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# The combination of modified antioxidant enzymes for anti-thrombotic protection of the vascular wall: the significance of covalent connection of superoxide dismutase and catalase activities

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## Abstract

Vascular wall protection can be achieved by preventive attachment to the vascular wall of antioxidants and elimination/neutralization of toxic products after their disproportioning. For this purpose we have prepared covalent conjugates between the vascular wall glycosaminglycan chondroitin sulfate (CHS) and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). The following conjugates were obtained: SOD-CHS, CAT-CHS and SOD-CHS-CAT. Their anti-thrombotic activity was compared in a rat model of arterial thrombosis by measuring the time of occlusion emergence and thrombus mass. It is noteworthy that the effectiveness of single bolus injections of SOD-CHS/CAT-CHS mixture was much lower than that of the bienzymic SOD-CHS-CAT conjugate. The conjugate SOD-CHS-CAT proved to be anti-thrombotically effective in doses two orders of magnitude lower than the native biocatalysts and an order of magnitude lower than SOD-CHS and CAT-CHS derivatives. For effective anti-thrombotic protection in oxidative conditions it is important to maintain the stable connection of SOD and CAT activity on the vascular wall and the large size of these conjugates. Covalent conjugate SOD-CHS-CAT is the best prospect for pharmaceutical development.

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## Introduction

It is generally accepted that excess free oxygen radicals produce damage to biological systems. However, the therapeutic use of antioxidants for preventing the damage is open to debate (Halliwell 2000; Gotto 2003). The defence of vascular endothelium against the damage caused by free oxygen radicals is probably an important factor of anti-thrombotic and anti-atherosclerotic protection (Rubanyi 1993; Sies 1993; You 1994). This can be achieved by preventive attachment of antioxidant enzymes to the vascular wall and elimination/neutralization of resulting toxic products after disproportioning of active oxygen metabolites (Fridovich 1997; Maksimenko 2002). For this purpose we have prepared covalent conjugates between the vascular wall glycosaminoglycan chondroitin sulfate (CHS) and the antioxidant enzymes superoxide dismutase (SOD, reaction 1 shown below) (Maksimenko & Tischenko 1997a) and catalase (CAT, reaction 2 mentioned below) (Maksimenko & Tischenko 1997b). The following conjugates were obtained previously: SOD-CHS, CAT-CHS and SOD-CHS-CAT. Their anti-thrombotic activity was compared in a rat model of the arterial thrombosis induced by the treatment of a vessel with ferrous chloride and measuring the time of the occlusion emergence and the obtained thrombus mass (Schumacher et al 1993). The nature of vascular injury in this case is otherwise as compared with the endothelial denudation model or arterial injury induced by electric current. An imbalance in the $Fe^{2+}/Fe^{3+}$ system facilitates the generation of free oxygen radicals with strong destructive potential in the organism (Halliwell & Gutteridge 1989). Since free oxygen radicals are involved in thrombus formation (Kumari et al 1993) and superoxide radical production is increased after successful thrombolysis in patients with acute myocardial infarction (Young et al 1993), the issue of vascular wall protection against free radicals becomes quite important (Maksimenko 2002). The ferrous chloride model (Schumacher et al 1993) proved to be

adequate for the investigation of the antioxidant effect since it implies the development of the Haber-Weis reaction in the focus injury according to Fenton chemistry (Halliwell 1994). It was found that the anti-thrombotic activity of the conjugates SOD-CHS and CAT-CHS were significantly higher than those of their components (namely, native enzymes and free CHS) used individually or as a mixture (Maksimenko et al 1998, 1999). The SOD-CHS conjugate markedly reduced the thrombus mass, while CAT-CHS predominantly prolonged the occlusion time. To increase the safety and efficacy of CAT derivatives it is reasonable to apply them together with SOD derivatives, when they interdependently convert the active oxygen metabolites to water and molecular oxygen (reaction 3), using the low doses of SOD preparations indicated.

$$O^{\cdot-}{}_2 + O^{\cdot-}{}_2 + 2H^+ \overset{SOD}{\rightarrow} H_2O_2 + O_2 \qquad \text{(Reaction 1)}$$

$$H_2O_2 + H_2O_2 \overset{CAT}{\rightarrow} 2H_2O + O_2 \qquad \text{(Reaction 2)}$$

as a sum:

$$4O^{\cdot-}{}_2 + 4H^+ \overset{SOD/CAT}{\rightarrow} 2H_2O + 3O_2 \qquad \text{(Reaction 3)}$$

The magnitude and duration of anti-thrombotic activity of modified enzymes allows one to expect a considerable protective effect from the mixture of SOD-CHS and CAT-CHS (due to stable coupling on the cell surface and possible formation of catalytic ensembles around the modified enzyme subunit anchored on the cell surface) as well as from the covalent bienzymic conjugate SOD-CHS-CAT (due to connected SOD and CAT activity and attachment of this derivative to the cell surface). Combined use of SOD with CAT seems biochemically quite reasonable, as far as the product of reaction 1 is the substrate of reaction 2. Such connection of SOD and CAT activity produces the safe products of reaction 3. Biomedical study of the above-mentioned combined action of the derivatives promises a new pharmacological way of simply and effectively protecting the vascular wall against various injuries during lifetime.

With this in mind we have compared the anti-thrombotic activity of SOD and CAT derivatives in the form of mixture of SOD-CHS and CAT-CHS, SOD-CHS-CAT bienzymic conjugate, as well as other combinations, after preventive single-bolus intravenous injection in a rat model of arterial thrombosis induced by ferrous chloride. The results are novel as we demonstrated first the high efficacy of anti-thrombotic protection of rat artery against oxidative injury with the help of stable connection of SOD, CAT and CHS on the vascular wall.

## Materials and Methods

### Reagents and chemicals

Preparations of Cu, Zn-superoxide dismutase (specific activity $5500 \pm 200$ U (mg of protein)$^{-1}$) isolated from rat liver (Simonyan 1984) and its modifier chondroitin sulfate A from bovine cartilage ($M_r$ 30 kDa) produced at Leningrad Meat Processing Plant were used. Benzoquinone, catalase from bovine liver (specific activity $17\,920 \pm 300$ U (mg of protein)$^{-1}$), its modifier chondroitin-4-sulfate from whale cartilage ($M_r$ 150 kDa) and dimethylformamide were obtained from Sigma (USA) and used in the study. Hydrogen peroxide was from Merck (Germany). Other reagents of analytically pure grade were Russian-manufactured (Rosreakhim).

### Enzyme derivatives with CHS

SOD-CHS (Maksimenko & Tischenko 1997a), CAT-CHS and SOD-CHS-CAT (Maksimenko & Tischenko 1997b) derivatives were obtained by the method of benzoquinone coupling, using chondroitin sulfate from whale cartilage for the preparation of the two latter derivatives. Protein content in the preparations was determined by the Bradford (1976) method. The preserved specific activity (U (mg protein)$^{-1}$) of the modified enzyme derivatives were 5900 for SOD-CHS and 5040 for CAT-CHS; for SOD-CHS-CAT, activity was 628 for SOD and 1390 for CAT. A unit of SOD activity was defined as the amount of the enzyme providing 50% inhibition of nitrotetrazolium blue reduction in the xanthine/xanthine oxidase system. A unit of CAT activity corresponds to the amount of the enzyme required for decomposition of 1 $\mu$M hydrogen peroxide (at an initial concentration of 20 mM) per min at 25°C (pH 7.0). Catalytic activity of enzyme preparations was determined as reported earlier (Maksimenko & Tischenko 1997a, b). Protein and CHS contents in the SOD-CHS-CAT conjugate were 3.4–3.6% and 14–16% by weight, respectively. The mixtures of SOD-CHS with CAT-CHS, as well as other combinations of SOD and CAT preparations, were prepared by mixing these reagents at the ratios indicated below.

### Experimental protocol

Thrombotic injury to rat arteries was induced by treatment of exposed vessel (carotid artery) with a saturated solution of ferrous chloride as described elsewhere (Schumacher et al 1993). Male rats weighing $450 \pm 20$ g were used. The carotid artery was dissected under calipsol anaesthesia (15 mg kg$^{-1}$ body weight intraperitoneally), and a Statham flowmeter sensor (1 or 2 mm in diameter) was installed on it. A catheter for drug administration was inserted into the jugular vein. Thrombotic damage was produced with saturated ferrous chloride solution (Schumacher et al 1993; Maksimenko et al 1999, 2003). Selected doses (according to catalytically active SOD and CAT) were used to study the anti-thrombotic action of the bienzymic conjugate SOD-CHS-CAT, (SOD-CHS) + (CAT-CHS) mixture, and other combination of SOD and CAT derivatives. The preparations (in 0.2 mL normal saline) were administered intravenously 10 min before the treatment of the artery by covering it for 10 min with a standard piece of filter paper soaked with a saturated solution of ferrous chloride. The blood flow was monitored in a flowmeter for an hour after application of ferrous chloride and the time of arterial

occlusion was recorded (when blood flow rate dropped to zero). Thrombus was excised from the assayed fragment of rat carotid artery after termination of the experiment and weighed. Control rats were given the same volume (0.2 mL) of normal saline. Thus, the experimentally determined parameters were the time of occlusion emergence (occlusion time) and the mass of formed thrombus (thrombus mass).

Experimental groups for each dose of preparation consisted of 4–6 rats (mean body weight 450 g). The data of this study were compared with the results obtained earlier for native SOD and CAT, SOD-CHS, CAT-CHS and free CHS individually (Maksimenko et al 1998, 1999, 2003). All rats received care in compliance with the Convention on Animal Care in Russian Cardiology Research-and-Production Complex and this study was approved by the institutional Ethics Committee.

### Statistics

Statistical analysis was performed using the Kwikstat 2.11 package of statistical programs (Elliott 1990). The values are presented as the mean ± s.e.m. The differences were considered as statistically significant at $P < 0.05$ (analysis of variance) according to corresponding recommendations (Wallenstein et al 1980; Glantz 1993).

## Results and Discussion

It should be noted that free CHS exhibits anti-thrombotic activity at doses (mg) much higher than native enzymes and than the content of CHS itself in conjugates. The main anti-thrombotic effect is stipulated by the action of antioxidant biocatalysts. This approach was realized in our study in-vivo with combinations of various derivatives of antioxidant enzymes. The parameters of the control experiment were 15.6 ± 1.2 min for occlusion time and 7.03 ± 0.62 mg for formed thrombus mass.

### Dose–effect dependence for SOD-CHS/CAT-CHS mixture

A single-bolus injection of (SOD-CHS) + (CAT-CHS) mixture produced no pronounced anti-thrombotic effect (Table 1) in comparison with the control. In the studied dose range statistically significant differences in the prolongation of occlusion time were observed at 1270 U SOD activity (for SOD-CHS) with 720 U CAT activity (for CAT-CHS) (designated as dose A) and at 1650 U SOD activity (for SOD-CHS) with 1970 U CAT activity (for CAT-CHS) (designated as dose B) per rat. Dose B produced a much more potent effect than dose A (Table 1) with respect to occlusion time. It should be noted that an individual dose of native SOD equal to 2440 U or native CAT dose equal to 14 000 U (Maksimenko et al 1999, 2003) produced an effect similar to that of dose B. Thus, administration of (SOD-CHS) + (CAT-CHS) mixture provides a 2- to 7-fold decrease in the effective dose in comparison with individual effective doses of native enzymes. When administered individually, SOD-CHS or CAT-CHS produced a similar effect on prolongation of occlusion time (Maksimenko et al 1999, 2003) in doses that were not significantly different from dose B for (SOD-CHS) + (CAT-CHS) mixture.

There were no statistically significant differences in the effects on the mass of forming thrombus in the studied dose range of (SOD-CHS) + (CAT-CHS) mixture (Table 1). Presumably, in a certain dose range the mixture reduces the thrombus mass by 20–30%, keeping the thrombus formation at a moderate level. This effect was observed earlier with SOD + CHS but not with CAT + CHS mixture (Maksimenko et al 1998, 1999). The latter mixture markedly decreased the thrombus mass (Maksimenko et al 2003). In light of this it is interesting to test the anti-thrombotic effect of (SOD-CHS) + CAT mixture as compared with other combinations of SOD and CAT derivatives.

Generally, in anti-thrombotic activity the (SOD-CHS) + (CAT-CHS) mixture is inferior to the individual effects of SOD-CHS and CAT-CHS. This may be due to different vascular distribution of the mixture's components

**Table 1** Anti-thrombotic activity of various doses of (SOD-CHS) + (CAT-CHS) mixture in a rat model of arterial thrombosis (mean body weight 450 g)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Dose of SOD activity (mg of catalytically active enzyme (U per rat)) | | 0.08 (440) | 0.08 (440) | 0.16 (880) | 0.16 (880) | 0.23 (1270)[a] | 0.23 (1270) | 0.30 (1650)[b] |
| Dose of CAT activity (mg of catalytically active enzyme (U per rat)) | | 0.02 (270) | 0.04 (720) | 0.03 (540) | 0.08 (1430) | 0.04 (720) | 0.08 (1430) | 0.11 (1970) |
| Occlusion time (min) | 15.6 ± 1.2 (control) | 19.7 ± 0.3 | 16.0 ± 0.5 | 19.3 ± 2.3 | 26.3 ± 1.5 | 39.1 ± 2.1 | 24.8 ± 2.8 | 48.4 ± 1.8 |
| Thrombus mass (mg) | 7.03 ± 0.62 (control) | 5.28 ± 0.61 | 6.13 ± 1.22 | 6.60 ± 1.14 | 4.85 ± 0.86 | 5.42 ± 0.58 | 5.19 ± 0.37 | 5.96 ± 0.29 |
| No. of rats in group | 6 (control) | 4 | 6 | 4 | 6 | 6 | 6 | 6 |

The differences are statistically significant at $P < 0.05$ (analysis of variance); data are means ± s.e.m. [a]Dose A; [b]dose B (see text for details). Control is intravenous administration of normal saline.

after their conjunctive administration, lack of an optimal ratio between SOD and CAT activity in the mixture or an adverse reaction of the vascular system to increased doses of protein in comparison with individual administration of the derivatives.

### Dose–effect dependence for covalent bienzymic SOD-CHS-CAT conjugate

The aforementioned suggestions were confirmed by the corresponding anti-thrombotic activity of the covalent bienzyme conjugate SOD-CHS-CAT after its injection in quite small doses (Table 2) in comparison with the other compounds. The maximum anti-thrombotic effect was achieved with an SOD-CHS-CAT dose containing 37 U SOD activity and 80 U CAT activity per rat. After administration of this dose the increase in occlusion time was significantly greater than after administration of any other dose of the conjugate (analysis of variance). There were no significant differences in the effect of the conjugate on the thrombus mass in a wide dose range: 12–85 U SOD activity and 28–195 U CAT activity per rat (Table 2). It is noteworthy that the anti-thrombotic activity of the enzyme derivatives was similar in the optimal dose range, although the optimal doses of bienzymic conjugate are much lower (Figure 1). By specific enzyme activity the conjugate was anti-thrombotically effective in doses two orders of magnitude lower than native enzymes and an order of magnitude lower than SOD-CHS and CAT-CHS. The optimal ratio between SOD and CAT activity in SOD-CHS-CAT conjugate (i.e. when the conjugate exhibited the maximum anti-thrombotic activity by occlusion time and thrombus mass) was determined experimentally (Table 2). The optimal enzyme activity for the conjugate was 25–50 U SOD and 55–110 U CAT per rat. Such small effective doses point to prospective use of the conjugate for further biomedical research and stress the importance of finding out the optimal ratio between SOD and CAT activity for pronounced anti-thrombotic effect.

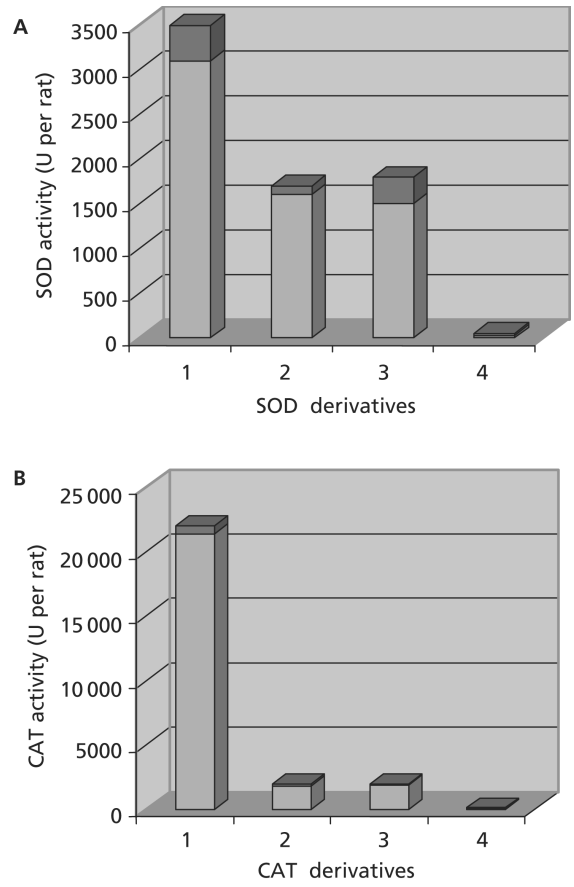


**Figure 1** The comparison of intervals for optimal doses (designated as dark areas at the top of bars) of the antioxidant enzymic derivatives with respect to the anti-thrombotic action by SOD activity (A) and CAT activity (B). 1, native enzyme; 2, covalent conjugate of enzyme with CHS; 3, mixture of SOD-CHS and CAT-CHS; 4, SOD-CHS-CAT bienzyme conjugate. The given doses (in units of enzymic activity per rat) determined the experimental therapeutic area for the maximum inhibition of arterial thrombosis by intravenous bolus injections (0.2 mL) of the corresponding derivative. The error values of the specific activity determination did not exceed 2– 4%.

**Table 2** Anti-thrombotic activity of various doses of SOD-CHS-CAT covalent conjugate in rat model of arterial thrombosis (mean body weight 450 g)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Dose of SOD activity (U per rat) | | 6 | 12 | 25 | 37 | 50 | 70 | 85 | 145 |
| Dose of CAT activity (U per rat) | | 14 | 28 | 55 | 80 | 110 | 160 | 195 | 320 |
| Weight dose of preparation (mg[a]) | | 0.27 | 0.55 | 1.10 | 1.70 | 2.20 | 3.33 | 3.90 | 6.66 |
| Occlusion time (min) | 15.6 ± 1.2 (control) | 22.5 ± 2.5 | 27.1 ± 2.7 | 29.8 ± 2.2 | 55.3 ± 6.1 | 34.6 ± 1.4 | 33.6 ± 1.4 | 19.1 ± 3.8 | 19.0 ± 3.0 |
| Thrombus mass (mg) | 7.03 ± 0.62 (control) | 6.27 ± 0.69 | 4.66 ± 0.85 | 3.78 ± 0.61 | 3.72 ± 0.74 | 4.04 ± 0.68 | 4.83 ± 0.36 | 5.09 ± 0.24 | 6.91 ± 0.29 |
| No. of rats in group | 6 (control) | 4 | 6 | 6 | 6 | 6 | 6 | 4 | 4 |

The differences are statistically significant at $P < 0.05$ (analysis of variance); data are means ± s.e.m.. [a]Protein content in preparation was 3.4–3.6% (see text for details). Control is intravenous administration of normal saline.

### Anti-thrombotic efficacy for combination of SOD and CAT derivatives in a dose of optimal action in-vivo for SOD-CHS-CAT conjugate

The highest anti-thrombotic effect of SOD-CHS-CAT conjugate (in the optimum dose range) was observed at 34–40 U SOD and 77–83 U CAT activity per rat, as follows from Table 2. As the optimal dose ratio for SOD and CAT activity, these dose intervals were chosen for comparison of anti-thrombotic effect produced by different combinations of SOD and CAT derivatives (Figure 2). The combinations (in 0.2 mL normal saline) were tested in a rat model of arterial thrombosis as described previously (Schumacher et al 1993; Maksimenko et al 1999, 2003). The effects of the combinations on the occlusion time did not differ from control (analysis of variance). The effect of native CAT with SOD-CHS mixture was insignificantly higher than that for the control (Figure 2). Presumably, due to the greater size of the CAT molecule in comparison with the SOD molecules the electrostatic complex forming between CAT and SOD-CHS is more stable than that forming between SOD and CAT-CHS. As mentioned above, the same was observed for CAT + CHS and SOD + CHS mixtures (Maksimenko et al 1998, 1999, 2003), confirming the stability of electrostatic complexes (Maksimenko & Tischenko 1997a, b) with native CAT. The stability of the complex between native CAT and SOD-CHS provides better attachment to the vascular wall and improves its anti-thrombotic activity. In fact in the assayed series, the CAT with SOD-CHS combination produced the greatest, although statistically insignificant, effect on the decrease of thrombus mass (Figure 2). It can be suggested that the stability of the complex facilitates the presence of SOD, CHS and CAT on the vascular wall in concentrations sufficient for their anti-thrombotic defence.

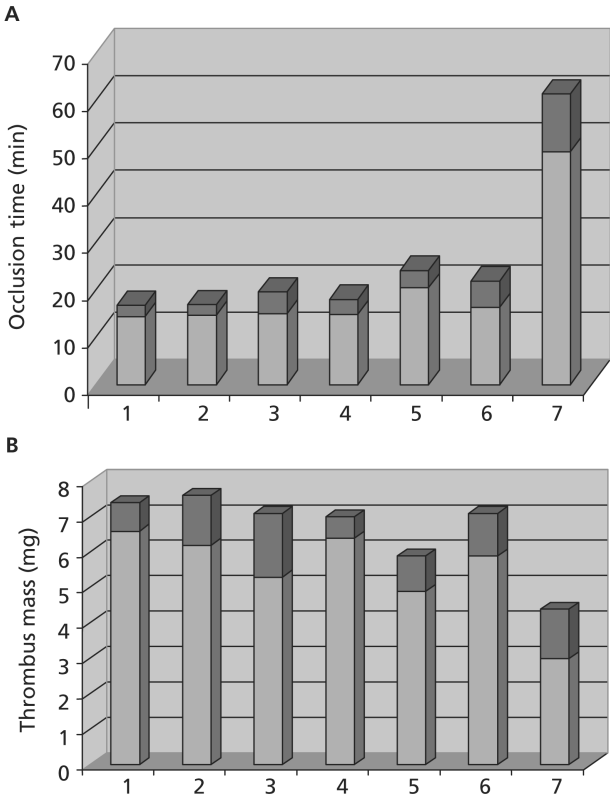


**Figure 2** Anti-thrombotic activity (intervals are shown as dark areas at the top of bars) of different combinations of SOD and CAT derivatives and of the SOD-CHS-CAT conjugate with respect to occlusion time (A) and thrombus mass (B). 1, control rats (normal saline); 2, native SOD with native CAT; 3, native SOD, native CAT with free CHS; 4, native SOD with CAT-CHS conjugate; 5, SOD-CHS conjugate with native CAT; 6, SOD-CHS conjugate with CAT-CHS conjugate; 7, SOD-CHS-CAT conjugate. Each combination was injected at the same dose of SOD ($37 \pm 3$ U) and CAT ($80 \pm 3$ U) activity. Each group consisted of 6 rats. The error values of the specific activity determination did not exceed 2–4%.

Optimal concentrations were achieved with the use of SOD-CHS-CAT conjugate (Table 2). The conjugate exerted a statistically significant (analysis of variance) anti-thrombotic effect concerning both the occlusion time and thrombus mass (Figure 2). This stresses the importance of covalent connection of SOD and CAT activity on the vascular wall for its anti-thrombotic protection and points to the significance of greater size of conjugates for extracellular antioxidant defence similar to that performed by extracellular SOD (Marklund 1982; Stralin et al 1995). According to our results, SOD-CHS-CAT covalent conjugate is the best prospect for further research and development. The accurate mechanisms responsible for the anti-thrombotic action of various combinations of antioxidant enzymes, as well as bienzymic SOD-CHS-CAT conjugate, remain undefined but clearly reflect the biocatalyst function of SOD and CAT and imply antioxidant decrease of cardiovascular events (Tepel et al 2003).

### Conclusions

These findings suggest that the modified antioxidant enzymes can be used in pharmacological and surgical manipulations. The bienzymic conjugate SOD-CHS-CAT could be a prospect for intravascular therapeutic injections (systemic vascular protection). The SOD-CHS with CAT-CHS or with native CAT mixtures could be useful for the surface modification of vascular prostheses and bypasses during surgical revascularization (local protection of vascular fragment). Thus, combined activity of antioxidant enzymes is a valuable element of biochemical engineering destined for effective medical application. The latter can be realized extracellularly and intracellularly by both direct (Muzykantov et al 1999) and indirect (Fukuo et al 2002) correction of metabolic pathways in the vascular wall cells.

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