Vol. 117, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1983 Pages 22-29

PSEUDO-INHIBITORS OF NEUTROPHIL SUPEROXIDE PRODUCTION: EVIDENCE THAT SOYBEAN-DERIVED POLYPEPTIDES ARE SUPEROXIDE DISMUTASES

Aaron S. Abramovitz, Jun-Yan Hong, and Verrell Randolph

Department of Biochemistry, UMDNJ-New Jersey Medical School and the Graduate School of Biomedical Sciences
100 Bergen St., Newark, NJ 07103

Received October 7, 1983

We have previously reported the purification of polypeptides from soybean which are potent inhibitors of superoxide production by human neutrophils. We now report that neither oxygen uptake nor hydrogen peroxide production by stimulated neutrophils is affected by these inhibitors. Furthermore, the E-1 and E-3 polypeptides inhibit ferricytochrome c reduction by a xanthine oxidase superoxide generation system. The inhibitory activity of E-3 in the model system is blocked by 1 mM KCN while E-1 is only slightly cyanide sensitive. Atomic absorption analysis of E-1 and E-3 polypeptides reveal copper in the latter and manganese in the former. Thus, E-3 is a copper-containing superoxide dismutase while E-1 appears to be a manganese-containing superoxide dismutase.

Stimulation of neutrophils by various agents including phorbol esters results in a burst of metabolic activity, increased oxygen uptake, and the production of reactive oxygen species including H_2O_2 and $O_2^{\cdot-}$ (1,2). The ability of legume-derived polypeptide protease inhibitors to block $O_2^{\cdot-}$ production by stimulated neutrophils has been used to support the hypothesis that these events are mediated by cellular proteases (3,4). However, while crude extracts of soybean do inhibit neutrophil $O_2^{\cdot-}$ production (5), we find that purified Kunitz trypsin inhibitor and the 8,000 dalton Bowman-Birk inhibitor are without effect, contrary to previous reports by others (3,6). We have recently shown that purified soybean polypeptides which appear to inhibit neutrophil $O_2^{\cdot-}$ production are weak protease inhibitors at best (7). Thus, it has become important to determine how these polypeptides exert an effect upon stimulated neutrophils.

This work was supported by grant 32-81 from the UMDNJ Foundation.
Abbreviations: $O_2^{\cdot-}$, superoxide radical anion; PMA, 4 β-phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; DFP, diisopropylfluorophosphate.

0006-291X/83 $1.50

MATERIALS AND METHODS

Unless otherwise stated, reagents were obtained from Sigma Chemical Co., St. Louis, MO. Ferricytochrome c (Sigma grade III or VI) was further purified by gel exclusion chromatography to remove superoxide dismutase contamination as described by Tritsch (8).

Cell Preparation. Human neutrophils were isolated from fresh, heparinized whole blood of volunteers by discontinuous Ficoll-diatrizoate gradients as described by Aguado, et al. (9).

Oxygen Uptake. Neutrophils were suspended at 3 x 10^6 cells/ml in PBS-glucose (5 mM KPi, 150 mM NaCl, 2 mM glucose, pH 7.3) and equilibrated at 37° in a thermostatted reaction vessel fitted with a Clark-type oxygen electrode and meter (Yellow Springs Instrument Model 53). An aliquot of inhibitor to be tested was added to the mixture and baseline oxygen consumption recorded on a 100 mV recorder. From 20-50 ng of PMA were added to stimulate the cells and the rate of oxygen consumption was recorded. Rates were evaluated as nmol O_2 consumed per minute per 10^6 cells.

Superoxide Production. The reduction of ferricytochrome c by stimulated neutrophils was measured as described previously (7).

Superoxide Dismutase Activity was assayed as described by McCord and Fridovich (10). Superoxide was generated in a reaction mixture containing 0.1 mM xanthine and 60 μM ferricytochrome c in 2.0 ml of Na_2CO_3/HCO_3^- buffer, pH 10.0. After the addition of 2.8 μg of xanthine oxidase, the reaction was continuously monitored spectrophotometrically at 550 nm (25°). Aliquots of solutions being tested for superoxide dismutase activity were included in the reaction volume prior to the addition of the xanthine oxidase. In some assays, aliquots of a freshly prepared stock solution of 200 mM KCN were added to a final concentration of 1 mM to suppress activity of any copper-containing dismutases which might be present.

H_2O_2 Production. Neutrophils were suspended in PBS-glucose with 1 mM sodium azide at 3 x 10^5 cells/ml. Suspensions were pre-incubated with and without aliquots of inhibitors at 37° and then stimulated with 20 ng PMA. After 15 minutes, the cells were immediately sedimented by centrifugation in a microcentrifuge, and the supernatant fluid recovered and stored on ice for H_2O_2 determination. Peroxide was measured essentially as described by Tsan, et al. (11). In brief, scopeletin was dissolved in 0.5 M KPi pH 7.1 at 5 μM and 15 μg horseradish peroxidase was added. The relative fluorescence was determined for λ_{ex} 350 nm and λ_{em} 460 nm using a Perkin-Elmer model 512 spectrophotofluorimeter. Aliquots of standardized H_2O_2 (UV extinction at 230 nm = 8100 (12)) were added, and decrease in fluorescence emission recorded, followed by aliquots of cell supernatants and notation of the corresponding fluorescence changes, all within the same cuvette.

Metal Analysis. The Cu and Mn content of polypeptide preparations was measured by atomic absorption analysis with a Perkin-Elmer Model 403 atomic absorption spectrometer. The spectrometer was calibrated with certified standards (Fisher Scientific) and all assays were performed in triplicate. Sample volume was uniformly maintained at 20 μl. The polypeptide samples were prepared for analysis either by dialysis against distilled and deionized H_2O (Millipore Corp.) with and without 1 mM EDTA, pH 7.3.

SDS-urea Polyacrylamide Gel Electrophoresis. The M_r of polypeptide subunits were estimated by the procedure of Swank and Munkres (13). Calibration standards were CNBr-treated preparations of myoglobin and ferricytochrome c.

Polypeptides from Soybean. The E-1 and E-3 polypeptides were purified from extracts of soybean flour by DEAE-cellulose and CM-cellulose ion-exchange chromatography and preparative (native) polyacrylamide gel electrophoresis as previously described (7). In some preparations, the initial crude extract was obtained by extraction with Tris buffer as described by Hwang, et al. (14).

RESULTS

Between 500 and 1000 μg each of the E-1 and E-3 polypeptides were purified from soybean extracts and subjected to SDS- urea polyacrylamide gel electrophoresis to determine the M_r of their subunits. The results are shown in Figure 1. The apparent M_r for E-1 and E-3 are 20,000 and 16,000, respectively. The polypeptides appear to be essentially homogeneous by this criterion. Both E-1 and E-3 appear active as inhibitors of neutrophil superoxide production. The ability of the E-3 polypeptide to interrupt neutrophil production of superoxide is independent of the time of addition relative to the initial stimulation of the cells. Thus, addition of E-3 polypeptide to neutrophils even 10 minutes after the addition of excess PMA, abruptly halts the reduction of ferricytochrome c (Figure 2).

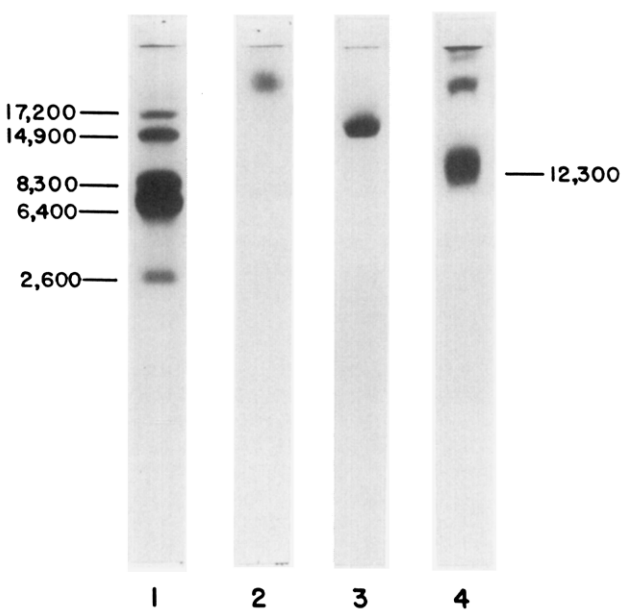

Figure 1. SDS-urea polyacrylamide gel electrophoresis determination of soybean polypeptide M_r. Protein bands were visualized by Coomassie blue stain. lane 1, molecular weight reference polypeptides, CNBr fragments of myoglobin; lane 2, E-1, 17 μg; lane 3, E-3, 20 μg; lane 4, cytochrome c, 15 μg.

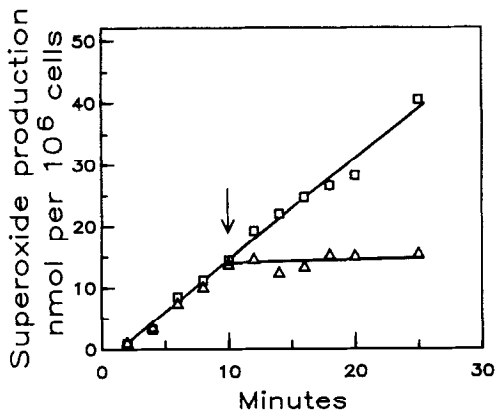

Figure 2. Kinetics of inhibition of neutrophil superoxide production by E-3 polypeptide. Duplicate suspensions of neutrophils (3 x 10^5 cells/1.3 ml) were incubated at 37° and stimulated with 20 ng/ml PMA at t=0 minutes. A control cell suspension containing 30 μg/ml superoxide dismutase was also stimulated with PMA. Aliquots were removed and centrifuged at timed intervals to allow measurement of reduced cytochrome c in the supernatant fluid. At t=10 minutes (arrow), 10^{-6} M E-3 polypeptide was added to one of the suspensions and removal of aliquots for assay of superoxide production was continued. All values of superoxide production were calculated based on superoxide dismutase inhibitable cytochrome c reduction. Uninhibited control cell suspension stimulated with PMA: □ ; neutrophil suspension inhibited 10 minutes after PMA stimulation, △ . Experiment has been replicated twice.

The ability of soybean extracts and purified polypeptides to affect neutrophil oxygen uptake and H_2O_2 production as well as superoxide production by a typical preparation of cells is summarized in Table 1. The $O_2^{\cdot-}$ production is attenuated by addition of either soybean extract of E-3 polypeptide, while oxygen uptake and hydrogen peroxide production are unaffected. The stoichiometry of oxygen uptake, hydrogen peroxide production and superoxide production is 1.0:0.95:0.80.

Table 1. Effect of inhibitors on stimulated neutrophil metabolism of oxygen.*

Inhibitor	Oxygen uptake	H_2O_2 production	Cytochrome c reduction ($O_2^{\cdot-}$)
none	3.04	2.89	2.44
crude soybean extract (400 μg)	3.24	3.03	0.49
E-3 protein (4 μg)	-	2.9	0.35
Soybean trypsin inhibitor (1000 μg)	-	-	2.44

*nmol/10^6 cells/min.

Table 2. Effect of inhibitors on xanthine oxidase production of $O_2^{\cdot-}$.

Inhibitor	Relative activity*	
	-KCN	+KCN
none	-	1.00
soybean trypsin inhibitor (1 mg)	1.01	-
Bowman-Birk inhibitor (250 μg)	0.97	-
E-1 (6.6 μg)	0.05	0.75
E-3 (0.4 μg)	0.43	0.92
superoxide dismutase (1 μg) (bovine)	0.24	0.98

*control rate: 0.02A per min at 550 nm

In order to determine whether superoxide production is actually affected by soybean-derived inhibitors or whether the observed effects were due to artefact involving the superoxide assay, a model superoxide generating system was employed. Xanthine oxidase oxidation of xanthine at pH 10.0 was utilized to generate $O_2^{\cdot-}$ and subsequently to reduce ferricytochrome c. The effect of soybean extracts and purified soybean polypeptides were determined and the results are summarized in Table 2. Crude soybean extract as well as purified E-1 and E-3 polypeptide inhibits xanthine oxidase mediated reduction of ferricytochrome c via superoxide. The addition of 1 mM KCN blocks the ability of E-3 to inhibit ferricytochrome c reduction but only partially affects the inhibitory activity of E-1. The Kunitz soybean trypsin inhibitor has no measurable effect on this system.

Since the soybean polypeptides which are active against neutrophils were thought to be protease inhibitors we further examined the effect of KCN upon the protease inhibitory activity of soybean extracts. As we previously reported, E-1 and E-3 have weak inhibitory activity against trypsin. Significantly although 1 mM KCN partially counteracts the ability of E-1 and fully blocks the ability of E-3 to inhibit the xanthine oxidase mediated reduction of ferricytochrome c, there is no cyanide effect upon protease inhibitory activity of these preparations (data not shown).

Atomic absorption analysis of the E-1 and E-3 polypeptides is summarized in Table 3. The samples dialyzed against H_2O reveal Mn and Cu in both

Table 3. Cu and Mn content of E-1 and E-3 polypeptides.

	Cu*		Mn*	
	dialyzed v.s. H_2O	dialyzed v.s. EDTA	dialyzed v.s. H_2O	dialyzed v.s. EDTA
E-1	1.55	0.81	0.92	0.11
E-3	2.41	1.74	0.30	0.03

*values are ng metal/μg protein.

preparations, although the Mn content of E-1 is approximately 3-fold greater than that of E-3. The inverse is true of the Cu content of E-1 and E-3, where E-3 has the greater value. However, after dialysis against EDTA, E-3 retains most of its Cu, while E-1 retains less. The Mn content of both polypeptides is reduced to trace levels upon EDTA treatment.

DISCUSSION

One line of evidence for the hypothesis that cellular proteases mediate the stimulated production of oxygen radicals by neutrophils has been the reported ability of polypeptide protease inhibitors to block the process (3,4). There are substances present in crude extracts of legumes which inhibit the reduction of ferricytochrome c by stimulated neutrophils. However, these substances have not been rigorously characterized as protease inhibitors. In this laboratory it has not been possible to reproducibly detect inhibition of neutrophil superoxide production by purified soybean trypsin inhibitor (Kunitz) or the Bowman-Birk inhibitors previously reported by others (3,6), despite meticulous attention to experimental detail. Instead, our strategy has been to purify those polypeptides in soybean which have the ability to block ferricytochrome c reduction by stimulated neutrophils. We have recently described the purification of two of these polypeptides, E-1 and E-3 (7). Since neither E-3 nor the crude soybean extract inhibits stimulated neutrophil production of H_2O_2 or the uptake of oxygen it is evident that these substances are active only with respect to blocking the $O_2^{\cdot-}$ assay, i.e. the reduction of ferricytochrome c. The use of a model system for $O_2^{\cdot-}$ production clearly

reveals that the inhibitory activity of the soybean polypeptides E-1 and E-3 as well as crude soybean extract is not uniquely directed against superoxide produced by neutrophils. Furthermore, KCN reverses most of the inhibitory effect of E-3 in the model system, which is characteristic of copper containing superoxide dismutases (15). The E-1 preparation is only partly sensitive to KCN, however. A manganese containing superoxide dismutase would be expected to exhibit the properties of E-1. The metal content of these preparations suggests that the E-3 polypeptide is probably a Cu/Zn superoxide dismutase while E-1 may be a manganese superoxide dismutase. The subunit M_r of E-3, 16,000 dalton is consistent with this conclusion. The E-1 polypeptide subunit whose M_r is 20,000, is smaller than that reported for a tetrameric Mn superoxide dismutase isolated from Pisum sativum whose subunit M_r is 23,500 dalton (16).

These results suggest that previous reports of apparent inhibition of neutrophil superoxide production by legume-derived polypeptide protease inhibitors can be attributed to contamination of these preparations by catalytic amounts of one or more of the dismutase enzymes. In fact, there is no convincing evidence that protease inhibitors of any kind affect neutrophil superoxide production by interaction with cellular proteases. Chemical reagents such as TPCK, which have been used to inactivate certain proteases and are active against neutrophils, are known to react with enzymes other than proteases (17,18). Similarly, DFP which phosphorylates serine-195 in chymotrypsin (19) also reacts with enzymes whose substrates are not proteins.

Thus, the mechanism of neutrophil inhibition by reagents known to affect proteases is uncertain. However, chemical reagents may yet prove to be valuable tools in the dissection of neutrophil function if we consider enzymes whose substrates are not proteins among the possible targets. Although, the soybean-derived superoxide dismutases E-1 and E-3 are not useful probes of the biochemical events within stimulated neutrophils, their presence in foodstuffs like soybeans raises interesting questions

for future exploration of any protective value these enzymes might have when ingested, against oxygen radical damage to host tissue.

ACKNOWLEDGMENTS

We wish to thank Ms. Marcia Lomedico for skillful technical assistance. We are also grateful to Dr. John Bogden for guidance and generous access to his atomic absorption spectrometer.

REFERENCES

1. Badwey, J.A. and Karnovsky, M.L. (1980). Ann. Rev. Biochem. 49:695-726.
2. Klebanoff, S.J. and Clark, R.A. (1978). The Neutrophil: Functional and Clinical Disorders (North Holland).
3. Goldstein, B.D., Witz, G., Amoruso, M. and Troll, W. (1979). Biochem. Biophys. Res. Comm. 88:854-860.
4. Kitagawa, S. and Takaku, F. (1982). Adv. Exp. Med. Biol. 141:441-451.
5. Yavelow, J., Gidlund, M. and Troll, W. (1982). Carcinogenesis 3: 135-138.
6. Kitagawa, S., Takaku, F. and Sakamoto, S. (1980). J. Clin. Invest. 65:74.
7. Abramovitz, A.S., Yavelow, J., Randolph, V. and Troll, W. (1983). J. Biol. Chem. in press.
8. Tritsch, G.L. (1982). Prep. Bioch. 12:289-293.
9. Aguado, M.T., Pujol, N., Rubiol, E., Tura, M. and Celada, A. (1980). J. Immunol. Methods. 32:41-50.
10. Crapo, J.D., McCord, J.M. and Fridovich, I. (1978). Methods in Enzymol. 53:382-393.
11. Tsan, M.F., Douglass, K.H. and McIntyre, P.A. (1977). Blood 49:437-444.
12. Homan-Müller, J.W.T., Weening, R.A. and Roos, D. (1975) J. Lab. Clin. Med. 85:198-207.
13. Swank, R.T. and Munkres, K.D. (1971). Anal. Biochem. 39:462-477.
14. Hwang, D.L.-R., Lin, K.-T.D., Yang, W.-K. and Foard, D.E. (1977). Biochim. Biophys. Acta 495:369-382.
15. Rotilio, G., Bray, R.C. and Fielden, E.M. (1972). Biochim. Biophys. Acta 268:605-609.
16. Sevilla, F., Gorge, J.L. and Del Rio, L.A. (1979). Dev. Biochem. 11B:185-195.
17. Witt, D.J. and Summers, D.F. (1980). Virology 107:34-49.
18. Kupfer, A., Gani, V., Jimenez, J.S. and Shaltiel, S. (1979). Proc. Natl. Acad. Sci. U.S.A. 76:3073-3077.
19. Schoellmann, G. and Shaw, E. (1963). Biochemistry 2:252-255.