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Dual-Substrate Plate Diffusion Assay for Proteases

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A plate diffusion assay for endopeptidases was developed. Proteases applied to plates containing 1% casein, 1% gelatin, and 1.5% agar caused distinct zones reminiscent of immunoprecipitation bands. The diameter of the zones was linearly proportional to the log of the enzyme activity applied over a range from 0.01 to greater than 100 IU/ml.

Research on the physiology of *Clostridium botulinum* grown at suboptimal pH (T. J. Montville, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, P7, p. 206) suggests that the activity of the extracellular proteases, which play an important role in the detection of botulinal toxin, may be dependent on the culture conditions used. Investigation of this area was hampered by the lack of a suitable protease assay. DasGupta and Sugiyama (4), in their isolation and characterization of a botulinal protease, used the liberation of *p*-nitroaniline from amino acid *p*-nitroanilides (5) as a protease assay. The need to analyze a large number of samples and to conduct the assays in an isolation chamber rendered this method unsuitable for my use. Methods which measure the increase in the amount of amino acids not precipitated by trichloroacetic acid (9, 13) were considered but were deemed unacceptable because of the high toxicity of the botulinal toxin which would be concentrated in the precipitate. A fluorescent assay for proteases (15) would carry active botulinal toxin through the assay procedure, possibly exposing laboratory workers to an unacceptable hazard.

The need for a method which required a minimum of sample handling, was sensitive and accurate over a large range of protease concentrations, and yet could be done in an anaerobic chamber containing a minimum amount of instrumentation led to the investigation of plate diffusion assays as a possible solution. Detection of enzymes such as amylase and gelatinase in agar plates is used as a systematic tool (6). Plate diffusion tests are also used to determine antibiotic activity (3), to screen for novel bacterial phenotypes (10), and as a rapid method for the dosimetry of mutagens (1). A qualitative plate diffusion assay for the detection of proteases in low-fat dairy products has recently been reported (7).

This paper reports a simple, quantitative, dual-substrate diffusion assay for proteases that is sensitive, linear over four logs of enzyme concentration, and is useful for examining a variety of bacterial endopeptidases.

MATERIALS AND METHODS

Protease assay plates containing 1% casein, 1% gelatin, or 1% casein and 1% gelatin in a solution of 1.5% agar (Difco Laboratories) were prepared. Because direct addition of casein to the media resulted in macroscopic casein aggregates and plates with poor readability, the casein was dissolved in 0.02 N NaOH and stirred until it formed a translucent solution. The other compounds were added, and the media were adjusted to pH 7.0 with 1 N HCl. The media were sterilized (121°C for 15 min), tempered at 60°C, and dispensed in 20-ml portions into sterile gridded square petri dishes (100 by 15 mm; Falcon Plastics). After the agar solidified, wells were cut into the plates with a sterile no. 2 cork borer.

The sensitivity, linearity, and readability of the different protease detection media were determined by using the protease standards described in Table 1. These protease solutions were freshly prepared in distilled water at a concentration of about 100 IU/ml. They were sterilized by filtration through a 0.45-μm membrane and diluted in sterile water to various concentrations down to approximately 0.01 IU/ml.

Wells on duplicate plates containing either substrate or both substrates were filled with 35 μl of protease standards or supernatants from 48-h cultures of *Bacillus cereus* or *C. botulinum* cultured in Trypticase soy broth (BBL Microbiology Systems) or cooked meat medium (Difco), respectively. The plates were incubated at 30°C.

The diameters of the zones formed were measured at predetermined incubation times. Plots of zone diameter (in millimeters) versus the log of the enzyme activity were constructed and subjected to linear regression analysis by the method of least mean squares.

RESULTS AND DISCUSSION

Within 3 h of incubation, the casein-gelatin plates developed precipitation zones indicative of enzyme activity when levels as low as 0.1 IU/ml were applied. By 24 h of incubation, these zones were large enough to allow quantitation of proteolytic activity as low as 0.01 IU/ml. Representative results are shown in Fig. 1. These

TABLE 1. Description of enzymes used

Enzyme	Information	Source
Protease type IX	From *B. polymyxa*	Sigma[a]
Protease type XII	EC 3.4.23.6 from *Aspergillus saitoi*	Sigma
Protease type IV	From *Streptomycetes caespitosus*	Sigma
Subtilopeptidase A	EC 3.4.21.14	Sigma
Papain type IV	EC 3.4.22.2	Sigma
Trypsin	EC 3.4.21.4	Worthington[b]
Carboxypeptidase A	EC 3.4.12.2	Worthington
Carboxypeptidase B	EC 3.4.12.3	Worthington

[a] Sigma Chemical Co., St. Louis, Mo.
[b] Worthington Diagnostics, Freehold, N.J.

distinct, well-delineated zones were in contrast to those formed on plates containing only gelatin or casein. On plates containing only gelatin, clear zones could be seen under oblique illumination, but these were smaller and developed later than the zones on the casein-gelatin plates. The *Bacillus polymyxa* protease (Fig. 1, row 3) was the only protease tested that gave a strong reaction on plates containing casein as the sole substrate. The other endopeptidases gave relatively smaller, diffuse zones of precipitation on the casein plates at high concentrations of ap-

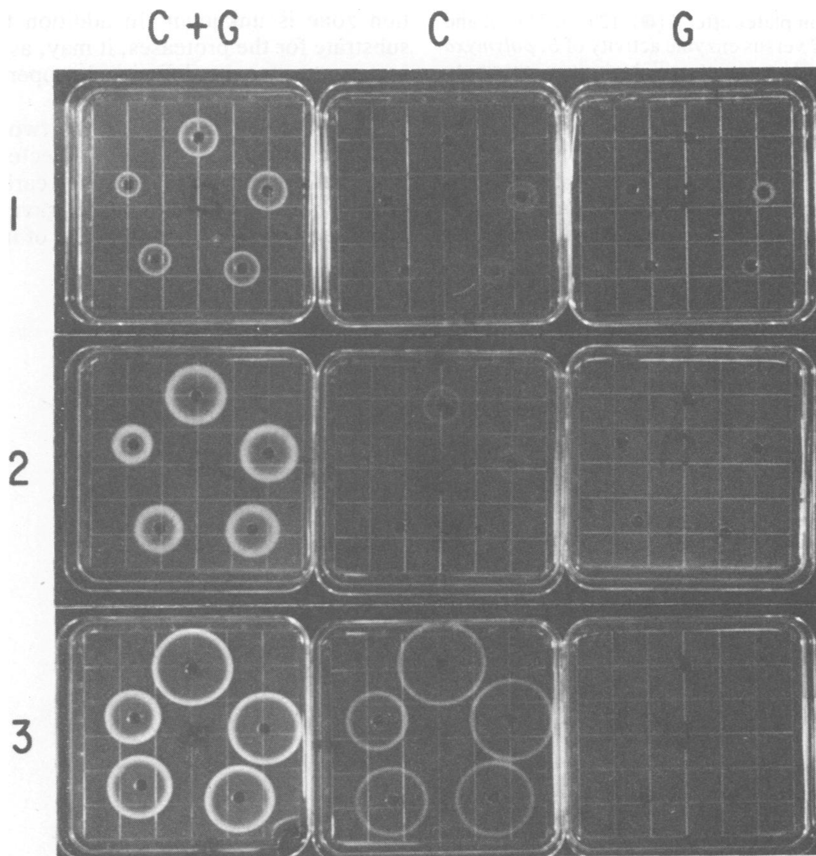

FIG. 1. Zones formed on plates containing casein (C), gelatin (G), or casein plus gelatin after 24 h of incubation with papain (EC 3.4.22.2) at 180, 90, 36, 18, and 3.6 IU/ml (clockwise from top) (row 1); *A. saitoi* protease (EC 3.4.23.6) at 32, 16, 6.4, 3.2, and 0.64 IU/ml (row 2); and *B. polymyxa* protease at 165, 82, 33, 16, and 3.3 IU/ml (row 3).

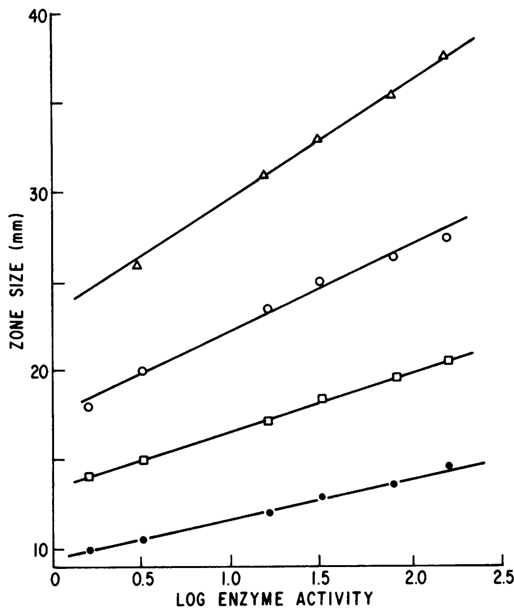

FIG. 2. Dose-response curves for zone size formed on casein-gelatin plates after 4 (●), 12 (□), 24 (○), and 48 (△) h at 30°C versus enzyme activity of *B. polymyxa* protease. A 1-IU amount will hydrolyze casein to produce 1.0 μmol of tyrosine per min.

plied activity. These hazy zones appeared similar to those observed by Hartman and Persson (7), who used casein as the sole substrate for enzymatic activity. Casein-gelatin plates could detect levels of enzyme activity that were 10- to 100-fold lower than the lowest detectable level on plates containing only one of the substrates.

Distinct precipitation zones, similar to those observed here, have been reported in studies on the action of rennet on casein. Cheeseman (2) suggested that these distinct zones might be due to a casein-agar interaction. The results in Fig. 1 for plates containing only casein and agar argue against this hypothesis. The formation of insoluble precipitates during casein hydrolysis has been investigated by Lawrence and Creamer (11), who demonstrated that the hydrolysis of κ-casein yields insoluble *para*-κ-casein. They suggested that the conversion of soluble κ-casein to insoluble *para*-κ-casein is due to the scission of a single bond and that continued hydrolysis results in the resolubilization of the *p*-κ-casein. This is consistent with the observation herein that a clear area developed within the area encircled by the precipitation zone on the casein-gelatin plates. The reason that gelatin sharpens and increases the size of the precipitation zone is unknown. In addition to being a substrate for the proteases, it may, as a solidifying agent, alter the diffusional properties of the assay plate.

The proteolytic activity of the two exopeptidases examined was not easily detected, even on the casein-gelatin plates. Neither carboxypeptidase A nor carboxypeptidase B gave any reaction after 24 h at 30°C. After 48 h of incubation,

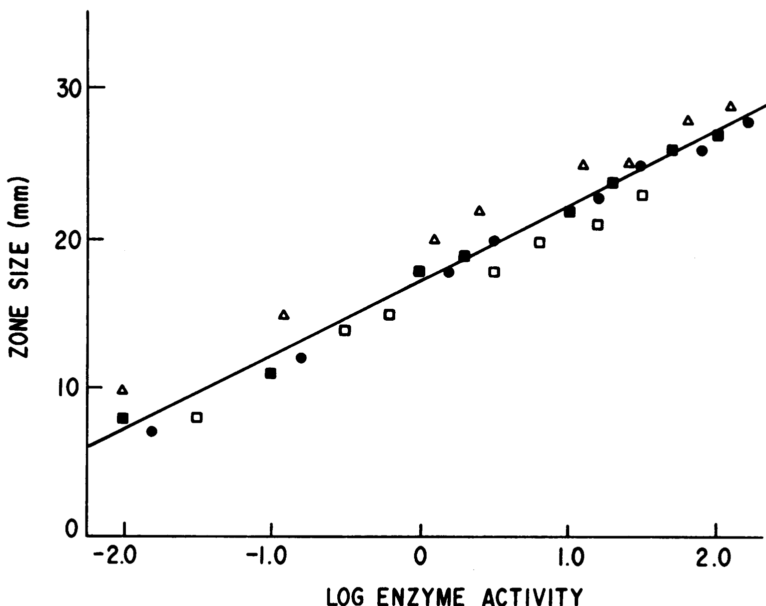

FIG. 3. Dose-response curves of proteases after 24 h at 30°C Symbols: ●, protease type IX; □, protease type XII; ■, protease type IV; △, subtilopeptidase A.

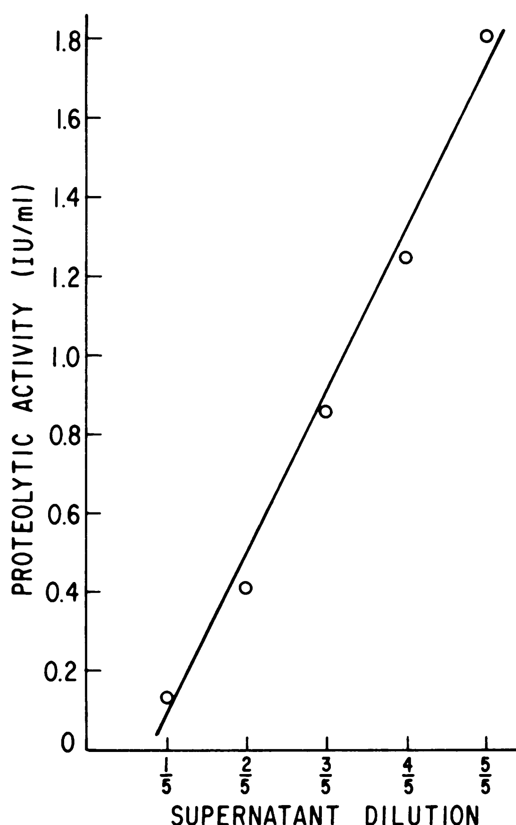

FIG. 4. Determination of proteolytic activity versus dilution for a supernatant from a 48-h cooked meat culture of *C. botulinum* 62A.

hazy zones could be detected only for carboxypeptidase A when activities as low as 1.7 IU/ml were applied. Even at higher activities, these zones were too diffuse to allow the measurement of zone size and quantitation of proteolytic activity. Obviously, one would not expect proteolysis from the carboxy terminus of a protein inward to yield insoluble *p*-κ-casein.

The reports previously mentioned (2, 7, 11) used dilutions of commercial proteases, which makes it impossible to determine the sensitivity of the methods. Most of the endopeptidases tested in this report could be detected at levels of about 0.01 IU/ml. The response between the zone diameter in millimeters and the log of the enzyme activity was found to be linear ($r > 0.98$) for all of the endopeptidases in Table 1. Representative results are shown in Fig. 2. The dose-response curves for *B. polymyxa* protease were linear ($r = 0.99$) regardless of when the zones were measured. However, as the incubation time of the casein-gelatin plates was increased, not only did the absolute zone size become larger, but the slope of the zone size versus log enzyme concentration curve increased, thereby improving the sensitivity of the assay. The other proteolytic standards gave similar results. Cheeseman (2) found a similar dose-response relationship, but with a shallow slope, which made his assay too insensitive to be useful. A microslide modification (12) of his method gives slopes approaching those shown in Fig. 2 at 24 and 48 h. In addition to being slightly less sensitive, the microslide method is more complex and can assay only one sample per slide. Up to 20 samples per plate could be assayed by using the dual-substrate plate diffusion method.

Limited studies with the dual-substrate assay indicate that the dose-response is independent of the bacterial protease being assayed. Figure 3 demonstrates that four of these protease standards could be plotted on the same curve of zone size versus log enzyme activity and coregressed with a correlation coefficient of 0.97. *B. cereus* and *C. botulinum* supernatants were diluted and assayed for proteolytic activity with *B. polymyxa* protease as a standard. The measured proteolytic activity was linearly correlated ($r = 0.99$) with the dilution factor for both the *C. botulinum* (Fig. 4) and *B. cereus* (data not shown) proteases. Supernatants which had been boiled for 10 min did not cause zones to form. In a more extensive study, Lawrence and Sanderson (12) found that a mechanistically similar assay could be used effectively with the proteases of 100 randomly selected microorganisms.

Although other bacterial proteases having different pH optima, temperature optima, or cofactor requirements might yield dose-response curves different from the protease standards, the assay as described is well suited for the determination of gross proteolytic activity in cultures in which the natures of the proteases are unknown. If the pH and temperature optima for a protease of particular interest are known, the assay can be easily modified accordingly. Effects from small-molecular-weight inhibitors and culture pH should be minimal because the sample volume (35 μl) is small compared to the volume of the assay plate (20 ml). In addition, these inhibitors would rapidly diffuse away from the much larger protease molecules.

The results of a time course experiment, in which the zone sizes of protease standards and undiluted *B. cereus* and *C. botulinum* culture supernatants were measured after 4, 7, 12, 24, 30, and 48 h of incubation, are presented in Fig. 5. Although the zone size versus time plots are curvilinear, the culture supernatant curves follow the curves of protease standards with similar proteolytic activity. Thus, the length of incubation does not effect the validity of the assay, provided that the unknown bacterial protease is assayed against a protease standard curve gener-

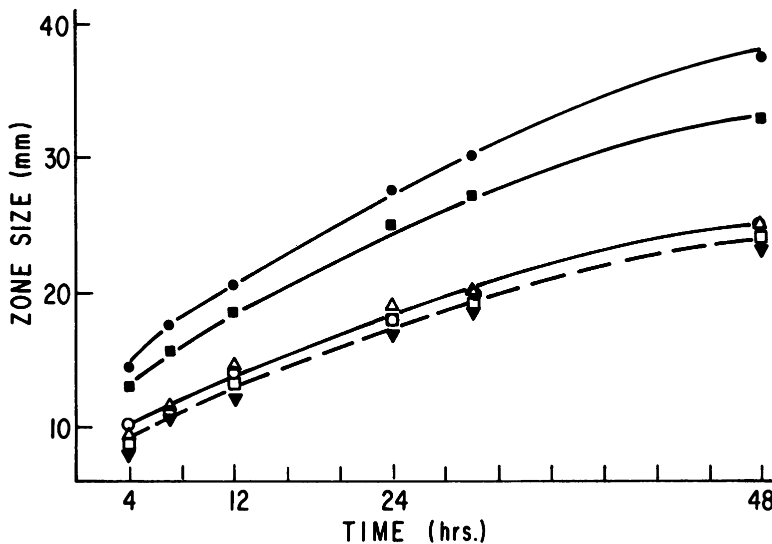

FIG. 5. The effect of incubation time (30°C) on the zone size formed by different protease concentrations. Symbols: ●, ■, and ○, *B. polymyxa* protease at 165, 33, and 1.6 IU/ml, respectively; □, *Streptomycetes caespitous* protease at 1.0 IU/ml; △, and ▼, supernatants from *C. botulinum* and *B. cereus* (assayed at 1.8 and 0.8 IU/ml, respectively).

ated under the same conditions and read at the same time. In Fig. 5, the spread between curves for samples with low and high proteolytic activity increased with time. This reflects the increased sensitivity of the assay at longer incubation times, as shown in Fig. 2.

This assay is being used to study the effect of culture media on botulinal proteolytic activity. For example, using protease type IX (Sigma Chemical Co.) as a standard and reading the plates at 24 h, I have found that six proteolytic strains of *C. botulinum* types A and B had protease activities of more than 1.0 IU/ml when cultured in cooked meat medium (Difco) but less than 0.1 IU/ml when cultured in botulinum assay medium (8, 14). These results will be reported more fully in a separate communication.

It is expected that, with minor modifications to suit individual requirements, the casein-gelatin plate diffusion assay will be useful in a variety of analytical, food, and pharmaceutical applications.

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