MULTIPLE MOLECULAR WEIGHT FORMS OF STAPHYLOCOCCAL NUCLEASE

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Staphylococcal nuclease, an exocellular, heat stable phosphodiesterase (Cunningham, Catlin, and de Garilhe, 1956) has been reported to occur in two forms with differing molecular weights (Taniuchi, Anfinsen, Heins, and Carrol, 1965). Thus, upon observing that the nuclease activity in the culture supernatants of several strains of Staphylococcus aureus displayed bimodal solubility in $(NH_4)_2SO_4$ (80-90% of the activity precipitated between 60 and 70% saturation, while the remainder of the activity was soluble even in saturated $(NH_4)_2SO_4$), we sought to establish if different molecular weight forms were being separated by the $(NH_4)_2SO_4$ treatment. This was found to be the case, but it was also found that the nuclease occurs in an unusually large number of molecular weight forms, the smallest of which possesses a molecular weight of less than 1,000, placing it among the smallest enzymatically active peptides occurring naturally.

Materials and Methods

Five strains of S. aureus were studied: the Wood 46 strain;

UNH-15, a strain obtained from a case of bovine mastitis; F.D.A. 234, a food poisoning strain; and two strains isolated from human infections.

For nuclease production, all strains were grown in 500 ml of a dialysable medium (Chesbro, Heydrick, Martineau, and Perkins, 1965) in a four L flask under an 80% O_2:20% CO_2 atmosphere. Following inoculation with 25 ml of a 24 hr culture in the same medium, the flasks were shaken at 35 C. The gas mixture was replenished after five hr. After ten hr, five ml of chloroform were added to the flask to kill the pathogen. The chloroform-treated culture was held 12 hr at 5 C and the cells then removed by centrifugation. The supernatant was boiled for 15 min (Alexander, Heppel, and Hurwitz, 1961) to inactivate any acid or alkaline phosphatase and deoxyribonuclease present and to activate the nuclease (Ohsaka, Mukai, and Laskowski, 1964). Boiling the medium with the cells still present, which would make the chloroform treatment unnecessary, results in production of an oily scum interfering with later steps of the procedure. The boiled medium was recentrifuged and the supernatant retained as the crude preparation.

For $(NH_4)_2SO_4$ fractionation, the crude preparation at 5 C was saturated with $(NH_4)_2SO_4$ and the precipitate collected after 24 hr by centrifugation (insoluble activity). The supernatant was made 0.3 M in trichloroacetic acid (TCA), the resulting precipitate collected by centrifugation, neutralized with 1 N NaOH was dissolved in distilled water (soluble activity).

Nuclease activity was measured by the method of Alexander,

et al (1961) using heat-denatured calf thymus DNA as substrate.

Ultracentrifugal analysis was performed on a 2-3% sucrose gradient by the sedimentation velocity method of Hogeboom and Kuff (1954). The samples were centrifuged at 39,500 x g for 10-12 hr in the SW39 rotor of a Spinco Model L preparative ultracentrifuge. After bottom puncturing the centrifuge tubes, three drop fractions were collected and assayed for nuclease activity.

Gel filtration was performed with Sephadex G75 and three spherical polyacrylamide gels, Biogel P10, P6, and P4 (Biorad Laboratories, Richmond, Calif.), with approximate molecular weight fractionating ranges of 3,000-70,000, 5,000-17,000, 1,000-5,000, and 500-4,000 respectively. The gels were equiliberated and eluted with the 0.18 M, pH 8.6, glycine buffer used in the enzyme assay system. Sephadex G75 and Biogel P10 were standardized with muramidase and ribonuclease, proteins whose basicity approximates that of the nuclease and which would reveal any tendency of the columns to retard polycations. Biogel P6 and P4 were standardized with bacitracin and raffinose. The void volume (Vo) and permeable volume ($Vo + V_1$) of the columns were determined with blue dextran (Pharmacia Chemicals, Piscataway New Jersey) and methyl red respectively.

Electrophoresis was performed on S. and S. 589 Green Ribbon C paper using an ammonium acetate buffer adjusted to pH 5.0 with acetic acid and diluted with distilled water to a conductivity of 1.0 millimho. A gradient of 16.5 V per cm was used.

One dimensional descending chromatography was performed

with S. and S. 507-C paper qualitatively, and S. and S. 589 Green Ribbon C preparatively, using butanol:acetic acid:water (55:15:20). The same solvent was used in the first direction for two dimensional chromatography and 80% phenol:concentrated NH_4OH (98:1) used in the second.

Results

Ultracentrifugal analysis of the insoluble activity of the UNH-15 strain indicated a major component having a molecular weight of 15,000, while analysis of the soluble activity indicated a major component of molecular weight 7,000. However, both activities were polydisperse.

The two types of activities were subjected to filtration through Sephadex G75 (Figure 1). The insoluble activity eluted in two major peaks, corresponding to molecular weights of 15,500 and 13,000. The soluble activity resolved into two peaks, one corresponding to a molecular weight of 6,500, while the other was a broad peak leaving the column at the termination of the permeable volume, indicating that it contained unresolved components with molecular weights of 3,000 or less.

To establish the size of the smaller members of this apparent series of molecular weight forms, and also to establish if these lower molecular weight forms could be demonstrated in the culture supernatants of other strains, the soluble activity from five strains was filtered through Biogel P6. Any activity eluting at the lower operational limit of this gel (approximate molecular weight 1,000) was refiltered through Biogel P4.

Except for the F.D.A. 234 strain, all strains produced

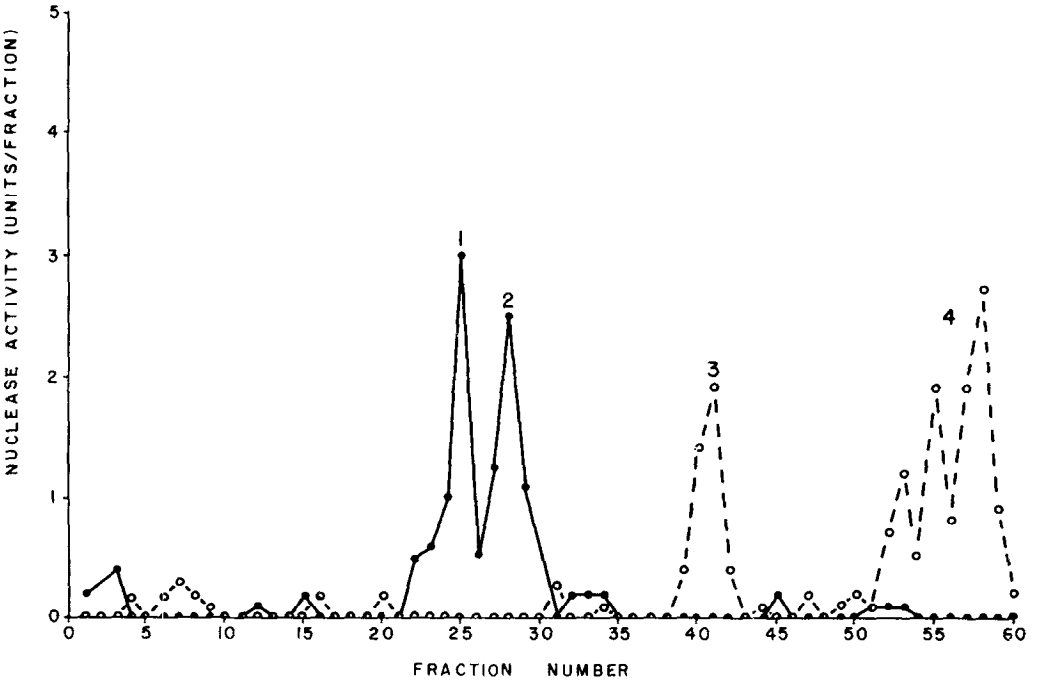

Figure 1. Sephadex G75 filtration of $(NH_4)_2SO_4$ - fractionated nuclease activity from Staphylococcus aureus UNH-15. The $(NH_4)_2SO_4$ insoluble activity (●——●) from two ml of culture supernatant, and the $(NH_4)_2SO_4$ soluble activity (0----0) from four ml of culture supernatant were applied at 4 C to a three cm ID column with a void volume (Vo) of 40.5 ml, a total permeable volume (Vo + V_1) of 121.5 ml, and a flow rate of 8.4 ml per hr. The eluting buffer is described in the text. Fractions of 1.4 ml were collected. The peaks correspond to forms with approximate molecular weights of 15,500(1), 13,000(2), 6,500(3), and 3,000 or less(4).

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nuclease activity having indicated molecular weights of 5,000 or less, with the minimal form having a molecular weight of 2,800. The F.D.A. 234 strain produced a form that, on refilteration through the P4 gel, had an indicated molecular weight of 900.

To determine if these multiple forms were present in the crude preparations, or were a result of the $(NH_4)_2SO_4$ and TCA treatments, crude preparations from the F.D.A. 234 and UNH-15

strains were filtered through Sephadex G75. The results, shown in Figure 2, demonstrated that the multiple forms were present in the culture supernatants before either precipitating treatment was employed. There were, in fact, more peaks in the crude UNH-15 than had been found in both precipitated fractions of this strain.

The observation that fewer forms were detectable in the precipitated fractions than in the crude supernatant suggested that the forms were interconvertible, and indicated the possibility

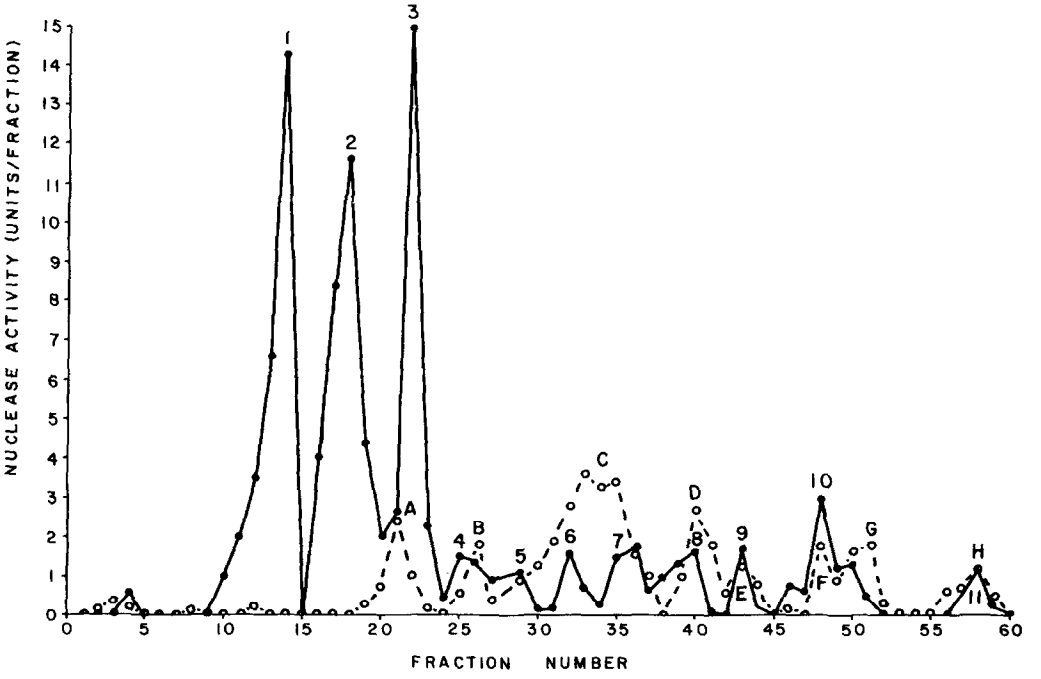

Figure 2. Sephadex G75 filtration of the nuclease activity in crude culture supernatants of Staphylococcus aureus strains F.D.A. 234 (●——●) and UNH-15 (0----0). Two ml of culture supernatant were filtered as described in Figure 1. The peaks correspond to forms with approximate molecular weights of 29,000(1), 23,500(2), 18,600(3), 15,500(4), 12,500(5), 10,500(6), 8,500(7), 6,600(8), 5,600(9), 4,200(10), 3,000 or less(11); and 19,700(A), 14,800(B), 10,000-8,500(C), 6,600(D), 5,600(E), 4,200(F), 3,500(G) 3,000 or less(H).

of providing evidence that the multiple forms observed were aspects of one enzyme, rather than several, by regenerating the forms of either fraction from the other.

Consequently, to establish that the higher molecular weight, $(NH_4)_2SO_4$ insoluble forms could be derived from the lower molecular weight, soluble forms, the soluble activity from one L of UNH 15 crude preparation was neutralized following TCA precipitation and brought to a volume of 10 ml with distilled water. At this point, gel filtration indicated the absence of any form with a molecular weight greater than 10,000. The syrupy solution remained fluid at -5 C and it was held at this temperature for two months. Part of the sample was then saturated with $(NH_4)_2SO_4$ and the amounts of insoluble and soluble activities present determined. A second portion of the sample was filtered through Sephadex G 75.

Sixty per cent of the activity had become insoluble in saturated $(NH_4)_2SO_4$, and the major portion of the activity in the gel filtered sample was associated with a peak having an indicated molecular weight of 17,000.

To establish that the lower molecular weight, $(NH_4)_2SO_4$ soluble forms could be derived from the higher molecular weight, insoluble forms, and to obtain enough of the putative monomer of molecular weight 900 for a determination of its amino acid residues, advantage was taken of the inability of DEAE cellulose to retain the lower molecular weight forms. Insoluble activity from the F.D.A. 234 strain (185,000 units) was dissolved in distilled water and kept at 5 C for two weeks. The solution was then ab-

sorbed on a 2x10 cm column of DEAE-cellulose in the chloride form. The column was eluted with distilled water and the nuclease activity in the effluent (17,000 units) precipitated with TCA. The precipitate was neutralized, redissolved in distilled water, and filtered through Biogel P4. The central fractions of the peak migrating through the column at a rate corresponding to a molecular weight of 900 were pooled, yielding 8,000 units of nuclease. It was thus possible to obtain the monomer from a mixture of the heavier forms.

The pooled activity was taken to dryness, redissolved in the electrophoretic buffer, and subjected to electrophoresis. Guide strips were used to locate the nuclease activity, which was then eluted from the paper and taken to dryness.

A portion of this material was chromatographed in one dimension. Five ninhydrin reacting components were found in a guide strip and the nuclease activity in a second guide strip had an Rf corresponding to that of one of the ninhydrin positive components (0.10). The balance of the electrophoretically purified activity was preparatively chromatographed in one dimension. The band containing the nuclease activity was located in guide strips and then eluted from the main body of the chromatogram. The eluted material was reduced to a volume of one ml, made 6 N HCl, and held for 16 hr at 120 C. After being taken to dryness and redissolved in 10% isopropanol, the hydrolysate, containing approximately 12 ug of amino nitrogen, was chromatographed in two dimensions.

Hydrolysis released eight amino acids from the active pep-

tide: alanine, aspartic and glutamic acids, glycine, leucine, lysine serine, and valine. The calculated molecular weight of the peptide, assuming the residues were present in equimolar amounts, was 818.

Discussion

Taniuchi, et al (1965) have reported that the nuclease occurs in two forms with molecular weights of approximately 10,000 and 20,000, and contains nearly the usual array of amino acid residues. Since these workers purified the enzyme from an $(NH_4)_2SO_4$ precipitate, they were presumably analyzing what corresponded to the insoluble fraction used in the present study, which contains only the higher molecular weight forms.

Obviously, however, polymers derived solely from the 900 molecular weight form could not display such an array of amino acids, indicating that there may be several active peptides, some of which are larger and possess a greater variety of amino acids. This is also suggested by the finding that four of the five strains we studied produced a minimal subunit of molecular weight 2,800. Alternatively, the higher molecular weight forms may be heterogenous aggregates, since the nuclease can be readily demonstrated to combine with other proteins: mixed with bovine serum, both the insoluble and soluble activity migrate through Sephadex G75 with the serum proteins.

Isolation of the minimal subunit from several strains in

amounts adequate for analysis should distinguish between these alternatives.

Acknowledgements

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