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Characterization of *Vibrio cholerae* Protease Activities with Peptide Digest Analysis

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A simple method for the analysis of microbial proteases is described that was used to characterize the proteolytic activities of various *Vibrio cholerae* isolates. This method utilized the unique peptides generated from the degradation of a standard protein by proteases of various specificities. These peptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The unique patterns of peptides seen in gels can be used to type proteases according to their relative specificities. Culture supernatants of *V. cholerae* isolates from a variety of environmental and human sources were analyzed for the presence of a protease previously isolated and characterized in this laboratory from *V. cholerae* strain CA401. Supernatants from most isolates showing dimethyl casein proteolytic activity exhibited the presence of enzymes similar to the CA401 protease in their peptide digest patterns against bovine serum albumin and in their immunological reactivities. The probable widespread presence of this virulence-associated protease in *V. cholerae* isolates is discussed.

Microbial proteases are increasingly recognized as important virulence factors for a variety of pathogens. Evidence exists that proteases may be important virulence factors in *Pseudomonas aeruginosa, Vibrio cholerae*, and possibly *Serratia marcescens* infections (6-9). Proteases are often difficult enzymes to characterize biochemically. A single protease may display heterogeneity in molecular weight or charge. This may be due to post-translational processing or auto- or heterocatalytic destruction during purification. With growing evidence of the importance of proteases in infections, it is important to develop simple methods which can be used to characterize proteases from clinical or environmental isolates. A frequently used technique for protease typing has been the electrophoretic separation of proteases in agarose, starch, or polyacrylamide gels followed by an activity stain (zymogram technique). This technique produces unique spots or zones of enzymatic activity on the gel, and has chiefly been used for taxonomic purposes (2, 5, 11). The zymogram technique is limited by a lack of specificity in the staining procedures and the electrophoretic heterogeneity which can be induced in a single precursor enzyme by various degrees of post-translational cleavage. Thus, a single enzyme may produce multiple activity spots which represent catalytically identical but electrophoretically heterogenous enzymes.

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The technique described here utilizes the catalytic specificity of proteases as a basis for typing strains of *V. cholerae*. A standard protein is used as a substrate for proteases found in culture supernatants. The unique peptides generated by cleavage are separated on the basis of relative molecular weight by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The unique banding patterns generated in the gels are used as the basis for catalytic typing.

This technique was inspired by the work of Cleveland et al. (1), who utilized SDS-PAGE to perform peptide digest analysis of phage proteins. We have in essence reversed this technique and utilized the various cleavage patterns of a reference protein by different protease preparations to establish the catalytic relations of the proteases to one another.

The protease of *V. cholerae* strain CA401 has recently been isolated and characterized (D. R. Schneider, Ph.D. thesis, University of Texas at Austin, 1978). Strain CA401 syncase supernatants appear to contain a protease of one catalytic specificity and antigenicity which exists in two forms of differing molecular weight. A modification of the catalytic typing technique described here was used in the aforementioned publication to help establish the identical catalytic specificities of these two forms of the enzyme. Due to the extensive characterization of the protease of strain CA401, it was chosen as the reference strain for this study. This paper

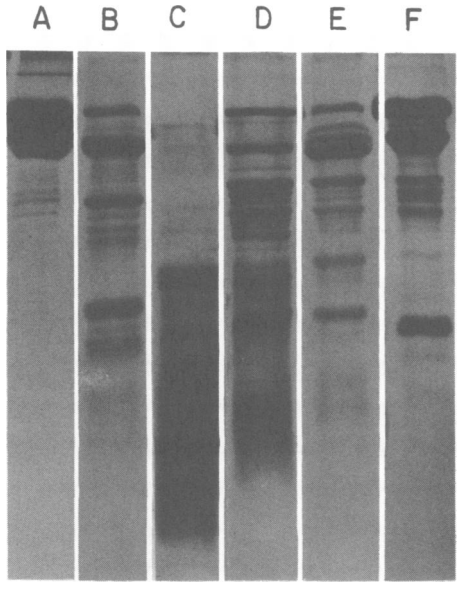

FIG. 1. *SDS-PAGE gels of BSA digestion products of various protease preparations. Lanes: A, BSA alone (no enzyme added); B, bovine trypsin treated (10 μg/100 μl of digestion mix, 30-min incubation); C, S. griseus protease (10 μg/100 μl, 15-min incubation); D, V. cholerae CA401 culture supernatant (15-min incubation); E, P. aeruginosa strain 2141 culture supernatant (2-h incubation); F, A. hydrophila culture supernatant (2-h incubation). Gels run with enzyme preparations alone showed no significant levels of staining.*

extends the applicability of this technique for protease typing.

MATERIALS AND METHODS

Bacterial strains. All strains are *V. cholerae* O-1, except for V40 which is *V. cholerae* non-O-1. Strains 1 to 34 have been described previously (12, 13).

Strains 1 to 9 were isolated from cholera patients in Calcutta, India. Strains 10 to 17 were isolated from cholera patients in Bahrain. Strains 18 to 21 were isolated from environmental sources in the western hemisphere. Strain 22 was isolated from a patient in Alabama with chronic gall bladder disease (a non-diarrheal illness). Strain 23 was isolated from a cholera patient in Texas. Strains 24, 25, and 27 to 33 were isolated from patients and sewers during an outbreak of cholera in Louisiana. Strains 26 and 34 were isolated from crustaceans in the area during the same outbreak. Crabs, which yielded strain 33, were eaten by some of the cholera patients. Strain 35 was isolated from a cholera patient in Vietnam by Finkelstein et al. (4). Strains 36 to 38 were isolated from cholera patients in Dacca, Bangladesh, by I. Huq. Strains 39 to 44 were isolated from cholera patients in Dacca, Bangladesh, by J. P. Craig. Strain 45 was obtained from J. P. Craig. *P. aeruginosa* strains were clinical isolates from the Texas Department of Health. The *Aeromonas hydrophila* strain was obtained from E. Matthew, Brackenridge Hospital, Austin, Tex.

TABLE 1. *Summary of protease analysis of Vibrio cholerae strains*

Strain	Dimethyl casein protease activity		Ouchterlony reaction[c]	BSA digestion pattern
	U/mg[a]	U/ml[b]		
1. CA401	0.22	0.81	Reference	Reference
2. CA321	0.18	0.65	Not done	Typical[d]
3. CA325	0.32	1.15	+	Typical
4. CA381	0.27	1.09	+	Typical
5. CA383	0.31	1.15	+	Typical
6. CA412	0.21	0.72	+	Typical
7. CA414	0.12	0.42	−	Typical
8. CA415	0.13	0.46	−	Typical
9. CA416	0.13	0.44	−	Typical
10. 7967	0.35	1.15	+	Typical
11. 7969	0.04	0.07	−	Not typable[e]
12. 7972	0.25	0.79	−	Typical
13. 7974	0.02	0.07	+	Not typable[e]
14. 7975	0.28	0.92	−	Not typable[f]
15. 7979	0.22	0.62	+	Typical
16. 7986	0.32	1.04	+	Typical
17. 7990	0.32	1.06	+	Typical
18. 1074-78	0.16	0.51	−	Typical
19. 2634-78	0.13	0.40	+	Typical
20. V40	0.71	1.46	+	Typical
21. V69	0.01	0.28	+	Not typable[e]
22. 1166-77	0.34	0.97	+	Not done
23. 479935	0.28	0.97	+	Typical
24. Lou 4	0.29	1.04	+	Typical
25. Lou 7	0.31	1.09	+	Typical
26. Lou 18	0.30	1.08	+	Typical
27. Lou 15	0.25	0.83	+	Typical
28. Lou 22	0.14	0.48	+	Typical
29. Lou 33	0.14	0.46	+	Typical
30. Lou 17	0.24	0.85	+	Typical
31. Lou 11	0.24	0.83	+	Typical
32. Lou 20	0.26	0.79	+	Typical
33. Lou 13	0.32	1.02	+	Typical
34. Lou 14	0.23	0.77	+	Typical
35. 3083	0.15	0.46	+	Typical
36. RV 47	0.12	0.37	+	Typical
37. RV 53	0.22	0.72	+	Typical
38. RV 59	0.22	0.72	Not done	Typical
39. Q3987	0.12	0.39	+	Typical
40. N15,503	0.23	0.67	+	Typical
41. N15,870	0.21	0.67	−	Typical
42. 17,486	0.12	0.37	−	Typical
43. 16,117	0.19	0.60	−	Typical
44. 27,459	0.24	0.76	+	Typical
45. Suny I	0.04	0.14	−	Typical[g]

[a] Milligrams (dry weight) of cell.
[b] Milliliters of supernatant.
[c] +, Reaction of identity with CA401; −, no reaction.
[d] Typical, Similar to reference
[e] Low degradation of BSA.
[f] High degradation of BSA.
[g] Gave only moderate degradation, but was typable.

Enzyme preparation and assay. Preparation of culture supernatants for the enzyme assay and the dimethyl casein assay for proteolytic activity were done as described previously (12). One enzyme unit equals 1 μmol of $-NH_2$ groups generated per min. The pseudomonas strains were grown in Trypticase soy broth (BBL Microbiology Systems) rather than in syncase.

Peptide digest preparation. The protein diges-

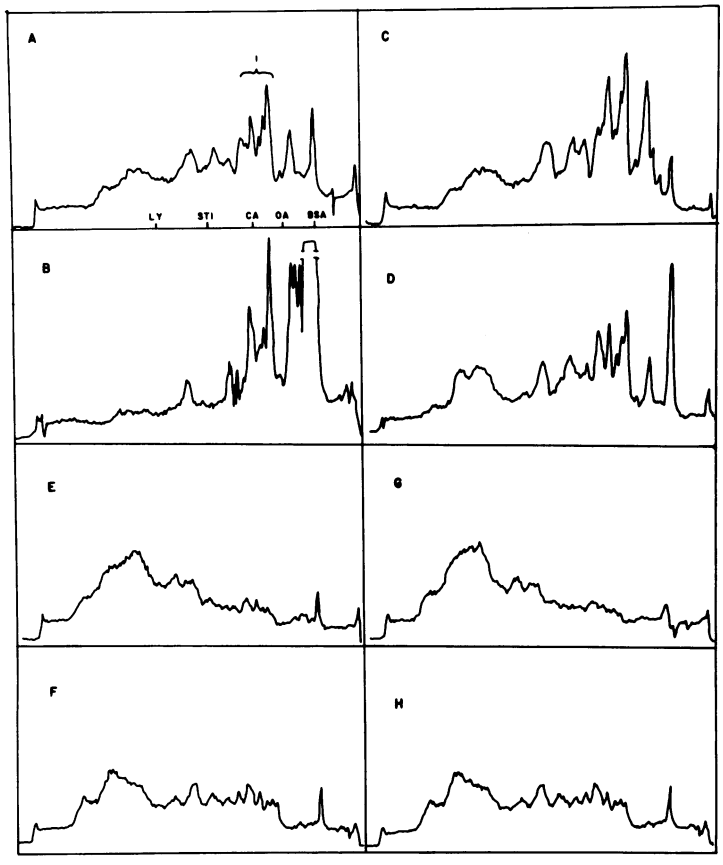

FIG. 2. *Gel scans of BSA digestion time courses. Strains and incubation times: A, CA401, 15 min; B, 7990, 15 min; C, CA401, 30 min; D, 7990, 30 min; E, CA401, 1 h; F, 7990, 1 h; G, CA401, 2 h; H, 7990, 2 h. Migrations of molecular weight (M_r) standards are marked in A: LY, lysozyme, 14,300; STI, soybean trypsin inhibitor, 21,000; CA, carbonic anhydrase, 30,000; OA, ovalbumin, 43,000; BSA, 68,000.*

tion stock consists of 4.0 mg of bovine serum albumin (BSA; Boehringer-Mannheim) per ml dissolved in a solution of 20% (vol/vol) glycerol (Difco Laboratories)–0.5% SDS (Sigma Chemical Co.)–0.1% bromophenol blue (Sigma) in 0.05 M borate buffer, pH 9.0. This digestion stock was heated for 5 min at 100°C and stored at − 20°C. The stock was warmed to 37°C before the addition of the enzyme preparation. A 1:10 dilution of the bacterial culture supernatants in 0.05 M borate buffer, pH 9.0, was used as the enzyme preparation. This enzyme preparation was mixed with an equal volume of the digestion stock (usually 40 or 80 μl) and incubated at 37°C. Incubation times varied as noted in text, but for routine screening experiments, 30-min and 2-h incubations were used and gave satisfactory results. After incubation, the enzyme was inactivated by boiling for 5 min after the addition of 2-mercaptoethanol and SDS to a final concentration of 10 and 1%, respectively. (For example, an 80-μl sample [40 μl of enzyme plus 40 μl of digestion stock] had 10 μl of 2-mercaptoethanol and 10 μl of a 10% SDS solution added before boiling.) Enzyme-treated stocks were then stored at − 20°C until use. They were stable as judged by SDS-PAGE patterns for at least 3 months.

Trypsin (Sigma) and *Streptomyces griseus* type VI protease (Sigma) digestions were done in a similar digestion stock prepared with 0.05 M tris(hydroxymethyl)aminomethane, pH 7.5, with 0.001 M $CaCl_2$ instead of borate buffer. This buffer was also used as a diluent for these enzymes.

SDS-PAGE was carried out in 15% gels essentially as described by Cleveland et al. (1). Electrophoresis was carried out in a vertical slab gel system by using plates measuring 1.5 mm by 10 cm by 15 cm. BSA (32 μg per slot, based on initial concentration by weight) was added. Power settings were 75 mA/gel using a "constant current" mode on an ISCO model 490 power source. Electrophoresis was performed until the tracking dye reached the bottom of the gel. Staining and destaining was performed with Coomassie blue G-250 by the method of Fairbanks et al. (3). Gel scans were done on a Joyce-Loebl model 200 densitometer with a 620-nm filter. Molecular weight standards were from Bio-Rad Laboratories.

Immunological analysis of supernatants.

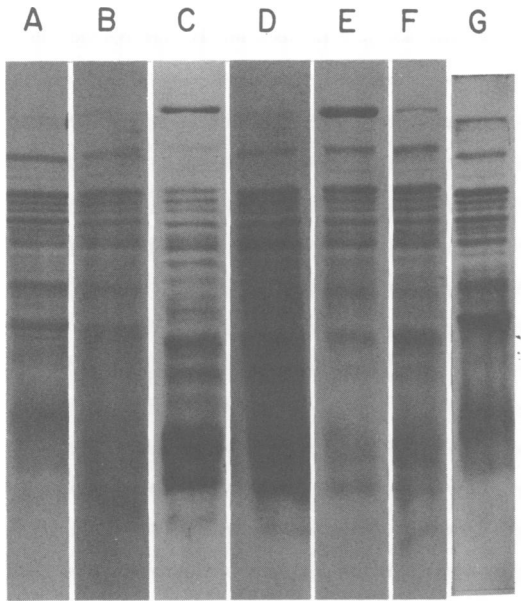

FIG. 3. *SDS-PAGE digestion patterns of various V. cholerae strains. Strains, serotypes, and incubation times: A, CA401, Inaba, 30 min; B, Q3987, Inaba, 2 h; C, RV53, Inaba, 2 h; D, 3083, Ogaba, 2 h; E, 479935, Inaba, 2 h; F, Lou 14, Inaba, 2 h; G, 1074-78, Ogawa, 2 h. The photograph of G was reduced slightly more than the other gels during photoprocessing.*

Ouchterlony gel immunodiffusion (10) was performed by using antiserum prepared against the purified major higher-molecular-weight form of the protease found in CA401 (EFIIA). This antiserum was described in earlier work (Schneider, Ph.D. thesis).

RESULTS

Figure 1 illustrates the specificity of the catalytic typing technique. Trypsin, *S. grieseus*, *V. cholerae*, *P. aeruginosa*, and *A. hydrophila* proteases all showed different peptide banding patterns in SDS-PAGE. Some similarity in banding patterns is evident in the last three organisms.

The importance of incubation time in this analysis is shown in the gel scans in Fig. 2. Some *V. cholerae* strains produced such high levels of degradation that only a smear in the 10,000- to 20,000-dalton range was evident at 2 h. No good comparison between strains was possible. By following the degradation with time, however, the complete identity of the degradation patterns of two strains could be seen. CA401 at 15 min (Fig. 2A) and 7990 at 30 min (Fig. 2D) are practically superimposable. Particularly striking in *V. cholerae* supernatant digests were the four peaks in the 29,000- to 35,000-dalton range (Fig. 2A, bracket 1). For routine screening of strains, 30-min and 2-h incubation periods were used.

The widespread presence of the peptide cleavage pattern of strain CA401 in *V. cholerae* isolates is shown in Fig. 3 and Table 1. Most strains produced proteolytic activity as measured by the dimethyl casein assay. Isolates from both Eastern and Western hemispheres of both Ogawa and Inaba serotypes show very similar peptide cleavage patterns. Of the 44 strains tested, 39 gave patterns similar to that of strain CA401. Of the five remaining strains, three (7969, 7974, and V69) did not produce sufficient levels of degradation to give a digest pattern, and one (7975) degraded the BSA too extensively for the peptide digest pattern to be determined (time course analysis was not performed). One strain (1166-77) was not tested.

Of the 39 strains giving the typical digest pattern, 28 reacted with immunological identity with strain CA401 when antiserum prepared against purified strain CA401 enzyme was used. Nine strains did not react (one strain was not tested). Of these nine, only one strain, Suny I, showed low (<0.15 U/ml) levels of enzyme.

Of the three low degradation strains from the five in the group not typable by BSA digest analysis, two (7974 and V69) were immunologically reactive, and one (7969) was not. Of the two strains producing high levels of degradation, one (V40) reacted immunologically, and one (7975) did not.

DISCUSSION

This study describes a simple method for determining the relative specificity of microbial proteases based upon the unique peptides generated by cleavage of a given protein. It is simple to perform and is readily reproducible. We have successfully used this technique with other proteins (rabbit aldolase and human immunoglobulin A) and also under nondenaturing conditions (absence of SDS in the digestion stock) (unpublished results).

The apparent widespread presence in *V. cholerae* isolates of protease similar to that found in strain CA401 is interesting. Previous work from this laboratory has suggested that the protease in strain CA401 is required for virulence and may be involved in either maintenance or growth of the organism in the gut. The frequent occurence of the enzyme in both environmental and clinical isolates may suggest that it plays a role in maintenance of *V. cholerae* in other environments as well as in the gut.

The failure of some strains to react immunologically despite positive peptide digest patterns identical to that of CA401 may be ex-

plained in several ways. It is known that the enzyme is very unstable in culture supernatants (Schneider and Parker, unpublished data) and may lose virtually all activity in a matter of hours at room temperature. The prolonged incubation required for the gel diffusion technique may mean that in these strains the protease may lose antigenicity (perhaps by degradation) along with activity. Alternatively, the sensitivitiy of the gel diffusion technique is not high. If such strains produce protease of significantly higher specific activity than that of CA401 protease, they may possess equivalent enzymatic activity but insufficient antigenicity to react in the Ouchterlony test. It is also possible that some strains of *V. cholerae* may possess an enzyme that is catalytically similar but serologically dissimilar.

The few strains that reacted immunologically but had low dimethyl casein protease and digestion activity are particularly interesting. These may be strains which produce inactive precursor enzyme (zymogen). Alternatively, they may produce enzymatically inactive, but still antigenic, degraded enzyme.

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