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EFFECT OF ACTIVE OXYGEN SPECIES SCAVENGERS ON FIBRINOLYTIC ACTIVITY OF SOME PROTEINASES

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Abstract Scavengers of different active oxygen species affect fibrin plate lysis, catalysed by various proteinases, only at relatively high concentrations (>10^{-2} M). Singlet oxygen scavengers change proteinase activity insignificantly except for strong inhibition of pepsin and papain by sodium azide, but pepsin - by histidine, and fibrinolytic urokinase activity - by all used $0_2^{\Delta 1}$ scavengers. Of all used scavengers of ·OH-radical only ethanol caused significant changes in the proteinases under study, except for α-chymotrypsin. The most strong inhibitory effect on proteinase activity was demonstrated by scavengers of superoxide radical. Thus, nitrotetrazolium blue strongly inhibited the activity of plasmin, urokinase (fibrinolytic activity), papain and pepsin. Catalase changed proteinase activity insignificantly, though it leads to total inhibition of pepsin activity at final 4.5×10^{-4} M concentration. These facts and our previous findings on generating of active oxygen species by proteinases give us grounds to suppose that minor active oxygen species, endogenous for the "proteinase-substrate" system, can participate in the catalytic function of some proteinases.

Key-words: fibrinolysis, proteinase, active oxygen species.
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Our earlier findings demonstrated that the treatment of human plasminogen samples by systems, generating superoxide radicals, or by H_2O_2 caused the increase in its fibrinolytic activity(1). Plasminogen activation by streptokinase, the protein without its own hydrolase activity, was strongly inhibited by superoxide radical scavengers, but not by those of singlet oxygen or ·OH-radical (2). The human plasminogen, turned out, to be able to generate active oxygen species including superoxide radical, during H_2O_2 decomposition as well as when molecular oxygen is reduced (3). At the same time, to judge by the results obtained, streptokinase possessed superoxide dismutase-like activity (3,4).

These results permitted us to substantiate the conception of oxygen-dependent plasminogen activation, which was realized without participation of activators of proteinase nature, but due to the O_2^--generating ability of zymogen and O_2^--converting function of streptokinase (3-5).

The described example of realization of proteolytic reaction due to inherent endogenous species of oxygen logically suggest the idea of possible participation of such oxygen in catalytic proteinase function as well.

Revelation of this aspect is associated with considerable methodics difficulties. But some data, elucidating this participation indirectly,can be obtained by inhibitory analysis.

The present study examines effect of scavengers of: $O_2^{\Delta 1}$ (NaN_3, histidine, tryptophan), ·OH (ethanol, mannitol, formate), O_2^- (nitrotetrazolium blue, adrenalin) and H_2O_2 (catalase) on activity of: trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), plasmin (EC 3.4.21.7), urokinase (EC 3.4.21.31), papain (EC 3.4.22.2), and that pepsin (EC 3.4.23.1).

MATERIALS AND METHODS

The following enzymes and reagents were used: urokinase (J.R.T.), papain (Fluka), bovine pepsin (Sigma), trypsin and chymotrypsin (Spofa), sodium formate, adrenalin (Serva), nitrotetrazolium blue, D-mannitol (Chemapol). Plasmin was isolated by affinity chromatography with Lys-Sepharose from Cohn $III_{2,3}$ fraction with high plasmin activity and not containing plasminogen (6). Activity of plasmin samples was determined by caseinolytic test (7) and comprised 25 caseinolytic units per mg of protein. These samples retained homogeneity during electrophoresis in 12.5% polyacrilamide gel with sodium dodecyl sulfate (8) in the absence of reductants, and produced two protein bands, corresponding to light and heavy plasmin chains, in the presence of 2-mercaptoethanol (8). Proteinase activity was determined by fibrine plates lysis method as described elsewhere (2). Parallel measurements differed by < 7%. The above method

offered lower fluctuation mobility and smaller changes of solubility of protein substrate, compared to those of globular proteins or low-weight substrates, when composition of solvent was altered. Plasminogen-activating function of urokinase was measured by means of plates, containing fibrine absorbed plasminogen. In all other cases this plasminogen was inactivated by UV lamp (18 W) irradiation of fibrin plates during 30 minute period (9). All tests were repeated at least four times and the data were analyzed statistically using Student`s test.

RESULTS

$O_2^{\Delta 1}$ scavengers at 10^{-6} - 10^{-2} M concentration slightly affected proteinase activity (Fig.1). Histidine and tryptophan (0.1 M final concentrations) inhibited activities of trypsin, urokinase and pepsin. It`s possible, that the above mentioned phenomena could not be explained just by $O_2^{\Delta 1}$ elimination, as the effect of amino acids on proteinase activity varies with degradation of different proteins. Thus, 5×10^{-2} M histidine inhibited the trypsin (or chymotrypsin) hydrolysis of serum albumin by 52% (or by 27%). At the same time, 1.8×10^{-2} M tryptophan inhibited these proteinases activity by 38% and 62% respec-

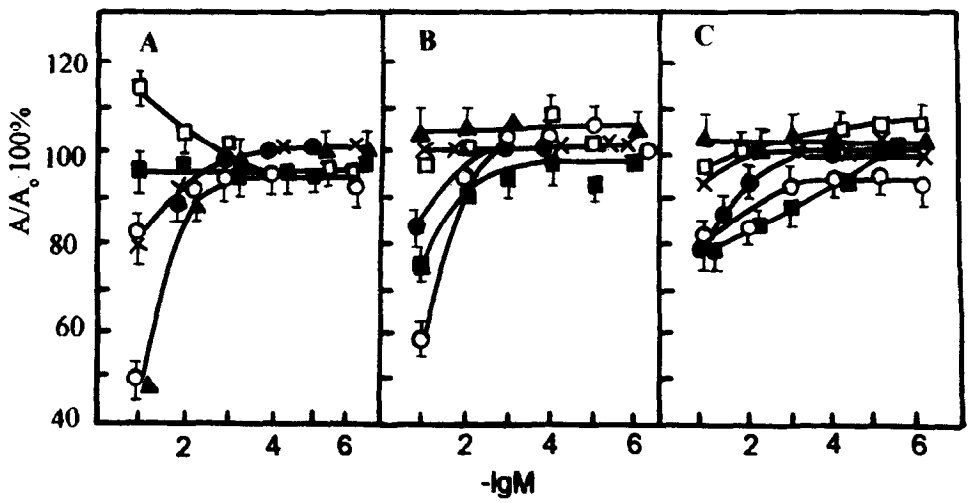

FIG. 1

Effect of NaN_3(A), L-histidine (B) or L-tryptophan (C) on the fibrinolytic activity (% to control) of trypsin (●—●), chymotrypsin (×—×), pepsin (○—○), papain (△—△), plasmin (□—□), and on the activating function of urokinase (■—■).

tively, while the pepsin proteolysis of hemoglobin and edestin was activated (10). 0.1 M NaN_3 inhibited pepsin and papain activity significantly, trypsin and chymotrypsin activity fell and slightly increased plasmin activity.

·OH scavengers. Partial substitution of water by ethanol depressed trypsin activity and increased pepsin, papain activity, and especially sharply increased plasminogen-activating function of urokinase (Fig. 2) along with streptokinase-dependent fibrinolysis (11)."Plasmin activity-ethanol concentration" dependence changes conversely to that of urokinase activity. The mechanism of ethanol action on proteinase activity is great complex. This mechanism is realized not only by ·OH interaction, but also by altering enzyme conformation, solvophobic interactions, and by playing the role of additional nucleophil in the catalysis. That is why a clarification of this mechanism requires additional supporting data. However, we presume certain analogy in the effects of ethanol and formate on activity of papain and urokina-

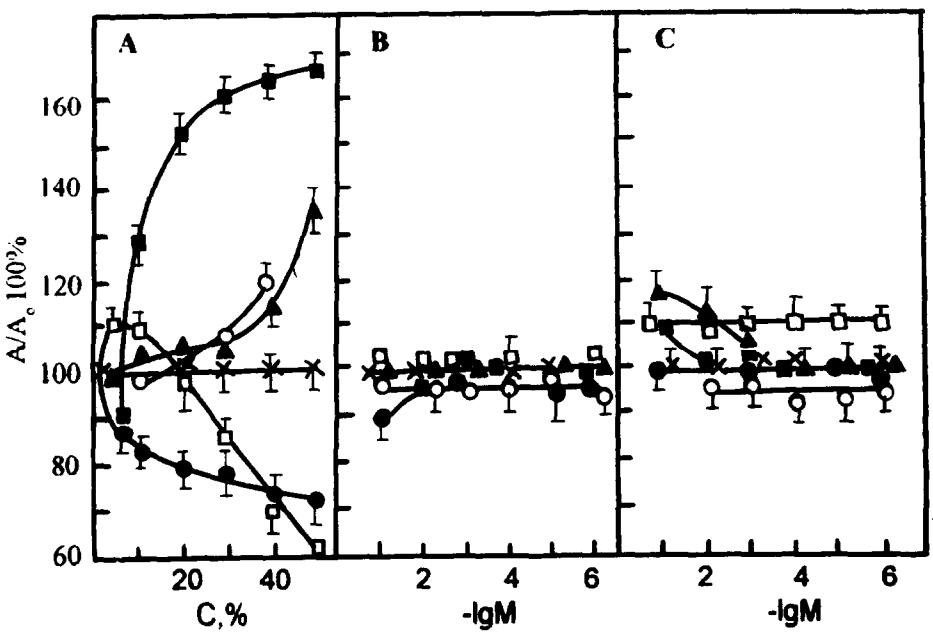

FIG. 2.

Effect of the water partial substitution by ethanol (A), of D-mannitol (B) or of sodium formate (C) on the fibrinolytic activity of trypsin , chymotrypsin, pepsin, papain, plasmin, and on the activating function of urokinase. The designations are the same as in Fig.1.

se. The lesser effect of formate could be explained by its anionic character, which is a hindrance to its penetration into enzyme molecule. There are no such limitations for ethanol. But, on the whole, mannitol and formate altered proteinase activity very weakly.

O_2^- scavengers considerably inhibited activity of papain, pepsin and plasmin(Fig.3), while their effect on other proteinases was rather low. Only adrenalin partially depressed trypsin and urokinase activity.

However, the effect of active oxygen species scavengers on urokinase activity was more complicated. Plasminogen-activating function of urokinase was sharply inhibited by nitrotetrazolium blue at the initial stage of fibrinolysis realization (Tabl. I); after that the process rate was restored, and the final results agreed with control ones. It was proved that urokinase break not only plasminogen, but other proteins as well (12). The effect of active oxygen species scavengers on fibrin degradation by urokinase or on its plasminogen-activating function differed considerably (see Fig. 1, 3; Tabl. II). Only mannitol

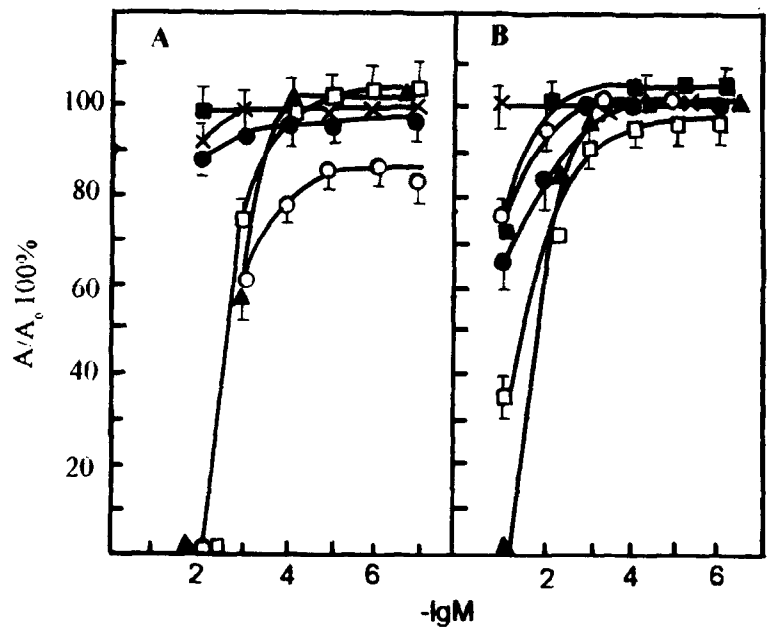

FIG. 3.

Changes in fibrinolytic activity of trypsin, chymotrypsin, pepsin, papain, plasmin and urokinase activating function after addition of nitrotetrazolium blue (A) or adrenaline (B). The designations are the same as in Fig.1.

TABLE I.

Nitrotetrazolium Blue (0.01 M) Effect on the Plasminogen-activating Function of Urokinase in the Fibrinolysis Dynamics (n=4).

Fibrinolysis Time, h	Urokinase Activating Function (mm^2 of Fibrinolysis Zones)		Inhibition, %
	without Nitrotetrazolium Blue	with Nitrotetrazolium Blue	
0	0	0	-
1	75±4	0*	100
2	145±10	70±3*	52
4.5	259±15	133±9*	48
20	1093±110	1026±95*	6

* - $P < 0.05$

and sodium formate did not alter direct fibrinolytic activity of urokinase (not shown). The inhibitory effect of histidine and tryptophan (>10^{-3} M) markedly expressed (Tabl.II), we found the inhibition by NaN_3 as well.

At lower concentrations NaN_3 and histidine enhance fibrinolytic activity of urokinase. O_2^- scavengers significantly retarded the urokinase fibrinolysis.

Additions of catalase (not shown) in concentrations of $4.5x(10^{-9} - 10^{-4}$ M) did not afffect activity of trypsin, chymotrypsin, plasmin, papain. Catalase in concentration of $4.5x$ ($10^{-7} - 10^{-4}$ M) increased activating function of urokinase by 10-14%, while at concentration of $4.5x10^{-4}$ M it completely inhibited pepsin activity.

DISCUSSION

The present results of the effect of active oxygen species scavengers on proteolytic activity enable us to assumption that these species can participate in catalytic function of some proteinases. This assumption is well conforms to the earlier revealed ability of trypsin, pepsin, and papain to generate active oxygen species in a solution. It was demonstrated by the method of chemiluminescence in "luminol-H_2O_2" system, that trypsin and pepsin decompose H_2O_2 with simultaneous formation of singlet oxygen and oxygen radicals. Moreover, in the absence of

TABLE II.

Effect of Active Oxygen Species Scavengers on the Direct Fibrinolytic Activity of Urokinase (n=4).

Experimental Conditions	Fibrinolysis Zones, mm^2	Experimental Conditions	Fibrinolysis Zones, mm^2
Control	413±20	+L-Tryptophan	
+Sodium Azide		10^{-3} M	293±20*
10^{-6} M	566±32*	10^{-2} M	116±9*
10^{-4} M	512±25*	10^{-1} M	0*
10^{-3} M	438±24	+Nitrotetrazolium Blue	
10^{-2} M	87±9*	10^{-6} M	359±35
10^{-1} M	78±10*	10^{-4} M	442±30
+L-Histidine		10^{-3} M	277±24*
10^{-6} M	496±22*	10^{-2} M	66±4*
10^{-4} M	438±20	+Adrenalin	
10^{-3} M	409±15	10^{-6} M	396±22
10^{-2} M	281±19*	10^{-4} M	396±19
10^{-1} M	0*	10^{-3} M	410±30
+L-Tryptophan		10^{-2} M	136±10*
10^{-6} M	388±27	10^{-1} M	0*
10^{-4} M	438±19		

exogenous H_2O_2 pepsin was able to catalyse the luminol oxidation, which was partially inhibited by catalase additions (13). Papain in aquaeous solution oxidized slowly adrenalin into adrenochrom, which allows to suspect the generation of superoxide radical in the system. Its concentration in the system is very low: the rate of adrenochrom formation was $6x10^{-3}$ $Mxmin^{-1}$ per 1 M papain (13). It is by two orders of magnitude lower than the rate of analogues process, catalysed by human plasminogen.

We believe, that exactly these active oxygen species, endogenous for the "proteinase-substrate" system can participate in the realization of proteinase catalytic function.

Moreover, it`s prematurely, however to assert firmly on the basis of our findings that $O_2^{\Delta 1}$ participates in proteinase function. The revealed inhibition of their activity by histidine or tryptophan may be due to the competition of amino acids with substrate, and the proteinases inhibition by sodium azide due to nucleophilic nature of compound or by interaction of

this reagent with metal-containing proteinase sites. Any certain data, explaining such a situation, are absent in the available literature.

However, it was established earlier that some chelators inhibited papain fibrinolytic activity (14). In our view such an effect is not quite usual, because the elimination of traces of heavy metals seems to activate cysteine proteinase. Taking into account these arguments we draw a conclusion that the mechanism of $O_2^{\Delta 1}$ scavengers effect on proteinase activity requires further studies.

More sharp impression was produced by the inhibition of the activity of pepsin, papain and soluble plasmin as well as urokinase fibrinolytic activity by O_2^- scavengers. Probably, it`s just $O_2^-$ that participates in proteinase catalytic function. The reason is that its lifetime in aqueous solutions is longer than that of $O_2^{\Delta 1}$. There are certain grounds for such an assumption, which can be demonstrated with papain. It`s common knowledge that SH-groups are very important for the realization of its catalytic function. Moreover, thiol autooxidation induces O_2^- generation (15). As it has been mentioned above, papain is actually able to generate this radical. Its concentration is not high, but, firstly, the applied methodical approach permits to register only those radicals which are released into solvent and secondly, it`s highly probable that not only the general radical concentration but rather their directed migration and transformation play a key role in proteinase catalysis.

The source of active oxygen species in pepsin or urokinase molecules is less clear. Special thorough studies on these proteinases molecule structure are necessary to determine this source. Moreover a participation of O_2^- in urokinase or plasmin catalysis may be realized in some other, more complicated pathways as compared to the case with papain. It is necessary to remember that a functional role of active oxygen species can be adequately established by means of specific scavengers only in two cases: 1) active oxygen species released into solvent are important; 2) scavengers reach radical-generating sites in protein macromolecule. It is probable, therefore, that soluble plasmin was sensitive to nitrotetrazolium blue (Fig. 3) while the proteinase, which was formed as a result of activation of zymogen, formerly sorbed in fibrine, was insensitive to this scavenger (2,14).

Of course, the stated views about a significant role of O_2^- above all in proteinase function is to some extent an assumption and it doesn`t exclude the participation of other active oxygen species.

The described above evidences present a new edge to participation of active oxygen species in the proteolysis, i.e. pos-

sible realization of endogenous oxygen-dependent mechanisms in catalytic function of some proteinases. Specific mechanisms of catalysis realization by means of active oxygen species in different proteinases are the subject of our future profound studies.

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