Structural Heterogeneity of the Protease Isolated from Several Strains of Staphylococcus aureus*

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REJEAN BEAUDET, SAMIR A. SAHEB, AND GABRIEL R. DRAPEAU†

From the Department of Microbiology, University of Montreal, Montreal, Quebec, Canada H3C 3J7

SUMMARY

The structural and biochemical properties of the extracellular protease purified from four different strains of Staphylococcus aureus are reported. All enzymes specifically cleave peptide bonds on the COOH-terminal side of either aspartic acid or glutamic acid, a specificity identical with that reported for staphylococcal protease V8 (DRAPEAU, G. R., BOLLY, Y., AND HOUARD, J. (1972) J. Biol. Chem. 247, 6720). However, differences in the electrophoretic mobilities and molecular weights for these enzymes were observed. Three of the enzymes have an estimated molecular weight of 12,000, whereas the fourth has a molecular weight in the range of 21,500 to 24,000. Immunological studies indicated that the larger protease is antigenically unrelated to the others. For all proteases, the NH₂- and COOH-terminal amino acids were found to be valine and glutamic acid, respectively.

Because of the presence of a glutamic acid residue at the COOH-terminal position in all proteases, it is concluded that the observed variations in electrophoretic mobilities and molecular weights may be the result of various degrees of autodigestion at the COOH-terminal end. In consequence, the proteases described in these studies are not necessarily the primary gene products.

The purification of a proteolytic enzyme from culture filtrates of Staphylococcus aureus, strain V8, has recently been reported (1). This protease specifically cleaves peptide bonds on the COOH-terminal side of either aspartic acid or glutamic acid. Further studies revealed that the enzyme can be rendered specific for glutamyl bonds, provided the digestion of protein substrates is carried out in ammonium acetate or ammonium bicarbonate buffer (2). Therefore, the striking difference between the staphylococcal protease and other extracellular proteolytic enzymes of bacterial origin is its high degree of substrate specificity. The possibility, however, that other strains of S. aureus liberate in the growth medium a proteolytic enzyme differing in substrate specificity from that of strain V8 cannot be excluded. In a survey involving 136 strains of S. aureus, Martley et al. (3) observed four classes of staphylococcal proteases which could be differentiated by the patterns of precipitation zones on a casein agar medium. The type of zone produced by any one strain was reproducible and it was suggested that the extracellular proteases produced by staphylococci were strain-specific.

In the present study the purification and partial characterization of the protease from four different strains of S. aureus are reported. It will be shown that although structural differences were observed among the various staphylococcal proteases, they all exhibited the same peptide bond specificity.

EXPERIMENTAL PROCEDURE

Organisms and Culture Conditions—S. aureus strains 152 and 155 which represent the zone types A and B, respectively (see Fig. 1), were kindly provided by Dr. R. C. Lawrence, New Zealand Dairy Research Institute (3); strains Wood 46 and 3A (zone types C and D, respectively) were from the collection of this Department. S. aureus strain V8, used previously (1, 2) and which also belongs to zone type C, has been included in the present study for comparison. The culture conditions for protease production were as previously described (1) with the exception that the casein concentration in the growth medium was lowered from 1.5 to 1%. The casein agar medium was prepared as described by Martley et al. (3).

Preparation of Proteases—The proteolytic enzymes from the different strains were purified as described earlier (1). Preparative polyacrylamide gel electrophoresis was omitted, since this procedure only served to eliminate a pigment which, in some preparations, was not completely removed by the step of chromatography on DEAE-cellulose (1).

Preparation of Antiserum—Antisera were prepared in rabbits. Each animal received a series of dorsal, subcutaneous injections at 1-week intervals. The protease was emulsified in an equal volume of Freund's adjuvant. The total amount of antigen administered to each rabbit was 8 mg (2.0 mg per week). Each animal was bled 1 week after the last injection. Immunodiffusion experiments were conducted by the Ouchterlony double diffusion technique using 1% agar gels in 10 mm phosphate buffer (4). The gels were developed at room temperature and photographs were taken after approximately 36 hours.

Other Methods—The methods used for the determination of the molecular weights of the proteases, their amino acid compositions, and substrate specificities have been described earlier (1). NH₂-terminal amino acid residues of enzymes and peptides were identified by the 5-dimethylamino-naphthalene-1-sulfonyl method as described by Gros and Labouesse (5). COOH-terminal residues were determined by hydrazinolysis as described previously (1).

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† To whom correspondence regarding this paper should be addressed.
FIG. 1 (left). Proteolytic zone types formed on casein-agar by different strains of Staphylococcus aureus. A, strain 152; B, strain 155; C, strain Wood 46; and D, strain 3A.

FIG. 2 (right). Polyacrylamide disc gel electrophoresis of the different proteases. The quantity of protein applied was from 50 to 200 μg. The origin is at the top, the anode at the bottom. Acrylamide concentration was 15%.

Proteolytic activity was measured by the method of Kunitz (6) using casein or hemoglobin as substrates (1). In a few instances, proteolytic activity was determined colorimetrically using N,N-dimethylcasein as substrate (7).

RESULTS

Fig. 1 shows the different zone types produced by the various strains of S. aureus chosen for the present study. The clear zone surrounding the colony of strain A indicates complete hydrolysis of the casein present in the medium into small, soluble peptides. A small transparent zone is also present around Colonies B and C, but this is surrounded by an outer white ring formed by the precipitation of larger, less soluble peptides. This suggests that the proteases secreted by these two strains, and particularly by strain D, are more specific and do not degrade casein as completely as does the protease from strain A. As previously reported by Martley et al. (3), these zone types do not change upon longer incubation periods and no relationship exists between the type and the diameter of the zone.

Purity of Proteases—All four proteases were purified to homogeneity as estimated by disc gel electrophoresis at pH 8.9 in 15% acrylamide (Fig. 2). It can be seen that protease A migrated more slowly than the others, although the electrophoretic conditions were identical in all cases. When all the different proteases were subjected to electrophoresis on a single gel plate (7.5% acrylamide), it was observed that not only protease A migrated more slowly, but differences were detected among the other proteases as well (Fig. 3). These differences were particularly significant between protease V8 and proteases C and D, whereas they were minor between proteases C and D and between proteases V8 and B.

The minor band in Gel D represents pigmented material which was not completely removed during the purification procedure.

Determination of Molecular Weights—The molecular weights of the four enzymes were estimated by disc gel electrophoresis in the presence of sodium dodecyl sulfate using proteases inactivated by oxidation with performic acid. The marker proteins

Fig. 3. Polyacrylamide disc gel electrophoresis of the proteases isolated from various strains of Staphylococcus aureus. The quantity of protein applied was approximately 6 μg. From left to right: proteases A (upper band) and V8 (lower band); C; B; D; V8; and B plus V8. The anode is at the bottom. The acrylamide concentration was 7.5%.
The molecular weight of protease A was also determined by sedimentation equilibrium in 50 mM sodium phosphate buffer, pH 7.5, and at a protein concentration of approximately 0.4 mg per ml. A partial specific volume of 0.728 was calculated from the amino acid composition of the enzyme, the aspartic acid hydrazides having undergone a partial conversion into aspartic acid.

**Other Properties**—Proteases B, C, and D exhibited maximal activity at pH 4.0 and 7.8 when tested with hemoglobin as the substrate. Protease A, however, had little activity on hemoglobin and therefore an optimum pH could not be determined. The reason for this poor activity on hemoglobin is not clear. All proteases exhibited maximal activity at pH 7.8 with casein as substrate. The specific activities with this substrate were 223, 581, 419, and 349 for proteases A, B, C, and D, respectively.

These results indicate significant differences in activities between these enzymes, particularly in the case of protease A which is the least active. Similar results were obtained on measuring the activities by the method of Lin et al. (7). However, the total number of bonds split by the proteases as determined after a 20-hour incubation period was approximately equivalent for the four enzymes, suggesting that they may have similar substrate specificities.

**Table I**

Amino acid composition of protease from Staphylococcus aureus strains A, D, and V8

| Amino acid     | Protease |
|----------------|----------|
|                | A        | D        | V8² |
| Lysine         | 10       | 6        | 6   |
| Histidine      | 6        | 3        | 3   |
| Arginine       | 6        | 1        | 1   |
| Aspartic acid  | 35       | 26       | 29  |
| Threonine      | 10       | 7        | 8   |
| Serine         | 16       | 6        | 4   |
| Glutamic acid  | 24       | 10       | 11  |
| Proline        | 14       | 10       | 11  |
| Glycine        | 24       | 9        | 9   |
| Alanine        | 8        | 7        | 7   |
| Cysteine       | 0        | 0        | 0   |
| Valine         | 20       | 8        | 7   |
| Methionine     | 2        | 1        | 1   |
| Isoleucine     | 19       | 6        | 6   |
| Leucine        | 10       | 4        | 4   |
| Tyrosine       | 11       | 3        | 3   |
| Phenylalanine  | 6        | 4        | 4   |
| Tryptophan     | 2        | 1        | 1   |
| Total          | 223      | 112      | 115 |

² Data taken from Ref. 1.
of alanine and valine was detected in addition to the other residues. Hydrazinolysis revealed only the presence of aspartic acid and glutamic acid in the digest prepared in phosphate buffer, while only glutamic acid could be detected in ammonium bicarbonate buffer. Since all the NH2-terminal residues determined in the peptides are adjacent to a glutamic acid or aspartic acid residue in ribonuclease, it is clear that the proteases have identical substrate specificities: cleaving only aspartyl and glutamyl bonds. They also appear to exhibit the same buffer-dependent specificity as observed for protease V8 (2); the cleavage of glutamyl bonds in the presence of ammonium bicarbonate buffer.

Immunological Studies—The proteases purified from strains C, D, and V8 gave a single continuous precipitin band with the antiserum directed against protease V8, indicating that these three proteases were structurally identical (Fig. 5, left). Protease B (not shown) was also immunologically indistinguishable from protease V8. Protease A, however, did not cross-react with the anti-protease V8 serum nor did anti-protease A form a precipitation line with any of the other proteases (Fig. 5, right). These observations suggest that protease A, although related to the others in a number of properties, namely its substrate specificity, must bear some important structural differences.

**DISCUSSION**

The present study clearly illustrates that important structural differences exist among the proteases produced by different strains of *S. aureus*. However, they all exhibit the same substrate specificity and share a number of other properties as well. Proteases B, C, D, and V8 cannot be differentiated immunologically and have similar if not identical molecular weights and amino acid compositions. The small electrophoretic variations could be accounted for on the basis of small differences in their primary structures.

All staphylococcal proteases examined contain a glutamic acid residue at their COOH-terminal positions. Since the presence of this residue at the COOH-terminal end correlates with the known specificities of these enzymes some autodigestion is strongly suggested. Since the molecular weight of protease A is approximately twice that of the other proteases, it could be that autodigestion is not as extensive in the case of this protease. The small differences in electrophoretic mobilities observed for proteases B, C, D, and V8 could also be accounted for if these enzymes have slightly different molecular weights, that is, if their polypeptide chains have varying lengths.

Since protease A is obtained by essentially the same purification procedure as that employed for the others and has an identical substrate specificity, there is ample evidence that these enzymes are structurally related. However, protease A does not share common antigenic properties with the others. Although this observation suggests that protease A is structurally dissimilar other possibilities should also be considered, however. For example, the additional portion of the polypeptide chain of protease A could be either exposed on the surface of the molecule covering up the possible common region or could induce conformational changes so that the enzyme is not recognized by the antiprotease V8 antibodies. Since our data suggest that the staphylococcal proteases investigated in this study might represent only a portion of an original proteolytic enzyme, the precursor enzyme may well be present in the culture filtrates. A new, higher molecular weight proteolytic enzyme which can be detected only during the early growth phase of the culture has been isolated from strain V8.

At present, the differences in zone types shown in Fig. 1 cannot be explained satisfactorily. Since these zone types give an indication of the degree of hydrolysis of the casein, it was originally expected that differences in specificities or activities would be encountered. However, no relation between the extent of casein hydrolysis in the casein agar medium and the activities of the enzyme is found to exist. It is suggested that perhaps the newly isolated protease present in the early phase of the culture might be responsible for the zone types produced by the various strains.

That structural differences can be observed in the protease isolated from various strains of *S. aureus* is not totally unexpected. Staphylococci represent a very heterogeneous group of microorganisms. The amino acid, purine, and vitamin requirements vary from strain to strain (9) and the presence of a mutator gene has been postulated in this organism (10). As another example, the amino acid sequence of the nuclease from strain V8 is known to differ from that isolated from strain Foggi by the replacement of a leucine by an isoleucine residue (11). It is therefore possible that various strains of *S. aureus* can produce proteases which differ slightly in their structure and consequently in their susceptibility to autodigestion.

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