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Detection of Microbial Proteolytic Activity by a Cultivation Plate Assay in Which Different Proteins Adsorbed to a Hydrophobic Surface Are Used as Substrates

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A screening technique for microbial proteases, the thin-layer enzyme assay cultivation technique, was developed. The inner surface of a polystyrene petri dish was coated with protein and then covered with a culture agar medium. The enzymes, produced during growth of the microorganisms, reach the protein-coated surface by diffusion in the agar. Degradation of the protein was visualized by condensation of water vapor on the surface after removal of the agar medium. The wettability of the enzyme-affected protein-coated polystyrene surface was decreased compared with the unaffected protein surface. Enzyme substrates used were fibrinogen, immunoglobulin G, egg albumin, human serum albumin, bovine serum albumin, hemoglobin, mucin, and gelatin. It was possible to use a variety of culture agar media, nonselective as well as selective, in the assay. The technique provides a sensitive, convenient, and inexpensive method for screening various microbial proteases. In addition, the technique can be used for screening proteolytic enzyme activity of specific microbial species in a mixed microbial sample as well as for studies of factors that influence the cultivation conditions for protease production and activity.

Plate assays that use the radial diffusion in agar gel technique have been found to be sensitive and convenient for quantitative determination of enzyme activity (5, 23, 24, 27–30, 35). Degradation of the enzyme substrate, incorporated in the agar gel, is seen as the development of a transparent zone in the gel.

Enzyme substrates have also been incorporated in solid culture agar media (7, 31–33). The enzymes produced by the microorganisms during growth, when cultivated on the surface of the medium, diffuse into the agar and make transparent zones around the colonies. Such assays allow principally qualitative determinations of the enzyme activity. Determination of enzyme activity on substrates incorporated in a culture medium requires the choice of a transparent medium on which optimal conditions for microbial growth, enzyme production, and enzyme activity cannot always be obtained. Moreover, a relatively high concentration of enzyme substrate in the agar gel is required.

It has previously been shown that proteins adsorbed to a polystyrene surface can be used as substrates for proteolytic enzymes (38). The wettability of the protein-coated surfaces was decreased after degradation of the proteins (38). In the assay developed, the thin-layer enzyme assay (TEA), the decreased wettability is visualized by condensation of water vapor on the surfaces (11). Compared with conventional plate assays, the substrate consumption is low (37).

TEA can be combined with the radial diffusion in agar gel technique (37). With this technique the gel is removed before the effect of the enzyme activity is visualized, which means that the opacity of the gel does not influence the results.

Accordingly, TEA should be well suited for screening microbial proteases produced during growth on a culture agar medium applied over protein-coated surfaces. Furthermore, it should be possible to use culture media of different compositions. The aim of the present study was to test these hypotheses.

MATERIALS AND METHODS

Microorganisms. *Pseudomonas aeruginosa* (EF551) and *Neisseria perflava* (EF11760) were kindly provided by E. Falsen (Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden). *Haemophilus parainfluenzae* (GH443), *Staphylococcus aureus* (GH4), *Staphylococcus epidermidis* (GH7), *Candida albicans* (GH11), and *Veillonella parvula* (H3) were isolated from oral samples in this laboratory. Other bacteria used were *Streptococcus faecalis* (Strep-MC4), *Peptostreptococcus anaerobius* (P-strep-MC5), *Fusobacterium necrophorum* (Fus-MC4) (12), *Streptococcus mutans* (B13) (9), *Lactobacillus*

casei (703) (Centre International de Distribution de Souches et d'Information sur les Types Microbiens, Lausanne, Switzerland), *Streptococcus sanguis* (ATCC 10556; American Type Culture Collection, Rockville, Md.), *Actinomyces viscosus* (ATCC 15987), *Propionibacterium acnes* (ATCC 11828), and *Bacteroides asaccharolyticus* (ATCC 25260). The microorganisms were selected to represent various species with different nutrition and incubation requirements as well as different abilities to produce proteolytic enzymes, according to *Bergey's Manual for Determinative Bacteriology* (3).

Culture agar media. The following nonselective media were used: HCMG agar according to Möller (22) (anaerobically prepared and sterilized with 0.05% $CaSO_4$ [wt/vol]; glucose concentration reduced to 0.05% [wt/vol]), blood agar (blood agar base no. 2, 0696 [Difco Laboratories, Detroit, Mich.], with 5% [vol/vol] defibrinated horse blood), and brucella agar (brucella agar 11086 [BBL Microbiology Systems, Cockeysville, Md.], with 2% [wt/vol] agar 0140 [Difco], 5% [vol/vol] defibrinated horse blood, 2% [vol/vol] hemolyzed erythrocytes, and 0.01% [wt/vol] menadione). The selective media used were staphylococcus agar (staphylococcus medium 110, 0297 [Difco]), enterococcus agar (12205 [BBL]), mitis salivarius agar (0298 [Difco]), mitis salivarius-bacitracin agar (15), veillonella agar (0917 [Difco] with 8 ppm [wt/vol] of vancomycin [26]), Rogosa SL agar (4080 [Difco]), actinomyces agar (18), Drigalski agar (G. Vahlne, Ph.D. thesis, University of Lund, Lund, Sweden, 1945), and Sabouraud-dextrose agar (0109 [Difco]). Tellurite-hematin agar and hematin agar were both prepared according to Falsen (personal communication). The tellurite-hematin agar consisted of brain heart infusion agar (0418 [Difco]) with 8% [vol/vol] horse blood and 0.4% [wt/vol] potassium tellurite added. The hematin agar consisted of chocolate agar, prepared as described by Cowan (6) and with the addition of 5% [vol/vol] horse blood and 1.5 ppm [wt/vol] of gentian violet.

Enzyme substrates. Human serum albumin (HSA; fraction V; Sigma Chemical Co., St Louis, Mo.), bovine serum albumin (BSA; fraction V; Sigma), hemoglobin (type II; Sigma), egg albumin (Kebo-Grave, Stockholm, Sweden), gelatin (Fisher Scientific Co., Fair Lawn, N.J.), and mucin (type II; Sigma) were each dissolved in distilled water to 10 g/liter. Fibrinogen and immunoglobulin G (IgG) (both obtained from Kabi, Stockholm, Sweden) were dissolved in distilled water to 1 g/liter.

TEA-diffusion technique. The TEA-diffusion technique has been described in detail previously (37). According to this method, the inner surface of a polystyrene petri dish is coated with a protein (38) and then covered with an agar gel. The enzyme solution is applied to wells punched in the gel. After incubation at 36°C for 18 h, the agar is removed and the surface is rinsed with distilled water and thoroughly dried with compressed air. Enzyme degradation of the protein is indicated by a decreased wettability of the protein-coated surface (38). This is visualized by condensation of water vapor on the surface, simply performed by placing the dish upside down for about 1 min on another dish filled with water at 50°C. The diameters of the zones with decreased wettability are measured. The TEA-diffusion technique was used in this study for determination of the sensitivity of the enzyme substrates to pronase (pronase P, type VI, from *Streptomyces griseus*; Sigma) and of the effect of microbial culture media on the activity of pronase. The pronase was diluted in 9 mM sterile "universal buffer" (2) at pH 7.8. Difco agar (0140) was used as the diffusion agar unless otherwise stated.

TEA-cultivation technique. The protein-coated surface was covered with a solid microbial culture medium. A 7-ml portion of the medium, adjusted to about 40°C, was used for one petri dish, 90 mm in diameter. One to four microbial strains were inoculated on each dish. When a heavy growth had been obtained (Fig. 1a), the culture medium was removed. The surface was then rinsed, dried, and exposed to water vapor as described above for the TEA-diffusion technique (Fig. 1b).

Tube assay for detection of gelatinolytic activity. The microorganisms were incubated for at least 14 days in tubes containing gelatin and a nutritive broth (17).

RESULTS

Sensitivity of the adsorbed proteins to pronase determined with the TEA-diffusion technique. The sensitivity of the proteins used as substrates was tested with pronase, a microbial enzyme preparation with quantitatively known activity. The protein most susceptible to digestion was gelatin (Fig. 2). Pronase at 0.2 mU/ml gave an enzyme-affected zone with a diameter of 16 mm. Other susceptible proteins, in order of decreasing sensitivity, were HSA, hemoglobin/BSA, egg albumin, IgG, and fibrinogen. When the diameters of the enzyme-affected zones were plotted against the enzyme concentrations in a semilogarithmic diagram, a linear relationship was obtained (Fig. 2). The mucin-coated surfaces showed differences in wettability between pronase-affected and unaffected surfaces that were too small to make reliable measurements of the diffusion zones possible.

Influence of microbial culture media on pronase activity. The culture agar media used for cultivation of the microorganisms, as well as some growth-stimulating medium components, were tested for influence on pronase activity with the TEA-diffusion technique (37). Thus, culture agars were tested by using them as diffusion agars. The growth-stimulating components were added to agar at concentrations commonly used in culture media (Fig. 3). The results were compared with those obtained with agar without any additives as the diffusion medium. BSA was used as the enzyme substrate.

The culture agar media all reduced the enzyme-affected zones (Fig. 4). These were substantially decreased with blood, brucella, tellurite-hematin, hematin, and Sabouraud agars. The enzyme-affected zones were also clearly decreased, although to a lesser extent, with HCMG, actinomyces, staphylococcus, mitis salivarius, and mitis salivarius-bacitracin agars.

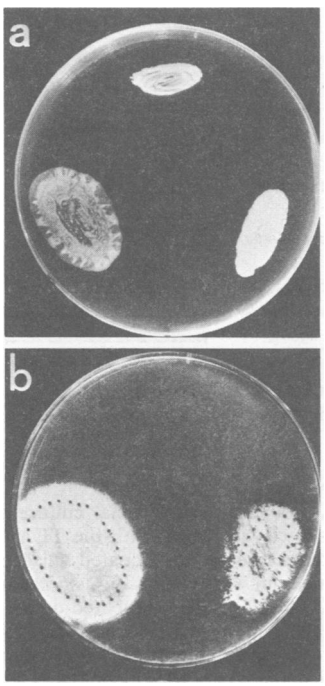

FIG. 1. (a) *Pseudomonas aeruginosa* (EF551) (left), *L. casei* (703) (top), and *Staphylococcus aureus* (OH4) (right) cultivated on HCMG agar applied over a BSA-coated polystyrene surface. (b) Proteolytic activity made visible by water vapor condensation on the BSA-coated surface after removal of the nutrition agar. Decreased wettability, indicating a degradation of the protein, is seen as whitish spots. The area for bacterial growth is shown by dotted lines. As can be seen *Pseudomonas aeruginosa* and *Staphylococcus aureus* organisms show proteolytic activity whereas, *L. casei* organisms do not.

The smallest reductions were obtained with enterococcus, veillonella, and Drigalski agars.

Rogosa agar decreased the wettability of the protein-coated polystyrene surfaces and thus could not be used in combination with TEA. The condensation pattern of the protein-coated surfaces that had been covered with Rogosa agar was clearly different from the enzyme-affected protein-coated surfaces.

Growth-stimulating components had varied effects on the apparent pronase activity as visualized by water vapor condensation (Fig. 3). Defibrinated horse blood, inactivated horse serum, liver extract (22), and potato juice (22) (anaerobically prepared) decreased the diameters of the enzyme-affected zones. Sodium oleate, however, increased the zones. Almost no effect on detected pronase activity was obtained with glucose, yeast autolysate (22), or horse erythrocytes in the agar.

Microbial proteolytic activity detected with the TEA-cultivation technique. Strains of 16 microbial species were tested for proteolytic activity on the adsorbed proteins. Each bacterial strain was cultivated on two different nonselective media and also on selective media. The *C. albicans* strain was cultivated on one nonselective medium and one selective medium. The results are given in Table 1. Strains of species known to be proteolytic, e.g., *Staphylococcus aureus* and *Pseudomonas aeruginosa*, degraded the proteins used as substrates when cultivated on both nonselective and appropriate selective media. The detection of proteolytic activity of *V. parvula* and *L. casei* strains, both belonging to species supposed to be nonproteolytic, was dependent on the enzyme substrate and culture medium used. No proteolytic activity was detected in *A. viscosus, H. parainfluenzae*, and *C. albicans* strains.

Gelatin, the protein most susceptible to pronase (Fig. 2), was degraded by all strains for which proteolytic activity was revealed. BSA, but not hemoglobin, was degraded by the *Peptostreptococcus anaerobius* strain despite an almost equal susceptibility to pronase. IgG, but not egg albumin, was degraded by the *V. parvula* strain. Both of these strains and the *N. perflava* strain showed proteolytic activity on fibrinogen-coated surfaces (the most pronase-resistant substrate) but not on egg albumin-coated surfaces.

The reducing effect of blood, serum, blood agar, and brucella agar on pronase activity is shown in Fig. 3 and 4. *V. parvula* and *F. necrophorum* strains showed proteolytic activity

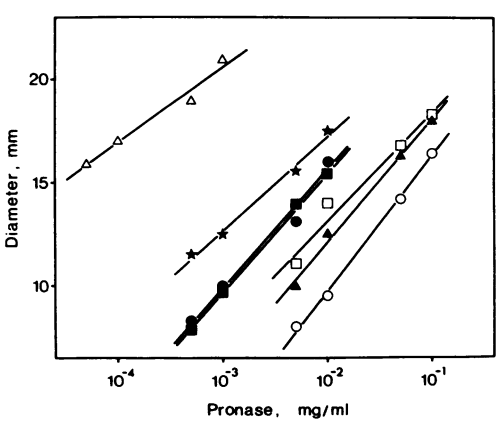

FIG. 2. Susceptibility of proteins to degradation by pronase tested with the TEA-diffusion in agar gel technique. The dishes were incubated with enzyme for 18 h at 36°C. The mean values (n = 4; difference between respective minimum and maximum values, ≤1 mm) of the diameters of the enzyme-affected zones were plotted against the logarithmic values of the enzyme concentrations. The correlation coefficients ranged between 0.98 and 1.0. The protein substrates were gelatin (△), HSA (★), BSA (●), hemoglobin (■), egg albumin (□), IgG (▲), and fibrinogen (○).

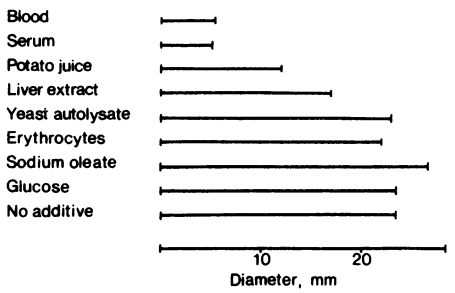

FIG. 3. Influence of microbial growth-stimulating nutritive medium components on pronase activity tested with the TEA-diffusion in agar gel technique. The components were added to agar at concentrations commonly used in cultivation media: 5% (vol/vol) blood, serum, liver extract, yeast autolysate, 5% (wt/vol) glucose, 2% (vol/vol) potato juice, 1% (vol/vol) erythrocytes, and 0.01% (wt/vol) sodium oleate. BSA was used as enzyme substrate. Pronase diluted to 100 μl/ml was incubated on the dishes for 18 h at 36°C. The mean values (n = 4; difference between respective minimum and maximum values, ≤1 mm) of the diameters of the enzyme-affected zones are given.

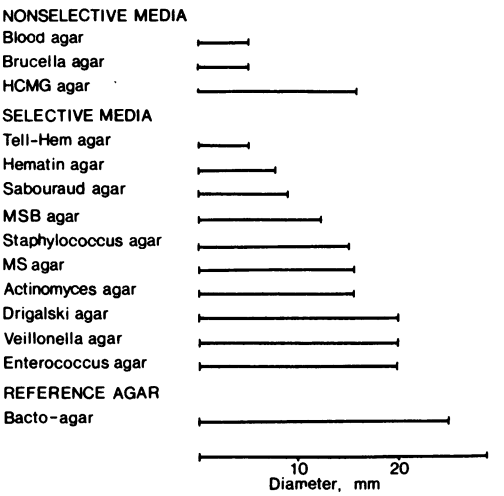

FIG. 4. Influence of microbial culture agar media on pronase activity tested with the TEA-diffusion in agar gel technique on BSA-coated surfaces. The respective medium was used as diffusion agar. Pronase, diluted to 100 μg/ml, was incubated on the dishes for 18 h at 36°C. The mean values (n = 3; difference between respective minimum and maximum values ≤1 mm) of the diameters of the enzyme-affected zones are given. Tell-Hem, Tellurite-hematin; MS, mitis salivarius; MSB, mitis salivarius-bacitracin.

when cultivated on HCMG agar (without blood and serum), but not when brucella agar was used. In contrast, proteolytic activity was detected for the *N. perflava* strain when cultivated

TABLE 1. Proteolytic activity detected on various proteins with the TEA-cultivation technique and on gelatin with the tube assay

Strain	Cultivation agar medium	TEA-cultivation technique								Tube assay (Gelatin)
		Fibrinogen	IgG	Egg albumin	Hemoglobin	BSA	HSA	Mucin	Gelatin	
Staphylococcus aureus	HCMG	+	+	+	+	+	+	+	+	+
	Blood	+	+	+	+	+	+	+	+	
	Staphylococcus	+	IW[a]	+	+	+	+	+	+	
S. epidermidis	HCMG	−	IW	−	+	+	+	+	+	−
	Blood	+	+	+	+	+	+	+	+	
	Staphylococcus	−	IW	+	+	+	+	+	+	
Streptococcus faecalis	HCMG	+	+	+	+	+	+	+	+	+
	Blood	+	+	−	+	+	+	+	+	
	Enterococcus	+	+	+	+	+				
	Tellurite-hematin	+	+	+	+	+	+	+	+	
S. sanguis	HCMG	−	−	−	+	+	+	+	+	+
	Brucella	IW	−	−	+	+	+	+	+	
	Mitis salivarius	−	−	−	−	+	+	+	+	
S. mutans	HCMG	−	−	−	−	−	−	+	−	−
	Brucella	−	−	−	−	−	+	+	+	
	Mitis salivarius-bacitracin	IW	IW	IW	IW	IW	IW	IW	IW	
Peptostreptococcus anaerobius	HCMG	−	−	−	−	+	+	+	+	−
	Brucella[b]	+	−	−	−	+	+	+	+	

TABLE 1—*Continued*

Strain	Cultivation agar medium	TEA-cultivation technique								Tube assay (Gelatin)
		Fibrinogen	IgG	Egg albumin	Hemoglobin	BSA	HSA	Mucin	Gelatin	
N. perflava	HCMG	–	–	–	–	–	–	–	–	–
	Blood	+	+	IW	IW	IW	IW	IW	+	
	Hematin	–	–	–	–	–	–	–	–	
V. parvula	HCMG	–	–	–	–	–	+	+	+	–
	Brucella	–	–	–	–	–	–	–	–	
	Veillonella	+	+	–	–	–	+	+	+	
L. casei	HCMG	–	–	–	–	–	–	–	+	–
	Blood	–	IW	IW	–	–	–	–	–	
A. viscosus	HCMG	IW	IW	IW	IW	IW	IW	IW	IW	–
	Brucella	–	–	–	–	–	–	–	–	
	Actinomyces[c]									
Propionibacterium acnes	HCMG	+	+	+	+	+	+	+	+	+
	Brucella	+	+	+	+	–	+	+	+	
Pseudomonas aeruginosa	HCMG	+	+	+	+	+	+	+	+	+
	Blood	+	+	+	+	+	+	+	+	
	Drigalski	+	+	+	+	+	+	+	+	
H. parainfluenzae	HCMG[c]									
	Brucella	–	–	–	–	–	–	–	–	–
	Hematin	–	–	–	–	–	–	–	–	
F. necrophorum	HCMG	+	+	+	+	+	+	+	+	+
	Brucella	–	–	–	–	–	–	–	–	
B. asaccharolyticus	HCMG	+	+	+	+	+	+	+	+	+
	Brucella	+	+	+	+	+	+	+	+	
C. albicans	HCMG	–	–	–	–	–	–	–	–	–
	Sabouraud	IW	IW	IW	IW	IW	IW	IW	IW	

[a] IW, Increased wettability.
[b] Without and with sodium oleate (0.01%, wt/vol).
[c] No growth.

on blood agar but not on HCMG agar. *Staphylococcus epidermidis, Streptococcus mutans*, and *Peptostreptococcus anaerobius* strains degraded more proteins when cultivated on blood agar or brucella agar.

In most cases cultivation on selective media gave the same results as that on nonselective media. Additional positive reactions were only revealed in the *V. parvula* strain cultivated on veillonella agar. The strains designated mucin degrading in Table 1 gave, in contrast to pronase, zones of clearly decreased wettability, indicating a degradation of the mucin.

Enterococcus agar gave a decreased wettability of gelatin-, mucin-, and HSA-coated surfaces, resembling the Rogosa agar-affected surfaces. Some of the strains repeatedly caused increased wettability (indicating an increased thickness of the protein layer). This could not be related to a specific strain, substrate, or culture medium.

Comparison of TEA-cultivation technique with tube assay for detection of gelatinolytic activity. Bacteria which revealed no proteolytic activity with the TEA-cultivation technique were also negative with the tube assay. However, gelatinolytic activity was detected in more strains with the TEA-cultivation technique than with the tube assay. Most of the strains giving divergent results with the two assays (Table 1) degraded more proteins when cultivated on blood agar or brucella agar than when cultivated on HCMG agar.

DISCUSSION

TEA has previously been shown to be a sensitive and convenient method for determin-

ing proteolytic enzyme activity in solutions. The sensitivity of the assay was found to be comparable to assays that use fluorescein- or radiolabeled enzyme substrates (37). The activity was determined either by the endpoint titration technique, in which drops of the test solution were applied directly to protein-coated surfaces (38), or by radial diffusion in agar gel (37). In the present study it was shown that TEA could be elaborated for screening microbial protease activity by cultivation of the microorganisms on a culture agar medium applied over the protein-coated surfaces.

A variety of proteases are produced by microorganisms (20). Both microbial protease production and activity of the proteases are regulated by cultivation factors such as medium composition (4). These factors have to be considered in protease assays. In addition, constituents of the nutrition media may influence the ability of an assay to demonstrate proteases. Consequently, it is an advantage if the screening assay permits the use of many different substrates, culture media, and incubation atmospheres, as was shown to be possible with the TEA-cultivation technique.

Proteolytic enzymes were classified by Hartley (16) in acid, serine, metallo, and thiol proteases, and Matsubara and Feder (20) grouped the microbial proteases in essentially the same way. Serine proteases are a widely distributed group of proteases in microorganisms (16). Pronase, a crude enzyme preparation from *Streptomyces griseus* which contains mainly serine proteases (20), was used in this study. Tested with the diffusion in agar gel technique, the proteins adsorbed to the polystyrene surface maintained their predicted sensitivity to pronase, which is in accordance with previous results obtained with the endpoint titration technique (38). The relationship between the diameters of the enzyme-affected zones and the enzyme concentrations was linear in semilogarithmic diagrams. Thus, TEA seems to be suitable for a preliminary screening of substrate-specific microbial proteases. This assumption is supported in that all proteins with an almost equal susceptibility to pronase were not degraded by some of the microbial strains. In addition, some strains showed no proteolytic activity on the proteins easily degraded by pronase, whereas the more pronase-resistant proteins were degraded.

One of the nonselective culture media, HCMG agar, was modified to improve the conditions for detection of proteolytic enzyme activity, rather than to be optimal for microbial growth. The glucose concentration was reduced since glucose may impede the production of proteolytic enzymes (8). Furthermore, the supplement to HCMG medium consisting of serum and extracts of potato, yeast, and liver, shown by Möller (22) to promote the growth of several species, was omitted. The inhibitory effect of serum on microbial proteases is well documented (13, 36). Protease inhibitors have also been isolated from potato and yeast (19, 25).

In the present study the effect of various nutritional components on proteolytic activity was tested with BSA as the enzyme substrate. BSA was used because it is a well-defined protein and gives homogeneous preparations. The degradation of BSA by pronase was shown to be inhibited by serum and extracts of potato and liver but not by yeast extract. Although modified, HCMG agar still reduced pronase degradation of BSA, but to a lesser extent than blood and brucella agars (Fig. 4). The microbial strains did not reveal positive reactions more often when cultivated on HCMG agar than when blood agar or brucella agar was used, and selective media did not affect the possibility of detecting proteolytic activity more than nonselective media. The results reflect the multifactorial interplay in microbial enzyme production and activity and stress the importance of the possibility of using culture media of various compositions, in addition to various enzyme substrates, in protease screening. It should also be noted that extrapolation of results obtained with pronase may not be valid for bacterial proteases.

Some combinations of strains and culture media repeatedly gave increased wettability compared with the wettability of the unaffected protein-coated surface (Table 1), indicating an increased thickness of the protein-coated surface (10, 38). The surface also retained its high wettability, as demonstrated with water vapor condensation, after additional washing procedures. Obtaining an increased wettability could not be related to a specific strain, culture agar, or enzyme substrate but rather to a combination of these factors. However, it was obtained most frequently with strains which degraded only a few or none of the proteins, e.g., with the tested strains of *Streptococcus mutans*, *N. perflava*, and *C. albicans* (Table 1). Strains of *C. albicans* were shown by Germaine et al. (14) to be proteolytic. Since an increased wettability may conceal production of proteolytic enzymes, it may be advisable to try some additional culture media or enzyme substrates or both.

The protein-coated surfaces which had been covered with Rogosa agar resembled the uncoated polystyrene surfaces, and denaturation or even desorption of the proteins cannot be excluded. The low pH of the agar (5.4) was thought to contribute to this effect. The pH may influence both the conformation of the adsorbed proteins (21) and the adsorption of the proteins to the polystyrene surface (1). However, the fact

that Rogosa agar adjusted to pH 7.0 gave the same result shows that pH was not the cause (data not shown).

The sensitivity of the tube assay for detection of gelatinolytic activity with pronase is 0.6 mU per ml of broth (34). The corresponding value for TEA is 0.04 mU per ml of test solution (37). The different degree of sensitivity between the tube assay and TEA probably explains why gelatinolytic activity was detected for some strains with TEA whereas the same strains were negative with the tube assay.

In summary, the experiments indicate that the TEA-cultivation technique provides a sensitive and easily performed method for screening microbial proteases. The assay can be used for screening proteolytic activity of microbial strains cultivated on selective media, for screening substrate-specific enzymes, and in studies of the regulatory effects of extrinsic factors on microbial protease production and activity.

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