Ester Production by *Pseudomonas fragi*

IV. Demonstration of Esterase Activity

M. C. REDDY, R. C. LINDSAY, and M. W. MONTGOMERY

*Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331*

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Assay of the esterase activity of sonically treated cell-free extracts, whole cell suspensions, and supernatant fluid of *Pseudomonas fragi* cultures with a differential respirometer revealed that the esterases were intracellular. Polyacrylamide-gel electrophoresis demonstrated six bands of esterase activity, which revealed substrate specificity differences. Band 1 exhibited slow mobility, bands 2, 3, and 4 moderate mobility, and bands 5 and 6 rapid mobility. Six bands were active with α-naphthyl acetate, four bands with α-naphthyl propionate, and 5 bands with α-naphthyl butyrate. These esterases appeared to be more active with aromatic esters than with aliphatic esters.

The fruity aroma in many dairy products has been attributed to *Pseudomonas fragi*. Reddy et al. (6) isolated and identified ethyl butyrate and ethyl hexanoate from mature cultures of *P. fragi* as the principal esters responsible for fruity aroma. Esterification can proceed via enzymatic or nonenzymatic pathways. These reactions are commonly mediated by living cells and can be accomplished through a direct esterification or through a pathway involving the activation of the fatty acid moiety by coenzyme A (4). Washam (Ph.D. Thesis, Oregon State Univ., Corvallis, 1968) demonstrated by gel electrophoresis that a wild type *P. aeruginosa* possessed two esterases, whereas Sierra (7) distinguished three esterases in an unpurified preparation from *P. aeruginosa*. Morichi et al. (3) reported that many of the esterases of lactobacilli were active with α-naphthyl acetate. However, no information is available regarding the possible enzyme systems involved in the ester formation or hydrolysis.

The purpose of this investigation was to determine the distribution of the esterase enzymes in *P. fragi* cultures and to determine their activity on different substrates.

**MATERIALS AND METHODS**

**Cultural procedure.** Two *P. fragi* strains, ATCC 4973 and one isolated from pasteurized milk by the method described earlier (6), were used in this investigation. Peptone broth (1%) was dispensed in 2-liter quantities into 4,000-ml Erlenmeyer flasks which were then plugged with cotton and autoclaved at 121 C for 10 to 12 min. A 0.5% inoculum from cultures grown in 1% peptone broth at 7 C for 9 days was added to the 2-liter broth samples. Each of the cultures was incubated in static conditions at 21 C for 4 days.

**Preparation of cell-free extract.** Cells were harvested by centrifugation in the cold (5 C) at 7,700 x g for 20 min. The supernatant was collected for the determination of esterases. After the cells were washed twice with 50-ml samples of chilled 0.025 M phosphate buffer (5 C and pH 6.8), they were suspended in 12 ml of the buffer. The bacteria were sonically disintegrated for 6 to 8 min in an ice bath, with Bronwill Biosonic III with an intermediate tip, and clarified by centrifugation at 100,000 x g for 1 hr. The supernatant fluid was freeze-dried, suspended in 20 ml of buffer, and subsequently assayed for esterase activity.

**Preparation of substrates.** The substrates used in the study of the distribution of esterases in *P. fragi* culture were 1 M ethyl butyrate, triacetin, and phenyl acetate. All substrates were prepared in 3% (w/v) Triton X-155 (Rohm and Haas) and 0.1% gum arabic. The esters and triglyceride were homogenized for 2 min in a water-cooled microblendor.

**Assay procedure.** Esterase activity of both the cell-free extract and the supernatant fluid of *P. fragi* was determined by manometric technique similar to those described by Norgaard and Montgomery (5) by using a Gilson differential respirometer at 25 C and pH 6.8. The volume of CO₂ liberated from the buffer system was recorded for each 10-min interval for 40 min. Blanks containing boiled enzyme preparations were used to determine nonenzymatic hydrolysis.

**Electrophoresis of esterases.** The cell-free extract, prepared by the procedure described above, was used in the electrophoretic study. Since the esterases were found to be intracellular by the assay procedure, the supernatant fluid was not electrophoretically analyzed.

**Electrophoretic procedure.** Gel preparation, electro-
phoresis, and esterase staining were performed by the procedure described by Montgomery et al. (2). Approximately 150 μl of the sample was applied with a 1-ml tuberculin syringe directly into the slots at the top of the gel to form a layer under the buffer, and electrophoretic separation was performed at 5 C for 1.5 to 2 hr.

**Zymogram development.** On completion of electrophoresis, the gel was removed from the cell and cut into 4 strips which were placed in 100 ml of 0.025 M phosphate buffer (pH 6.8) containing 70 mg of fast blue RR salt and 40 mg of the ester dissolved in 2 ml of acetone (2). Depending on the substrate used, the time of incubation at 25 C ranged from 45 min to 2 hr.

**RESULTS AND DISCUSSION**

**Assay of *P. fragi* esterases.** The distribution of the esterase enzymes was established by assaying the esterase activity in different fractions of the culture. The data presented in Table 1 clearly indicate that the greatest esterase activity with phenyl acetate as substrate was in sonically treated cell preparations. There was an equal amount of cellular material in the sonically treated cell fraction and in the whole cell suspension. However, the esterase activity observed for whole cell suspension was only about 25% of the activity exhibited by the sonically treated cell preparation. The enzyme activity of the whole cell suspension could have been due, partially, to some ruptured cells. The esterase activity shown by the culture supernatant fluid was negligible when compared to the activity of the sonically treated cell fraction. The results indicate that in *P. fragi* the esterases are intracellular.

**Respirometer assay of substrate specificity.** The substrate specificity of esterases of *P. fragi* was examined by using phenyl acetate, ethyl butyrate, and triacetin. The volume of gas liberated during 40-min incubation by 1 ml of the sonically treated cell preparation was 290 μl when phenyl acetate was the substrate, 35 μl when ethyl butyrate was the substrate, and 47 μl when triacetin was the substrate. Therefore, the enzymes were more active with aromatic esters than with aliphatic esters. A 12-hr incubation period was required to liberate 250 μl of CO₂ by 1 ml of sonically treated cell preparation with ethyl butyrate as the substrate. Since substrates other than triacetin were hydrolyzed, these intracellular esterases appear different from the extracellular lipases described by Mencher and Alford (1).

**Electrophoretic pattern of *P. fragi* esterases.** A diagram of a zymogram of replicate analyses of sonically treated cell preparations of *P. fragi* strain 9165 is presented in Fig. 1. Evidence for the presence of six esterase enzymes was provided by using α-naphthyl acetate as the substrate. The bands with esterolytic activity had quite different electrophoretic mobility. Band 1 had slow mobility, bands 2, 3, and 4 showed intermediate mobility, and bands 5 and 6 were fast-moving.

**Substrate specificity observed in gel electrophoresis.** The activity of the esterases was studied with α-naphthyl propionate and α-naphthyl butyrate in addition to α-naphthyl acetate. Examination of zymograms indicated that five bands were well developed in 50 min of incubation with α-naphthyl acetate. However, with α-naphthyl propionate as substrate, only bands 1, 4, 5, and 6 were active. When α-naphthyl butyrate was used, 2 hr of incubation was required for the development of bands 1, 2, 4, 5, and 6.

Bands marked 5 and 6 were active with all of the substrates used, and hence did not show acyl

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**TABLE 1. Esterase activity of *Pseudomonas fragi* culture fractions with phenyl acetate as substrate**

| Incubation time | Culture fractions |
|-----------------|-------------------|
|                 | Sonically treated | Whole cell | Culture supernatant |
| min             | cell prepn | µlitter | µlitter | µlitter |
| 0-10            | 68       | 23      | 3       |
| 10-20           | 85       | 18      | 3       |
| 20-30           | 82       | 20      | 1       |
| 30-40           | 68       | 15      | 1       |

* Esterase activity was defined as the microliter of CO₂ released per 10 min interval per milliliter of culture preparation.

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**FIG. 1. Polyacrylamide-gel electrophoresis of esterases from *Pseudomonas fragi* with various substrates. (A) α-Naphthyl butyrate; (B) α-naphthyl propionate; (C) α-naphthyl acetate.**
chain-length specificity. Since α-naphthyl acetate and propionate were hydrolyzed equally by bands 1 and 4 and showed less activity with α-naphthyl butyrate, these bands were more specific for the shorter acyl-side chains. Band 3 did not hydrolyze α-naphthyl propionate or butyrate and was specific for α-naphthyl acetate. In contrast to this, band 2 revealed specificity for α-naphthyl butyrate and acetate but not the propionate ester. Therefore, these results demonstrate that the esterases of P. fragi show marked differences in specificity toward the acyl side-chain of the substrates.

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