Assay for Lipolytic and Proteolytic Activity Using Marine Substrates

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Nondestructive assay procedures for determining microbial lipolytic and proteolytic activity on marine substrates were developed and tested with 287 isolates of bacteria, filamentous fungi, and yeasts. A definite substrate specificity was noted when the enzymatic activities on marine and nonmarine substrates was compared. Of 170 lipolytic isolates, 14 were only active on menhaden oil, 11 could hydrolyze menhaden oil and Tween 80 and/or tributyrin, and 145 isolates could only hydrolyze one or both of the nonmarine lipids. Of the 198 proteolytic isolates, 10 were specific for codfish extract, 152 were active against the marine substrate plus casein and/or gelatin, and 36 were specific for nonmarine substrates.

New or underutilized marine resources can provide an alternate source of food protein. Studies have demonstrated the value of fermentation as a means of increasing the nutritive value and acceptability of various marine products (2, 8; Chu and Roels, personal communication). The wide acceptance and use of fermented foods in the Orient suggests that fermentation might be a practical method of adding underutilized marine protein to the world food supply. A study was initiated to evaluate the ability of organisms isolated from various fermented foods to provide enzymes active against the lipid and protein components of marine products. To achieve this goal, however, it was necessary to develop assay methods specific for enzymes active against lipids and proteins of marine origin.

Existing methods for determining the lipolytic and proteolytic activity of microorganisms generally involve assaying such activity on substrates of nonmarine origin. Preliminary studies indicated that a microorganism's ability to degrade such nonmarine substrates was not necessarily indicative of its activity against marine lipids or proteins. Due to this enzyme-substrate specificity, the converse was also true, and activity against marine substrates was not always accompanied by comparable activity against nonmarine substrates.

MATERIALS AND METHODS

Organisms. Twenty-two samples of fermented foods and related products analyzed by Sands and Crisan (10; unpublished data) provided the 287 microbial isolates used in this study. The isolates represented 49 species of 25 genera of bacteria, yeasts, and filamentous fungi. Bacterial and yeast cultures were maintained on Standard Methods agar (BBL) and filamentous fungi on potato dextrose agar (BBL). All cultures were incubated at 27°C during initial isolations and the studies following. The bacterial and fungal isolates were identified as described by Sands and Crisan (10) and the yeasts were identified by M. Miranda (Dept. of Food Science and Technology, University of California at Davis). *Candida lipolytica* NRRL Y-1094 (Northern Regional Research Laboratory, Peoria, Ill.) and *Bacillus cereus* (ICPB Collection, University of California at Davis) were used as lipolytic and proteolytic reference strains, respectively.

Lipolytic assay. The direct substitution of a marine substrate for a nonmarine substrate in a conventional lipolytic assay procedure was difficult because of problems encountered in preparing such assay media with marine substrates. Lipolytic assays are usually based on detecting the free fatty acids formed from neutral lipids in the medium surrounding lipolytic colonies. Characteristically, lipids of marine origin are highly unsaturated and contain a large amount of free fatty acids. Such lipids often give a false-positive indication of lipolysis if used in conventional lipolytic assay media without prior treatment.

To assay lipolytic activity against marine substrates, it was possible to modify the method developed by Knaysi (7). The assay substrate was prepared from winterized, alkaline-refined, clay-bleached Atlantic menhaden (*Brevooirta tyrannus*) oil (no. HTM-0,5, Zapata-Haynie Products, Inc., Baltimore, Md.). The oil was further winterized for 24 h at 4°C to remove high-molecular-weight, saturated fats before use in the assay medium. After winterization, a clear supernatant oil was decanted from the gummy, semi-solid residue. This oil was dyed with nile blue sulfate (39:1, wt/vol; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and then carefully washed with 200 ml of a 50%
ethanol-water solution in a separatory funnel to minimize emulsification. After the oil-ethanol-water mixture was allowed to stand at room temperature for 24 h, a supernatant layer of reddish-purple oil formed and was removed by decanting. The reddish-purple oil was mixed with distilled water (1:3, vol/vol) and centrifuged at 1,560 × g in a refrigerated centrifuge for 10 min to separate the purple aequous solution containing free fatty acids from the red supernatant oil containing the neutral lipids. The red oil layer was decanted from the centrifuge tubes and stored at 4 °C in a nitrogen atmosphere until used. Other assay methods using dye indicators did not work because the specific indicators gave immediate lipolytic color reactions when incorporated into the treated oil.

A double-layer assay medium was prepared by pouring a basal layer of nutrient agar (BBL) prepared with 0.2 M sodium phosphate buffer, pH 7.1, and containing 2% agar. After solidification, this agar layer was overlayered with a thin layer of nutrient agar containing 2.5% agar and 10% red oil, also prepared with the buffer solution. To compare relative lipolytic activities on marine and nonmarine substrates, two conventional lipolytic assay media containing either Tween 80 or tributyrin (5) were evaluated. The Tween 80 medium was prepared with 1% peptone, 0.5% NaCl, 0.2% CaCl₂, 2% agar, and 4.7 ml of Tween 80 per liter (Atlas Powder Co., Wilmington, Del.), and the tributyrin medium was prepared with 0.5% peptone, 0.3% yeast extract, 2% agar, and 1% tributyrin (Fisher Scientific Co., Pittsburgh, Pa.). The media were sterilized by autoclaving at 131 °C for 15 min.

Lipolytic microorganisms were detected by their ability to hydrolyze the lipid substrates to produce free fatty acids. Colonies of lipolytic microorganisms on the menhaden oil assay medium were surrounded by blue zones of water-soluble free fatty acids against a red background of unhydrolyzed lipid. On the Tween 80 medium, lipase-positive colonies were surrounded by cloudy zones of precipitated calcium salts of free fatty acids and on the tributyrin medium by clear zones against a turbid background of emulsified unhydrolyzed lipid.

Proteolytic assay. Since a definite enzyme-substrate specificity was observed when these organisms were assayed for lipolytic activity, it was assumed that this might also be encountered in assaying for proteolytic activity. Conventional proteolytic assay media generally use proteins of nonmarine origin as substrates; therefore, it was necessary to devise a medium using a marine protein substrate.

Preliminary studies using several commercially available soluble fish protein extracts were not successful, since the low-molecular-weight peptides comprising such extracts could not be precipitated or otherwise made visible to permit detection of enzymatic hydrolysis. Conversely, insoluble fish protein concentrates were not readily attacked and dissolved by proteolytic enzymes, and the results of hydrolysis could not be determined. Simple extracts of several varieties of fish available commercially were prepared in the laboratory and tested as potential substrates for proteolysis. An extract of cod (Gadus macrocephalus) was found to be a satisfactory substrate and was used in this study.

The cod extract was prepared by homogenizing 40 g of a cod fillet with 150 ml of distilled water in a Waring blender (Waring Product Division, Dynamics Corp. of America, New Hartford, Conn.). The homogenized suspension was filtered through a double layer of cotton gauze and then twice through milk filter disks (no. 5614, Sears, Roebuck, and Co., Chicago, Ill.). The filtrate was autoclaved and cooled and then re-homogenized aseptically in a sterile Omnimetixer (Ivan Sorval, Inc., New York, N.Y.) to obtain a uniformly opaque suspension of the fish particles.

A double-layer assay medium was prepared containing 0.3% yeast extract, 0.5% ammonium nitrate, 0.5% glucose, and 2% agar. A basal layer of sterile medium was poured and after solidification was overlayed with a thin layer of the same medium but containing the cod extract (2.2%, vol/vol) in place of the ammonium nitrate. It was necessary to cool the overlay medium to 48 °C in a water bath before adding the fish extract to prevent the clumping of the proteinaceous material. The cod extract medium was gently swirled to obtain an opaque suspension before the overlay was poured. To compare the relative proteolytic activities of the isolates on marine and nonmarine substrates, two conventional proteolytic assay media containing either casein or gelatin (5) were evaluated. The casein medium was prepared with 4% casein and 3% agar, and the gelatin medium contained 0.6% gelatin, 0.5% glucose, 0.05% yeast extract, and 2% agar. The media were sterilized by autoclaving as noted above.

Proteolytic microorganisms growing on the cod extract assay medium were characterized by their ability to produce clear zones against an opaque background of unhydrolyzed protein. Similarly, microorganisms active against casein also produced clear zones against an opaque background. To detect proteolysis on the gelatin assay medium, it was necessary to flood the plates with a 20% aqueous solution of sulfosalicylic acid (J. T. Baker Chemical Co., Phillipsburg, N.J.) to precipitate unhydrolyzed protein. Proteolytic colonies were surrounded by clear zones.

RESULTS AND DISCUSSION

The lipolytic and proteolytic activities of the bacterial isolates are shown in Table 1. Of the 228 bacterial strains tested, 170 exhibited some degree of lipolytic activity. Only four strains were active against all three lipid substrates, whereas seven hydrolyzed the marine substrate plus one or the other nonmarine lipid substrate and 14 were only lipolytic on the menhaden oil. Nonmarine lipids were the only substrates hydrolyzed by 145 isolates, which included 26 hydrolyzing only Tween 80 and 52 hydrolyzing only tributyrin.

The bacterial isolates were more versatile in their ability to hydrolyze protein substrates.
LIPOLYTIC AND PROTEOLYTIC ASSAY

TABLE 1. Lipolytic and proteolytic activities of bacterial isolates tested on substrates of marine and nonmarine origin

| Organism                  | No. of isolates tested | Lipid substrate | Protein substrate |
|---------------------------|------------------------|-----------------|-------------------|
|                           |                        | Fish oil | Tween 80 | Tributyrin | Cod extract | Casein | Gelatin |
| Bacillus badius           | 2                      | -  | +  | + | - | - | + |
| B. brevis                 | 1                      | -  | -  | - | - | - | + |
| B. cereus                 | 17                     | +  | -  | + | + | + | + |
|                           | 4                      | -  | -  | - | + | + | + |
|                           | 2                      | -  | +  | + | - | + | + |
| B. cereus var. mycoides   | 3                      | -  | +  | + | - | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| B. circulans              | 2                      | -  | +  | + | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| B. coagulans              | 1                      | -  | +  | + | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| B. firmus                 | 1                      | -  | +  | + | + | + | + |
|                           | 3                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| B. licheniformis          | 2                      | +  | +  | + | + | + | + |
|                           | 4                      | -  | +  | + | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
|                           | 4                      | -  | +  | + | + | + | + |
|                           | 2                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| B. macerans               | 1                      | -  | +  | + | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| B. pantothenticus         | 1                      | -  | +  | + | + | + | + |
| B. polymyxa               | 2                      | -  | +  | - | + | + | + |
|                           | 1                      | -  | -  | + | + | + | + |
|                           | 1                      | -  | -  | - | + | + | + |
| Organism                          | No. of isolates tested | Lipid substrate | Protein substrate |  |
|----------------------------------|------------------------|-----------------|------------------|---|
|                                  |                        | Fish oil | Tween 80 | Tributyrin | Cod extract | Casein | Gelatin |  |
| *B. pulrifaciens*                | 1                      |           |           |            |             |        |        | + |
| *B. pumilus*                     | 1                      |           |           | +          | +           |        |        | + |
| *B. sphaericus*                  | 1                      |           |           | +          | +           |        |        | + |
| *B. subtilis*                    | 2                      | +         | +         | +          | +           | +      | +      | + |
|                                  | 5                      | +         |           | +          | +           | +      | +      | + |
|                                  | 1                      |           | +         | +          | +           | +      | +      | + |
| *B. subtilis var. aterrimus*     | 6                      |           |           | -          | +           | +      | +      | + |
| *Corynebacterium rathayi*        | 1                      |           | -         | +          | +           | +      | +      | + |
| *Escherichia coli*               | 2                      |           |           | -          | -           | +      | +      | + |
| *Flavobacterium lutescens*       | 1                      |           | -         | +          | +           | +      | +      | + |
| *Micrococcus candidus*           | 1                      |           | +         |            | +           | +      | +      | + |
| *M. caseolyticus*                | 1                      |           | -         |            | +           | +      | +      | + |
| *M. colpogenes*                  | 2                      |           | +         |            | +           | +      | +      | + |
|                                  | 1                      |           |           | +          | +           | +      | +      | + |
| *M. conglomeratus*               | 2                      |           |           | -          | +           | +      | +      | + |
| *M. flavus*                      | 1                      | +         |            | -          | +           |        |        | + |
| *M. luteus*                      | 1                      |           | +         |            | -           |        |        | + |
| *M. roseus*                      | 1                      |           |           | +          | -           | +      |        | + |
| *M. varians*                     | 4                      | +         | -         | -          | -           | +      |        | + |
|                                  | 1                      |           |           | -          | -           | +      |        | + |
|                                  | 1                      |           |           | -          | +           |        |        | + |
| *Proteus mirabilis*              | 1                      |           |           | -          | -           | -      |        | + |
| *P. spp.*                        | 1                      |           |           | -          | -           | -      |        | + |
| *Serratia indica*                | 1                      | +         | -         | -          | -           | +      |        | + |
| *Staphylococcus aureus*          | 1                      | +         |           | -          | -           | +      |        | + |
Some degree of proteolytic activity was exhibited by 198 strains, 140 of which could hydrolyze all three assay substrates. Ten isolates hydrolyzed only cod extract, whereas 11 hydrolyzed the cod and one or the other nonmarine substrate. Thirty-six proteolytic strains were only active against nonmarine substrates.

The lipolytic activity of filamentous fungi was particularly difficult to determine by conventional assay procedures. The spreading nature of the mycelial growth tended to obscure the precipitate formed on the Tween 80 assay medium and interfered with the observation of the clear zones formed on the tributyrin medium. The results obtained on the menhaden oil assay medium were easily discernible due to the color reaction noted when lipolysis occurred. The lipolytic assay of the yeast isolates presented little difficulty due to the discrete nature of the yeast colony. Nile blue sulfate exhibited no toxicity towards the isolates when used in the manner described.

The ease of using the cod extract assay medium for evaluating proteolytic activity was comparable to the casein medium since it is necessary to observe clear zones around proteolytic organisms in both assay procedures. As noted above, it was more difficult to make these observations with cultures of filamentous fungi due to the spreading growth of the mycelium. The yeasts were easily evaluated on the cod extract medium. The cod extract medium provided an advantage over conventional assay procedures with media containing substrates such as gelatin which must be chemically precipitated by flooding the assay plates before proteolysis can be determined. The cod extract assay procedure is nondestructive, and the assayed organisms can be recovered without contamination if desired.

The results obtained in the assay of yeasts and filamentous fungi for lipolytic and proteolytic activity against the marine substrates are shown in Table 2. The filamentous fungi exhibited a variety of lipolytic and proteolytic reactions. Ten fungal isolates were active against the menhaden oil alone, eight against the cod extract alone, and 14 were both lipolytic and proteolytic against the marine substrates. Fourteen strains were neither lipolytic nor proteolytic on the marine substrates. The yeasts were quite limited in their enzymatic ability. One strain exhibited both lipolytic and proteolytic activity, whereas the remaining strains were negative for both enzymatic reactions.

| Organism                      | No. of isolates tested | Substrates |                |
|-------------------------------|------------------------|------------|----------------|
|                               |                        | Fish oil   | Cod extract    |
| Filamentous fungi             |                        |            |                |
| Absidia spp.                  | 1                      | +          | -              |
|                               | 2                      | -          | +              |
|                               | 1                      | -          | -              |
| Alternaria spp.               | 6                      | +          | +              |
|                               | 2                      | -          | -              |
|                               | 2                      | -          | -              |
| Cladosporium spp.             | 2                      | -          | -              |
| Epicoccum spp.                | 2                      | +          | -              |
| Neospora sp.                  | 1                      | +          | +              |
| Penicillium spp.              | 3                      | +          | -              |
|                               | 2                      | +          | -              |
|                               | 2                      | -          | -              |
| Rhizopus spp.                 | 5                      | +          | +              |
|                               | 4                      | +          | -              |
|                               | 2                      | -          | +              |
|                               | 1                      | -          | +              |
| Syncephalastrum sp.           | 1                      | +          | -              |
| Trichoderma sp.               | 1                      | -          | +              |
| Yeasts                         |                        |            |                |
| Candida clauisenii            | 1                      | -          | -              |
| C. lambica                    | 1                      | -          | -              |
| C. solani                     | 1                      | -          | -              |
| Debaryomyces hansenii         | 1                      | -          | -              |
| Endomycopsis burtonii         | 1                      | -          | -              |
| Geotrichum candidum           | 1                      | +          | -              |
| Hansenula anomala             | 2                      | -          | -              |
| H. polymorpha                 | 1                      | -          | -              |
| Rhodotorula glutinis          | 1                      | -          | -              |
| Saccharomyces cerevisiae       | 1                      | -          | -              |
| Trichosporon cutaneum         | 1                      | -          | -              |

| TABLE 2. Lipolytic and proteolytic activities of filamentous fungi and isolates tested on substrates of marine origin |
|---------------------------------------------------------------|

nisms but none involve the use of marine substrates (1, 3, 4, 6, 9, 11). The results of this study re-emphasize the critical importance of considering substrate specificity in selecting methods for evaluating the enzymatic capacity of microorganisms. For example, if Tween 80 and tributyrin were used as substrates to evaluate the bacterial isolates studied here for their ability to hydrolyze menhaden oil, 8.2% of the isolates active against the fish oil alone would have been missed and 85.3% of the isolates active against either or both of the nonmarine substrates would not be capable of hydrolyzing the marine substrate; the assays would have only detected seven of the 25 bacterial isolates active against the fish oil.

The value of using fermentation in the devel-
opment of new marine food sources will be determined by the organisms ability to enzymatically induce the desired changes in the marine substrate. By using the assay methods described here, it will be possible to identify those organisms which can selectively hydrolyze the lipid and/or protein components of a marine product. The preparation of the assay media and the procedures used in the assay are relatively simple. Both assay procedures are nondestructive and can be used to selectively isolate lipolytic or proteolytic organisms from a mixed population.

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