

Isolation and Properties of Stachyrase A, a Chymotrypsin-Like Serine Proteinase from *Stachybotrys chartarum*

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A strain of the common mold *Stachybotrys chartarum* has been isolated from the lung of a child with pulmonary hemorrhage. We report the purification of stachyrase A, a new serine chymotrypsin-like proteinase from *S. chartarum*. This enzyme cleaves major protease inhibitors, several biologically active peptides, and collagen, all of which are found in the lung.

Neutrophil degranulation and release of powerful proteolytic enzymes, including elastase and cathepsin G, comprise one of the major mechanisms of defense against air-carried pathogens in the lung (14). Since such enzymes can also be destructive for the lung itself (6), specific proteinase inhibitors, including α_1 -proteinase inhibitor (α_1 -PI), are continuously present in fluids bathing the lung (17). However, invading pathogens releasing their own proteinases can cleave these inhibitors.

Stachybotrys chartarum is a common fungal organism that normally grows on decomposing organic material but has recently been found to colonize the lung (3). In the 1930s it was held responsible for the outbreak of a new disease in animals (4, 16). The first reports of stachybotryotoxicosis in humans appeared in 1940 in Russia where individuals afflicted with infection with *S. chartarum* have developed dermatitis, pain and inflammation of mucous membranes, cough, fever, headache, and fatigue (5). However, this organism attracted public attention as a result of acute idiopathic pulmonary hemorrhage-hemosiderosis and death occurring in infants from Cleveland, Ohio (2). The correlation between *S. chartarum* and acute idiopathic pulmonary hemorrhage-hemosiderosis has recently been questioned (1, 8), but simultaneously experimental lung mycotoxicosis was found in mice exposed to spores of *S. chartarum* (13). *S. chartarum* produces several mycotoxins, immunosuppressive agents, and hemolysin activity (9, 18). We hypothesized that this fungal pathogen might also produce proteinases that could lead to destruction of lung tissue.

S. chartarum strain JS5106 derived from the lung of an infant with pulmonary hemosiderosis (ATCC 201859) was grown in Luria-Bertani medium (15) containing 0.2% glucose and 0.2% elastin with moderate shaking for approximately 3 to 4 weeks. Cultures were assayed for amidolytic activity against 1 mM *N*-Suc-Ala-Ala-Pro-Phe-pNA in 50 mM Tris (pH 7.5) at 37°C. This analysis indicated that the medium contained proteinase, whereas the fungus itself contained limited amounts of the enzyme. To purify the enzyme 40% ammonium sulfate precip-

itation was applied, the pellet was redissolved in buffer A (50 mM Tris [pH 7.5] and 10 mM CaCl₂) and dialyzed, and ammonium sulfate was added to 4%. To remove dark pigment the sample was applied to a DE-52 column, the flowthrough was collected, ammonium sulfate was added to 15%, and the sample was applied to a phenyl-Sepharose column. The activity was eluted in a linear gradient from 10% to 0% ammonium sulfate in buffer A. Two separate peaks of activity were found that contained stachyrase A and another enzyme with different properties (unpublished data). Stachyrase A was further purified on a Mono-Q column (Amersham Pharmacia Biotech) equilibrated with buffer A, with elution in a linear gradient of 0 to 500 mM NaCl.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (11) of the purified enzyme revealed a protein band with a molecular mass of 32 kDa (Fig. 1). The enzyme possessed a gelatinase activity and was easily radiolabeled with [1,3-³H]diisopropylfluorophosphate (7, 12), indicating that it belongs to the serine proteinase family (Fig. 1). Several attempts of amino-terminal sequence analysis yielded an N-terminal sequence of ATQTGA (Microchemical Facility, Emory University School of Medicine, Atlanta, Ga., and Bio-

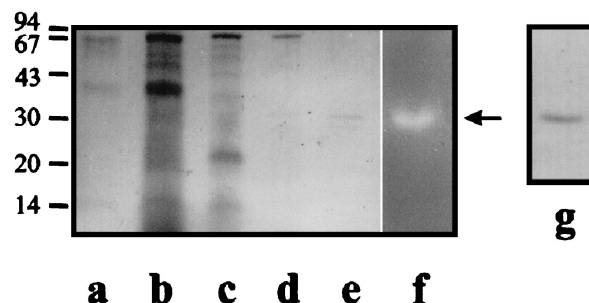


FIG. 1. SDS-PAGE of fractions from purification of stachyrase A and zymography of purified enzyme. Lane a, culture medium; lane b, 40% ammonium sulfate precipitate; lane c, DE-52 flowthrough; lane d, phenyl-Sepharose column eluate; lane e, purified stachyrase A eluted from MonoQ; lane f, gelatin zymography of purified stachyrase A; lane g, fluorography of purified stachyrase A labeled with [1,3-³H]diisopropylfluorophosphate. Samples were reduced and boiled (lanes a to e and g) or only boiled (lane f) before PAGE analysis.

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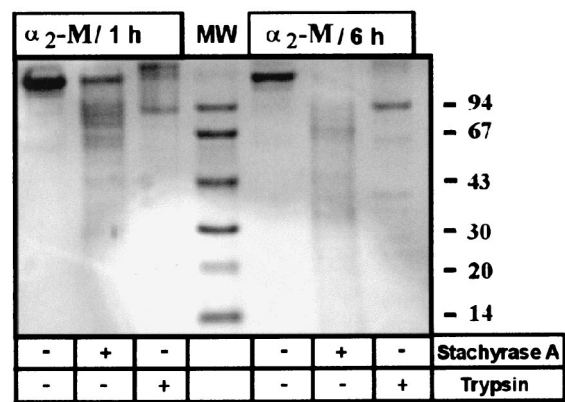


FIG. 5. Time-dependent degradation of α_2 -M by stachyrase A. One microgram of α_2 -M was incubated with or without stachyrase A or trypsin (200:1) for 1 or 6 h (as indicated) at 37°C. Samples were separated by SDS-PAGE.

whereas incubation with trypsin, as expected, yielded one major band of cleaved α_2 -M. In equimolar concentrations, neither α_1 -ACT, α_1 -PI, nor α_2 -M had any substantial effect on enzyme activity (data not shown). At very high concentrations of inhibitors inhibition could be detected, suggesting that they act as competitive substrates. In comparison, *Aspergillus*-derived seaprose was found to degrade α_1 -PI, form complexes, and inactivate α_1 -ACT but was inhibited by α_2 -M (10). In addition, we tested the effect of stachyrase A on secretory leukocyte protease inhibitor normally present in the lung (17). This protein was also cleaved by the fungus-derived enzyme (data not shown).

Based on the presented results we postulate that the role of stachyrase A should now be considered together with mycotoxins and hemolysin when pathological effects of colonization by *S. chartarum* are discussed.

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REFERENCES

- Centers for Disease Control and Prevention. 2000. Update: pulmonary hemorrhage/hemosiderosis among infants—Cleveland, Ohio, 1993–1996. *Morb. Mortal. Wkly. Rep.* **49**:180–184.
- Dearborn, D. G., I. Yike, W. G. Sorenson, M. J. Miller, and R. A. Etzel. 1999. Overview of investigations into pulmonary hemorrhage among infants in Cleveland, Ohio. *Environ. Health Perspect.* **107**(Suppl. 3):495–499.
- Elidemir, O., G. N. Colasurdo, S. N. Rossmann, and L. L. Fan. 1999. Isolation of *Stachybotrys* from the lung of a child with pulmonary hemosiderosis. *Pediatrics* **104**:964–966.
- Forgacs, J. 1972. Stachybotryotoxicosis, p. 95–128. In A. C. S. Kadis and S. J. Ajl (ed.), *Microbial toxins*, vol. VIII. Academic Press, Inc., New York, N.Y.
- Fung, F., R. Clark, and S. Williams. 1998. Stachybotrys, a mycotoxin-producing fungus of increasing toxicologic importance. *J. Toxicol. Clin. Toxicol.* **36**:79–86.
- Gadek, J. E. 1992. Adverse effects of neutrophils on the lung. *Am. J. Med.* **92**:27S–31S.
- Goldstein, J. M., A. Banbula, T. Kordula, J. A. Mayo, and J. Travis. 2001. Novel extracellular x-prolyl dipeptidyl-peptidase (DPP) from *Streptococcus gordonii* FSS2: an emerging subfamily of viridans streptococcal x-prolyl DPPs. *Infect. Immun.* **69**:5494–5501.
- Hagmann, M. 2000. Public health. A mold's toxic legacy revisited. *Science* **288**:243–244.
- Jarvis, B. B., J. Salemm, and A. Morais. 1995. Stachybotrys toxins. 1. *Nat. Toxins* **3**:10–16.
- Korzus, E., M. Luisetti, and J. Travis. 1994. Interactions of alpha-1-antichymotrypsin, alpha-1-proteinase inhibitor, and alpha-2-macroglobulin with the fungal enzyme seaprose. *Biol. Chem. Hoppe-Seyler* **375**:335–341.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Nelson, D., J. Potempa, T. Kordula, and J. Travis. 1999. Purification and characterization of a novel cysteine proteinase (periodontain) from *Porphyromonas gingivalis*. Evidence for a role in the inactivation of human alpha1-proteinase inhibitor. *J. Biol. Chem.* **274**:12245–12251.
- Rao, C. Y., J. D. Brain, and H. A. Burge. 2000. Reduction of pulmonary toxicity of *Stachybotrys chartarum* spores by methanol extraction of mycotoxins. *Appl. Environ. Microbiol.* **66**:2817–2821.
- Reynolds, H. Y. 1985. Phagocytic defense in the lung. *Antibiot. Chemother.* **36**:74–87.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sarkisov, A. K. A. O. 1944. Laboratory diagnosis of toxic strains of *Stachybotrys alternans*. *Veterinariya* **21**:38–40.
- Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52**:655–709.
- Vesper, S. J., D. G. Dearborn, O. Elidemir, and R. A. Haugland. 2000. Quantification of siderophore and hemolysin from *Stachybotrys chartarum* strains, including a strain isolated from the lung of a child with pulmonary hemorrhage and hemosiderosis. *Appl. Environ. Microbiol.* **66**:2678–2681.