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OZONE INACTIVATION OF ANTI-ELASTASE ACTIVITY OF CHICKEN OVOINHIBITOR AND HUMAN α-1-PROTEINASE INHIBITOR

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SUMMARY: Treatment of chicken ovoinhibitor with ozone inhibits the anti-elastase activity more than anti-trypsin and anti-chymotrypsin. Treatment of diluted human serum with ozone also shows that anti-elastase is inactivated more than anti-trypsin and anti-chymotrypsin. Treatment of purified human α-1-proteinase inhibitor with a 9 fold molar excess of ozone, inhibited anti-elastase 55%, anti-chymotrypsin 29% and anti-trypsin 18%.

It has been suggested that in the development of emphysema, the activity of proteolytic enzymes is in excess of the anti-protease capacity of the lung. Inherited forms of emphysema in humans are associated with a deficiency of α-1-proteinase inhibitor throughout the body (1). The development of emphysema as a consequence of cigarette smoking possibly is caused by inactivation of lung anti-protease. Janoff and Carp (2) have demonstrated the suppression by cigarette smoke condensate of anti-elastase activity in human serum. The active compounds in the cigarette smoke were apparently quite stable since they could be used to impregnate agar plates which were subsequently used for a diffusion assay. Since glutathione can prevent the effect of cigarette smoke condensate it has been suggested that the agents responsible for suppression of anti-elastase are oxidants such as peroxides.

We have considered the possibility that oxidants in polluted air, such as ozone, may inactivate anti-proteases (3). The anti-trypsin activities of protease inhibitors from bovine pancreas, ovomucoid, and human serum, were

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Abbreviations used: ATEE, N-acetyl-L-tyrosyl ethyl ester; BAME, N-benzoyl-L-arginyl methyl ester; NCS, N-chlorosuccinimide; $S(Ala)_3NA$, N-succinyl-L-alanyl-L-alanyl-L-alanine-P-nitroanilide.

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suppressed by ozone, but only at large molar excesses. During the suppression, methionine, histidine and tyrosine residues of the protease inhibitors were oxidized. These oxidations were consistent with results for oxidation of pure amino acids (4).

Sheckter et al. (5) found that the selective oxidation of methionine residues of ovoinhibitor resulted in loss of anti-elastase activity, some loss of anti-chymotrypsin activity, but no loss of anti-trypsin activity. Johnson and Travis [6] used the same oxidant, N-chlorosuccinimide (NCS[1]), to suppress the anti-elastase activity of human α-1-proteinase inhibitor. Trypsin was inactivated only after prolonged incubation of trypsin with the NCS treated proteinase inhibitor, showing that the modification slowed the rate of binding. Anti-chymotrypsin was little affected by oxidation of the methionine residues.

These results suggested the use of ozone to suppress the anti-elastase activities of ovoinhibitor and human α-1-proteinase inhibitor. Oxidation of methionine residues of proteins by ozone is by no means selective, however it is, with cysteine and tryptophan, one of the three most susceptible amino acid residues (4).

MATERIALS AND METHODS: Ovoinhibitor from chicken egg white, α-1-proteinase inhibitor from human serum, elastase (2 x crystallized from porcine pancreas), α-chymotrypsin (3 x crystallized from bovine pancreas), and trypsin (2 x crystallized from bovine pancrease), all were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Human serum was prepared from outdated blood kindly supplied by Mr. Ted Kott, San Bernardino Blood Bank. The substrates BAME, ATEE and S(Ala)$_3$NA were purchased from Sigma Chemical Company.

Trypsin and chymotrypsin activities were measured by the methods of Kassel (7) except that borate buffer was used instead of Tris, BAME was used as the trypsin substrate, and ATEE was used as the chymotrypsin substrate. Preliminary experiments established the appropriate concentrations of trypsin, chymotrypsin and the anti-proteases. Typical reaction mixtures contained 16 μg trypsin or 14 μg chymotrypsin, and 14 μg ovoinhibitor or 175 μg α-1-proteinase inhibitor in a final reaction volume of 3.0 ml. The acid liberated changed the absorbance of m-nitrophenol which was measured at 395 nm. Results are expressed as residual inhibitory activity of anti-protease after it had been exposed to ozone.

Elastase activity was measured by the method of Bieth et al (8) using S(Ala)$_3$NA as substrate. The reaction mixture contained 0.50 μg elastase, and 0.66 μg ovoinhibitor or 0.83 μg α-1-proteinase inhibitor.

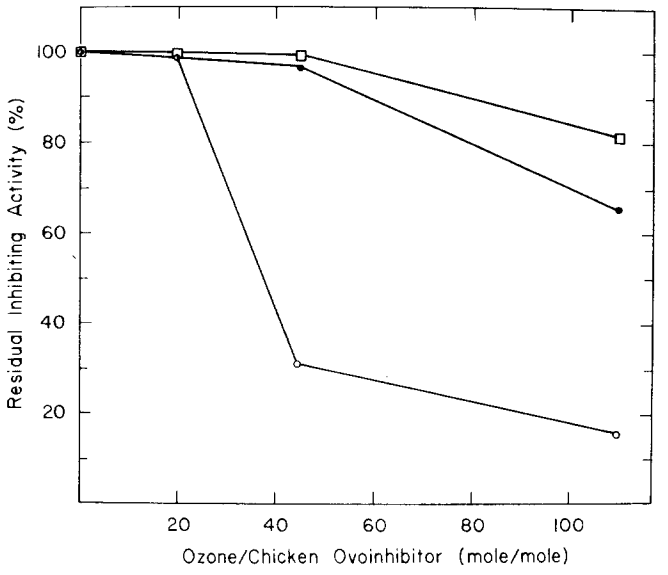

Fig. 1. Inactivation of ovoinhibitor by ozone. Ovoinhibitor was exposed to ozone and assayed as described in Materials and Methods. Data presented are averages of triplicate determinations. The experiment was done three times with similar results. Symbols: O, anti-elastase; ● , anti-trypsin; □ , anti-chymotrypsin.

Ozone was generated by passing an oxygen stream over a mercury lamp at 20 ml/min. Ozone concentration in the gas stream was assayed by liberation of iodine from a buffered potassium iodide solution. Delivery of ozone to test solutions was 23-32 nmol/min. It was bubbled from a perforated tip through 5 ml solution of ovoinhibitor (20 μg/ml) or human α-1-proteinase inhibitor (250 μg/ml) in 6 mM borate buffer pH 8.2. Aliquots of these ozone treated anti-proteases were assayed with trypsin, chymotrypsin and elastase. Human serum was diluted 1/15 with 6 mM borate buffer pH 8.2 treated with ozone, and assayed as described above. The protease inhibitors were incubated with the proteases for 10 min before measurement of residual proteolytic activity with the appropriate substrates.

RESULTS: The effect of increasing amounts of ozone on the anti-protease activity of ovoinhibitor are presented in Fig. 1. Anti-elastase activity was much more susceptible than anti-trypsin and anti-chymotrypsin activity. At a ratio of 40 mol O_3/mol ovoinhibitor 69% of anti-elastase was suppressed while only 1.2 and 3.7% of anti-trypsin and anti-chymotrypsin was suppressed. Higher ratios of ozone to protein mask the selectivity of ozone for the anti-elastase activity. The high molar excess of ozone required indicates that the ovoinhibitor is not particularly susceptible to ozone, but it should be noted that not all the ozone administered reacts with the protein.

Table I. Oxidation of human α-1-proteinase inhibitor by ozone.

Molar ratio O_3/ α-1-proteinase inhibitor	Residual inhibitory activity, %		
	anti-elastase	anti-chymotrypsin	anti-trypsin
9.2	44.8	71.2	82.1
18.6	0	19.4	57.8

Ozone exposure and assay are described in Materials and Methods. Data presented are averages of triplicate determinations. The experiment was repeated three times with results similar to those presented.

Response of the α-1-proteinase inhibitor to ozone is shown in Table I. Anti-elastase was most susceptible to inactivation, anti-chymotrypsin somewhat sensitive, and anti-trypsin fairly resistant. It appears at first sight that α-1-proteinase inhibitor is more susceptible than ovoinhibitor, but the difference in protein concentration at the time of ozone administration may be a factor.

Figure 2 shows the results of exposing diluted serum to ozone. The anti-elastase activity is suppressed while the anti-trypsin and anti-chymotrypsin are unaffected. The molar ratio of ozone to α-1-proteinase inhibitor was calculated using normal values for human serum (1). It is notable that the anti-elastase activity can be diminished by ozone even in the presence of a large excess of proteins which could also react with ozone.

DISCUSSION: Oxidation of ovoinhibitor by ozone gives results similar to, but not identical with oxidation by NCS (5). Differences are probably caused by the lack of specificity of amino acid oxidation by ozone. Treatment with ozone of α-1-proteinase inhibitor from human serum also gives results similar to those obtained when NCS is used as the oxidant (6). In the latter case the rate of binding of the anti-protease to trypsin was affected by the oxidation: there was little suppression of anti-trypsin if binding was allowed for 30 min. In our experiments, binding of ozone treated anti-protease was allowed for 10 min and little suppression of anti-trypsin was seen.

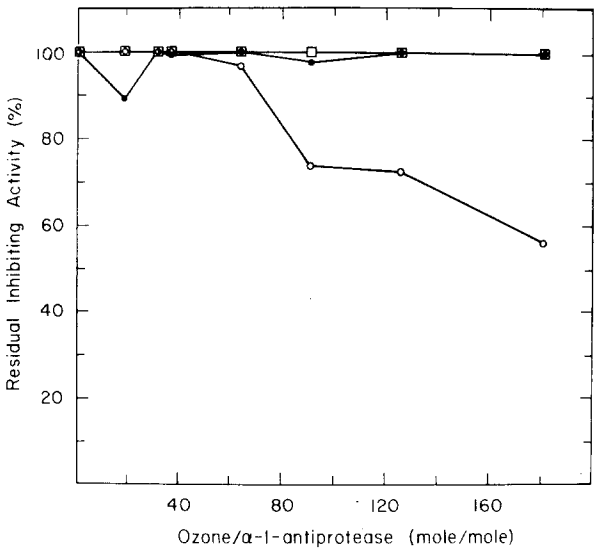

Fig. 2. Effect of ozone on anti-protease activity of human serum. Serum was diluted 1/15 in 6 mM borate buffer pH 8.2 and exposed to ozone and assayed as described in Materials and Methods. The experiment was done four times with similar results. Data presented in this experiment are averages of duplicate determinations. Symbols are the same as for Fig. 1.

Figure 1 and Table I suggest that α-1-proteinase inhibitor is more susceptible to ozone oxidation than is ovoinhibitor. The α-1-proteinase inhibitor (elastase) was 50% inhibited by a molar ratio of 8 whereas ovoinhibitor required a molar ratio of 40. However, protein concentration was 12.5 fold greater for the α-1-proteinase inhibitor exposure to ozone. In the case of glyceraldehyde-3-phosphate dehydrogenase and lysozyme, reaction of ozone is proportional to protein concentration up to 2 mg/ml (K. L. Knight, M. M. Dooley and J. B. Mudd, unpublished observations).

Although the reaction of ozone with ovoinhibitor is of academic interest, the reaction with α-1-proteinase inhibitor may have physiological significance. The test with serum indicates that ozone can suppress anti-elastase activity even in the presence of a large excess of other proteins. It is conceivable that anti-proteases in the lung can be oxidized by inhaled ozone, just as has been suggested for cigarette smoke (9).

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References

1. Kueppers, F. (1973) Environ. Res. 6, 403-423.
2. Janoff, A. and Carp, H. (1977) Am. Rev. Resp. Dis. 116, 65-72.
3. Leh, F., Warr, T. A. and Mudd, J. B. (1978) Environ. Res. 16, 179-190.
4. Mudd, J. B., Leavitt, R., Ongun, A. and McManus, T. T. (1969) Atmos. Environ. 3, 669-682.
5. Shecter, Y., Burstein, Y. and Gertler, A. (1977) Biochemistry 16, 992-997.
6. Johnson, D. and Travis, J. (1979) J. Biol. Chem. 254, 4022-4026.
7. Kassel, B. (1970) Meth. Enzymol. 19, 890-906.
8. Bieth, J., Spiess, B. and Wermuth, C. G. (1974) Biochem. Med. 11, 350-357.
9. Johnson, D. and Travis, J. (1978) J. Biol. Chem. 253, 7142-7144.