as antibody-drug interactions, construction of polyenzyme nanoensembles producing extra- and intracellular effects, and enzyme modification for targeted delivery to the damaged area. Modified enzyme derivatives have been became the effective agents against injurious action of oxidative stress *in vivo*. Research promotion is highly noticeable for covalent bienzyme superoxide dismutase-chondroitin sulfate-catalase conjugate. The obtained data contribute the investigation of salable conjugates produced by methods of chemical and biological synthesis. A broad research front and collective biomedical effort increase the probability of successful breakthroughs in the control over different types of damage to biological tissues.

Keywords: vascular injuries, oxidative stress, inflammation, enzymes, modification, targeted drug delivery, nanoconjugates.

Introduction

Progress in medicine implies the use of advanced diagnostic technologies, therapeutic techniques and highly effective new-generation drugs. A breakthrough in the development of new therapeutic agents was provided by molecular construction on the basis of proteins and other high-molecular-weight compounds. Examination of proteins as specifically folded macromolecular chains has revealed functional groups of amino acid residues on enzyme surface which could be modified to obtain derivatives with high stability for subsequent use in clinical practice. This review concerns with water-soluble modified protein compounds for medical purposes.

The development of approaches to protein modification was initiated within the concept of targeted drug delivery. Expansion of the

research led to interdisciplinary studies of biological conjugates for medical use.[17, 19, 21]

Methods for Bioconjugate Production

Bioconjugates have been obtained using various methods, such as chemical modification, site-specific mutagenesis, their combination, and construction of therapeutic nanosystems. With accumulation of modified protein derivatives a group of antibody-drug conjugates has formed. Further developments of this group were provided particularly by GlycoConnect, a technique consisting of a two-stage process: enzymatic remodeling of the N-glycan at Asp-297 in an IgG antibody and attachment of an azide, followed by ligation of the desired pharmacological agent using copper-free click chemistry.[40] The resulting conjugates with monoclonal antibodies demonstrate high stability and efficiency, promising their predominant therapeutic index. Galactose-conjugated antibodies are used in regionally specific and systemic immunotherapy.[33] Conjugation did not interfere with two antibody functions in addition to antigen binding: complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. Conjugation of egg-white proteins and lysozyme by covalent attachment to galactomannan via Maillard reaction potentiated antioxidant activity of ovalbumin (by increasing its lipid affinity) and antimicrobial activity of lysozyme.[31] The prospects of biomedical investigation of a given protein conjugate are strongly determined by the retained activities of its constituents. Therefore, a methodology for reactivation of conjugate components has been proposed.[41] Site-directed mutagenesis was used to generate mutant pyrophosphates with a substituted Cys residue for the specific Lys-148 site which is within a conserved sequence near the active site and exposed to the surface of the protein for chemical reaction. The mutated Cys residue was modified with p-chloromercuribenzoate (PCMB) or poly(2-hydroxyethyl methacrylate) (pHEMA), which resulted in obvious decrease or complete loss of the enzyme activity. However, the protein activity can be restored to different extents by reductive reagents leading to dissociation of the modifiers (PCMB or pHEMA) to recover the enzyme activity. This strategy provides an efficient control over enzyme activity at different levels of site-specific conjugation of small molecules and polymers.[41]

Bovine pancreatic ribonuclease conjugated with hydrophilic poly[N-(2-hydroxypropyl) methacrylamide] (classic or star-like) produced antitumor effect in nude mice bearing various human tumors after 10 daily intravenous or intraperitoneal doses of 2.5 and/or 1 mg/kg, respectively.[39]

Effects of Low- and High-Molecular Weight Modifiers on Enzymes

The perspectives of unifying small molecules with biological drugs are hampered by the necessity of simultaneous analytical characterization and regulatory considerations for the resulting antibody-drug conjugates.[6] The following aspects remain open to discussion: identity parameters for test and quality control samples, efficiency of the conjugates and their heterogeneity, restriction for additives, reference standards, definitions for various types of stability, etc. The developments in the antibody-drug conjugate technologies depend on the use of short linker molecules, the number of drug molecules (between three and seven) attached to the antibody, and the safety of manufacturing.[42] Conjugation with synthetic nanocarriers can be facilitated by using polyethylene glycol (PEG) functional nanostructures coupled to a bispecific antibody.[8] Dual specificity of these antibodies to methoxy PEG epitopes and cancer targets, such as epidermal growth

factor receptor provide better accumulation of the conjugate compared with linear methoxy PEG. Various modifying techniques have been employed to improve pharmacological characteristics of conjugated enzymes: half-life in the circulation, accumulation in the damaged area, "grafting" useful therapeutic activities, and to eliminate their drawbacks: low stability *in vivo*, high inhibitability and immunogenicity, etc. Methoxypolyethylene glycol-p-nitrophenyl carbonate (MW 5 kDa) was used for modification of lysozyme with preservation of its activity (77% its original enzyme activity), molecular structure, resistance to proteolysis and stability at 50ºC.[5] Overexpression of hyaluronan synthase 3 promoted the growth of cancer pancreatic tumor with abundant extracellular hyaluronan accumulation.[13] Treatment with PEGylated human recombinant hyaluronidase inhibited these processes (reduced adhesion of epithelial cells) and suppressed tumor growth. PEG coupling based on microbial transglutaminase has been proposed to increase molecular homogeneity of the resulting conjugate by specific interaction with one or two Gln residues of a given protein.[28] A targeted drug delivery system for cytostatics is based on a combination of the drug carrier Zn-porphyrin-cyclodextrin and immunoglo-bulins forming supramolecular coordination complexes.[10] Therapeutic superiority of the coordination assembly nanosystem over those of building blocks used for construction of the system was shown in a mouse model of human C32 carcinoma. Bovine serum albumin (BSA) was conjugated to poly(N-isopropylacrylamide-co-acrylic acid) by using water-soluble carbodiimide for application as a novel immunogenic system in vaccine technology.[3] Two types of conjugate particles formed depending on the mass ratio of albumin and polymer carrier: the protein molecules in the structure of conjugate particles are densely covered as a shell by polymer chain and practically "fenced off" from water environment and the conjugate-forming particles possess more friable structures in which protein molecules are practically exposed to the solvent. Covalent binding of Cu,Zn-superoxide dismutase (SOD) with low-molecular-weight heparin enhanced thermostability, acid and alkali resistance, and anti-trypsin stability of the enzyme.[45] Recombinant arginine deiminase (rADI) was conjugated with the C terminus of heparin-binding hemagglutinin adhesion protein via succinimidyl 3-(2-pyridyldithio)propionate (SPDP), and a recombinant fusion protein was produced to deliver this enzyme to rADI-resistant human breast adenocarcinoma cells.[43] Increased uptake of the conjugate restored the sensitivity of MCF-7 cells to rADI treatment. Similar conjugates can be used as antitumor drugs with an intercellular mechanism of action independent of argininosuccinate synthetase expression. In order to improve intravenous delivery of L-asparaginase the enzyme was covalently linked to fatty acids having different chain lengths (C_{12}, C_{16} and C_{22}).[1] The asparaginase bioconjugates showed higher resistance to proteolysis, higher stability at different pH and prolonged plasma half-life compared with native enzyme, which offers good biomedical prospects for this strategy.

An interesting method based on chemical modification with 3-maleimidopropionic acid (MPA) has been suggested to produce an *in vivo* conjugate between serum albumin and inhibitory peptide[44] with rapid and irreversible conjugation of the peptide to serum albumin thiol groups at a 1:1 molar ratio. After intravenous injection into rats the conjugate exhibited a remarkably extended *in vivo* half-life. This lays a basis for conjugation of efficient, safe and prolonged peptide inhibitors to endogenous proteins *in vivo*. The approach may decrease the cost

and improve compliance of the treatment and patient's quality of life.[44]

The great diversity of chemical and gene engineering techniques for enzyme modification led to a wide range of protein pharmacological agents. Covalent binding of proteins with polymers and small-size molecules allows production of conjugates with high stability[5,17,21,40], expanded retention in the body/circulation[3, 13, 28] and therapeutic efficiency in a wide range of pathologies. The first-level effects, i.e., those revealed at the initial stages of a consecutive research, manifest themselves due to multicontact interaction between protein molecule and modifier. After modification with high-molecular-weight compounds an enzyme obtains polymeric sheathing which increases its stability. Modification with low-molecular-weight compounds may provide additional interactions potentiating therapeutic effects of the resulting derivative. Site-directed mutagenesis offers optimization of amino acid chain in the protein molecule, and nanoparticles allow a dose-regulating release of therapeutic agent, thus improving its biomedical parameters. So far, there are no versatile methods to achieve these goals, and the effects produced by modified enzymes are tested empirically. The contribution of computer modeling to the development of general principles of enzyme drug construction has considerably increased in recent years. Experimental evaluation of modified enzymes is associated with stabilization (which generally results from the modification) or inactivation of the enzyme caused by modifiers (Figure 1).

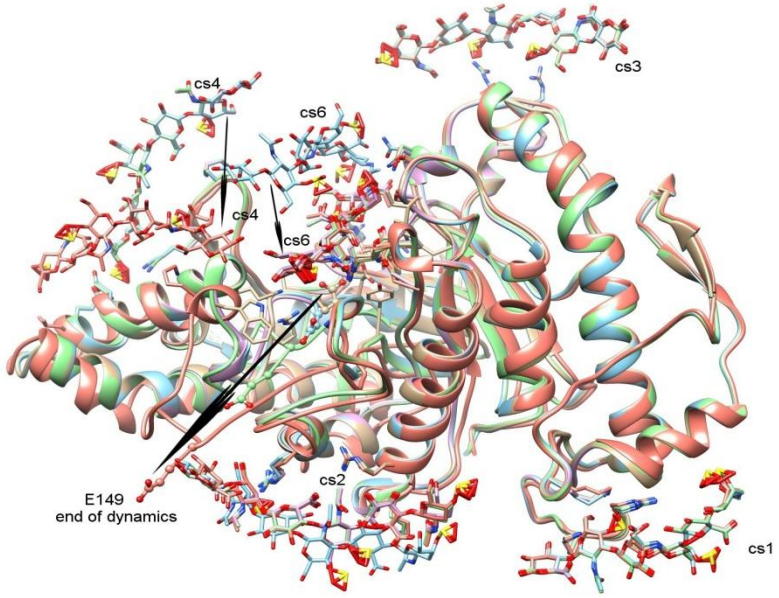

Figure 1. **A schematic drawing for irreversible conformational changes in bovine testicular hyaluronidase after insertion of hexasaccharides (trimers) of chondroitin sulfate.**

Insertion of chondroitin sulfate trimers (cs4 and cs6) in active center area of the enzyme (their location is shown before and after insertion) results in relocation of E-149 and D-147 amino acid residues responsible for catalysis at the periphery of the enzyme molecule, deformation of its active structure and inactivation.

Calculational studies *in silico* have demonstrated that the structure of native bovine testicular hyaluronidase (Figure 2) modified by polymeric chondroitin sulfate

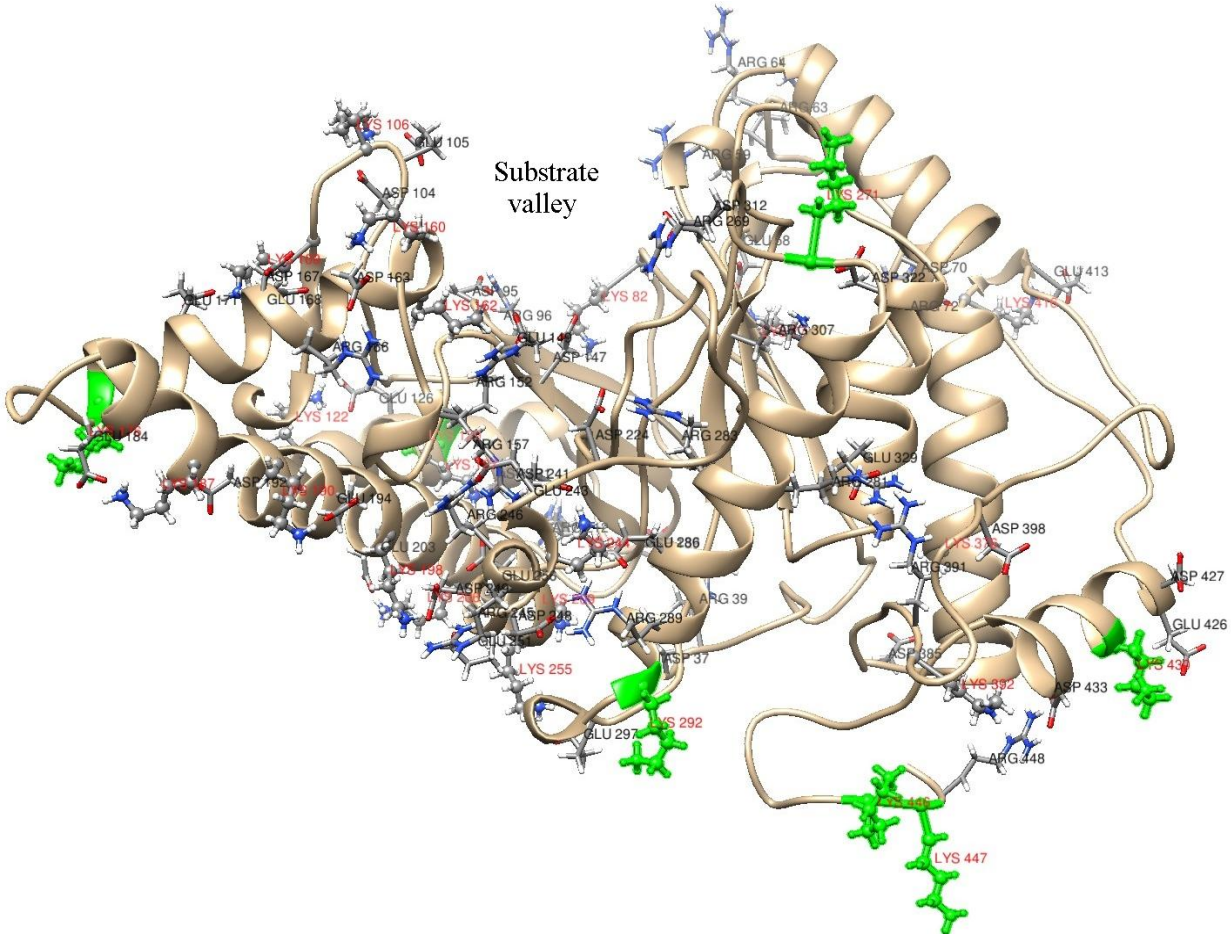

Figure 2. Tree-dimensional structure of native bovine testicular hyaluronidase.

Locations of lysine, arginine, glutamic and aspartic amino acid residues are indicated. Six lysine residues of the first-level availability are stained green/dark.

(Figure 3) becomes highly stable and shielded from inhibitors.[24] Depending on the molecular topography of the protein structure, the centers that bind with a modifier can be divided according to several levels of availability to the modifying agent.

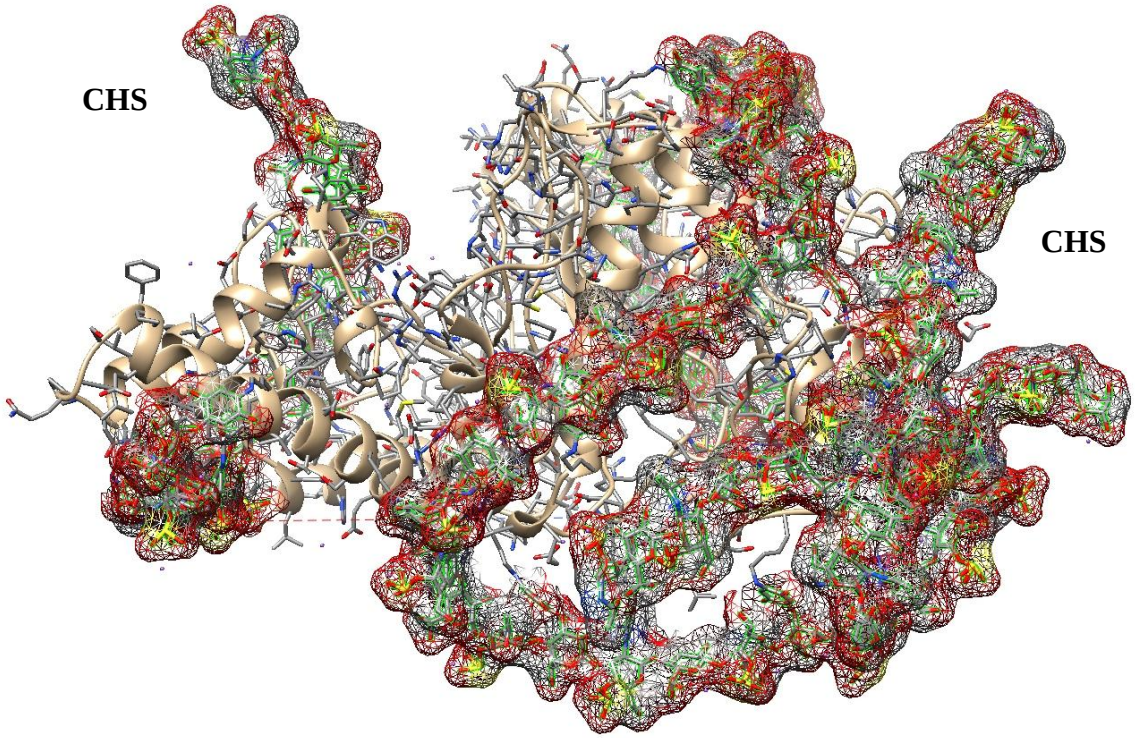

Figure 3. **3D model of bovine testicular hyaluronidase covalently modified by two chains of chondroitin sulfate.**

The enzyme structure is stabilized and shielded by chondroitin sulfate (CHS). The conjugate results from profound modification of the biocatalyst at 19 surface lysine residues.

New strategies have suggested accurate and reversible modulation of enzyme activity at various stages of site-specific[41] and *in vivo* (in the body)[44] conjugation with reliable activity preservation of the resulting therapeutic agent. The prospects for the development of a modified protein agent are largely determined by its therapeutic activity.

Enzyme Derivatives Preventing Oxidative Stress

Numerous pathologies are initiated and accompanied by oxidative stress, which urges an extensive research into antioxidants and their clinical application. High effectiveness of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase[17, 22] renders them into potential drugs for treatment of cardiovascular diseases associated with vascular wall inflammation, endothelial dysfunction and oxidative stress.[38] Oxidative stress has a pathophysiological role in systolic and diastolic heart failure[29] and acts as a key mechanistic mediator in hypertension.[2] Reduction of oxidative stress can be useful in the therapy of ST-elevation myocardial infarction.[4]

Antioxidant effects of SOD and CAT were potentiated by folic acid modification with carbodiimide activation. This increased activity and uptake of these enzymes by activated macrophages.[14] Modification of

SOD with low-molecular-weight heparin enhanced its heat stability and pH resistance.[45] Treatment of diabetic rats with SOD-polymer conjugate improved their antioxidant status, which demonstrates the advantages of enzyme modification for clinical application.[26] These approaches are aimed at production of modified derivatives based on one type of antioxidant enzyme.

Nano-size supramolecular antioxidant ensembles were suggested for prevention and reduction of oxidative stress.[35] They serve to deliver antioxidant enzymes, exert their own activity and reduce the area of antioxidant interactions. Combinations of biomolecules in these ensembles provide their mutifunctionality, rapid effect and targeting at the damaged area in order to achieve high therapeutic effect. Nanoparticles containing antioxidants have shown promise as high-performance therapeutic agents for attenuating oxidative stress with potential applications in treating and preventing neurodegenerative conditions.[36] This approach demonstrates a new development in antioxidant therapy and prevention of diseases associated with oxidative stress and offers new prospects for high-definition medicine.

Interestingly, IgG of patients with multiple sclerosis display CAT and SOD activities as compensatory response to decreased activity of intercellular antioxidant enzymes.[12] It should be stressed that the effective dose range for antioxidant therapy is crucially important, as evidenced by the finding that at high doses the antioxidants slow down the initiation of DNA synthesis at G0/G1 phase and block G1 phase without genotoxic effects in cultured human endometrial mesenchymal cells.[16] Genotoxic effect on proliferating cells allowed the authors to coin the term antioxidant stress which indicates the condition when a decrease in physiological concentration of active oxygen species with a parallel intrinsic increase in antioxidant content impairs systemic intracellular signaling and regulation of vital processes. Realization of these effects at the entire body level in the norm and pathology requires further investigations.

The specific nanocarrier PACkET was used for endothelium-targeted delivery of SOD and CAT.[7] Catalase-loaded PACkET protects endothelial cells against killing by H_2O_2 and alleviates pulmonary edema and leukocyte infiltration in a mouse model of endotoxin-induced lung injury. SOD-loaded PACkET mitigates cytokine-induced endothelial pro-inflammatory activation and endotoxin-induced lung inflammation. A nanozyme produced by electrostatic coupling of SOD1 with the cationic block copolymer poly(L-lysine)-poly(ethylene-glycol) followed by covalent cross-linking of the complexes with 3,3′-dithiobis(sulfo-succinimidylpropionate) sodium salt reduced uveitis in a rabbit model.[11] The nanozyme can be regarded as a potential therapeutic agent for the treatment of ocular inflammatory disorders. Generally, nanocarriers proved to be effective in experimental therapy with the use of individual enzymes or their combinations.

Combined application of antioxidant enzymes is more effective than their individual use. These combinations produce greater antioxidant effect, remaining safe pharmacological agents. For instance, when used together SOD and CAT neutralize superoxide radical and detoxify hydrogen peroxide to molecular oxygen and water (scheme)

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

In sum :

$$4\,O_2^{\cdot -} + 4\,H^+ \xrightarrow{SOD\,/\,CAT} 2\,H_2O + 3\,O_2$$

S c h e m e

Conjugation of SOD to CAT via the endothelial glycocalyx glycosaminoglycan chondroitin sulfate (CHS) generates SOD-CHS-CAT bienzyme derivative with extracellular activity.[25] The conjugate displays SOD and CAT activities, and CHS promotes its affinity for potential atherosclerotic lesion foci/zones in the vascular wall. Supramolecular structure of the conjugate allows one to regard it as a nanoparticle. These particles acquire unique physical, chemical and biological properties resulting from quantum mechanical interactions. Thus, the characteristics of the conjugate were determined by its size. In contrast to individual SOD and CAT, SOD-CHS-CAT inhibits platelet aggregation induced by ADP, serotonin or thrombin receptor agonist peptide (TRAP).[25] Both enzyme activities and the acquired nanostructure contributed to this process. Indeed, regarding platelet aggregation the conjugate displayed the properties of a nanoparticle. It should be noted the high antithrombotic action of conjugate: its effect was produced at doses two orders of magnitude lower than those for native SOD and CAT and an order magnitude lower than for their CHS-modified derivatives injected individually or in combination. Moreover, covalent conjugation of these enzymes provided simultaneous presence of SOD and CAT activities in the lesion, which was impossible with the use of their mixtures.[23] Higher survival rate in rats with lipopolysaccharide-induced endotoxic shock was observed after preventive and therapeutic administration of SOD-CHS-CAT conjugate.[20] These findings enhance substantially the frontiers for medical application of enzyme derivatives similar to SOD-CHS-CAT conjugate.

The interest into production of multienzyme ensembles has its history. Trypsin and chymotrypsin (molecular ratio 1:1) were conjugated using N-succinimidyl pyridyl dithiopropionate as a cross-linking reagent,[34] which increased its resistance to trypsinolysis. Enolase and phosphoglycerate mutase were conjugated using a similar technique with glutaraldehyde.[32] SOD-CAT conjugate obtained with glutaraldehyde as a cross-lining agent displayed protective activity in an isolated rat heart with ischemia-reperfusion model.[27] Hemoglobin conjugates with SOD and CAT obtained by employing dicarboxymethylated poly-ethylene glycol were designed to protect hemoglobin against free radicals upon cell transplantation with oxygen support by proteins.[30] The resulting conjugate had a high molecular weight (appr. 1000 kDa) and high SOD (70 %) and CAT (90%) remained activities. Gene and protein engineering technologies have been employed to produce protein ensembles which can be internalized by cells. A tri-functional protein with SOD, CAT and cell-permeable activities was obtained by coexpression of joined/fused genes.[15] A recombinant chimeric protein with peroxidase and SOD activities

produced cardioprotective effect in an isolated rat heart with modeled oxidative stress induced by hydrogen peroxide.[9, 37] The effect manifested itself in heart rate normalization, improved contractile function and oxidative stress reduction. These findings illustrate the development of approaches to extra- and intracellular protection against oxidative stress by modified protein derivatives obtained by biological and chemical synthesis.

Conclusion

Intensification of the research and development of modified protein derivatives for medical purposes led to considerable success in the production of antibody-drug conjugates and multi-enzyme ensembles. Practical application of enzyme thrombolytic agents[18] boosted the study of modified enzyme antioxidants which proved to be effective in correction of metabolic cardiologic disorders. Being dependent on social and economic conditions, the breakthrough in the investigation of biopharmaceuticals is largely determined by close cooperation between life science researchers and medical specialists.

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