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Glycerol Ester Hydrolase Activity of Lactic Acid Bacteria

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Seventeen strains of lactic acid bacteria were assayed for their glycerol ester hydrolase activity by using an improved agar-well technique, and eight strains by determining the activity in cell-free extracts using a *p*H-stat procedure. All cultures tested showed activity and hydrolyzed tributyrin more actively than they did tricaproin. The cell extract studies demonstrated that the cells contained intracellular esterases and lipases. The culture supernatant fluid was without activity. The lipase and the esterase differed in their relative activity to each other in the different extracts and in the ease by which they could be freed from the cellular debris. It is suggested that the lipase of these organisms is an endoenzyme and the esterase an ectoenzyme.

Glycerol ester hydrolases are enzymes catalyzing the hydrolysis of triglycerides or fats. These enzymes are important to the dairy and food industry not only because of their ability to produce undesirable flavors in milk and other food products, but also because of their role in the development of desirable and characteristic flavors in many ripened cheeses. However, the origin of fat hydrolysis in cheese such as cheddar cheese, where lactic acid bacteria constitute the main microflora, still remains unknown.

The ability of lactic acid bacteria to hydrolyze triglycerides or fats is a controversial subject. Early work on the lipolytic activity of lactic acid bacteria by Long and Hammer (9), Peterson and Johnson (13), and Wolf (Proc. Soc. Agr. Bacteriologists, p. 48, 1940, and p. 21, 1941) suggested that certain lactobacilli and lactic streptococci showed some lipolysis, but only after prolonged incubation of several months. More recently, Stathouder and Mulder (15) concluded that lactic acid bacteria produced only small amounts of lipase, if any, and that these microorganisms had no influence on fat hydrolysis in cheese ripening. However, lactic acid bacteria do contain an appreciable amount of lipid which is, in part, neutral lipid containing triglycerides (7, 8). Since these microorganisms do have the necessary enzyme system to synthesize triglycerides or fats, it is reasonable, although not mandatory, to assume that they may also have the enzymes required to break down such compounds.

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In a previous communication (11), we described an improved agar-well assay method for detection of microbial lipolysis. This technique enabled us to detect low levels of lipolytic activity and to evaluate the lipolytic properties of lactic acid bacteria. All of the organisms tested were shown to possess detectable lipolytic activity. The recent report by Fryer, Reiter, and Lawrence (5) confirmed these results.

The present work was undertaken to obtain further information concerning the glycerol ester-hydrolyzing properties of lactic acid bacteria and to differentiate their ability to hydrolyze substrates in emulsion (lipase) as compared to substrates in solution (esterase; 4, 6, 17).

MATERIALS AND METHODS

Test organisms and cultural conditions. The lactic acid bacteria used in these studies were supplied through the generosity of the National Institute for Research in Dairying (NIRD), Shinfield, England; Chr. Hansen's Laboratory, Inc. (CHL), Milwaukee, Wis.; the Marschall Dairy Laboratory, Inc. (MDL), Madison, Wis.; and the Unilever Research Laboratory (URL), Bedford, England. Additional cultures were obtained from the American Type Culture Collection (ATCC), Rockville, Md. Detailed information about the cultures is given in Table 1. Unless otherwise indicated, all organisms were grown in Lactobacillus (Difco) MRS broth (2) containing 1% of 10% cream at their approximate optimal growth temperatures. The cells were harvested at the end of logarithmic growth.

Stock cultures were maintained on an agar medium obtained by adding 1.5% agar and 0.3% calcium carbonate to MRS broth. Agar butts, inoculated by stabbing, were incubated for 2 to 3 days at optimal

temperatures for growth and then stored at 0 to 2 C. Cultures for daily use were prepared by inoculating MRS broth with a freshly grown culture (1% inoculum), then freezing and storing the inoculated broth at −20 C. These cultures were used to inoculate fresh broth as needed.

Preparation of cell-free extract. After harvest by centrifugation, the cells were washed twice in 0.01 M ammonium chloride buffer, *p*H 7.2, and resuspended in the same buffer. Cell-free extracts were then prepared by repeated disruption of the cells by a French press (at 8,000 psi), followed by centrifugation of the homogenate at 25,000 × *g* for 20 min. The protein content of the cell-free extracts was determined by the method of Lowry et al. (10) with crystalline serum albumin as the standard.

Esterase assay. The esterase activity (esterolytic activity of a preparation directed toward a substrate in solution) of lactic acid bacteria or their cell-free extracts was determined by titration of the liberated acids with an automatic recording *p*H-stat (E. H. Sargent & Co., Chicago, Ill.). The initial velocity of hydrolysis of triacetin was followed by continuous titration as described for the assay of milk lipase by Parry, Chandan, and Shahani (12). The reaction mixture containing 0.2 M aqueous solution of triacetin was brought to 35 C before the enzyme was added, and the mixture was maintained at *p*H 7.2 by continuous addition of 0.1 N CO_2-free KOH. Blanks containing boiled enzyme were similarly titrated.

Lipase assay. The lipolytic activity (esterolytic activity of a preparation directed toward a substrate in emulsion) of lactic acid bacteria or their cell-free extracts was also determined by a *p*H-stat titration procedure. The substrate, emulsion of tributyrin in 10% gum arabic described by Desnuelle, Constantin, and Baldy (3), was prepared by ultrasonic treatment of the reaction mixture with a Branson Sonifier (model S125; Heat System Co., Melville, N.Y.) at full power for 3 min. Sonic treatment for longer periods did not increase utilization of substrate. During sonic treatment, the beaker containing the substrate mixture was kept in an ice-water bath to prevent excessive heating of the emulsion. After incubation of the assay mixture for 10 hr or more, the liberated fatty acids were determined by back titration to the initial *p*H. Blanks containing boiled enzyme, substrate without enzyme, and enzyme alone, were similarly titrated.

The improved agar-well assay technique (11) was also used in the evaluation of lipolytic activity of lactic acid bacteria. Whole cells were washed in 0.01 M ammonium chloride buffer (*p*H 7.2), resuspended in the same buffer, and then placed in the agar wells. The zones of hydrolysis were measured after 24-hr incubation of the plates at 32 C. Although a strict comparison of esterolytic activities would require measurements of cell dry weight, the cell suspensions of the different organisms were simply adjusted to the same optical density, equivalent to about 10^{10} organisms/ml.

For both esterase and lipase, one unit of activity is defined as the amount of enzyme catalyzing the formation of one millimicro-equivalent of acid per minute.

RESULTS

Lipase activity. All of the lactic acid bacteria screened for lipolytic activity by the improved agar-well assay technique were active (Table 1). Further, all of the bacteria listed in Table 1 were substantially more active toward tributyrin than toward tricaproin. When other triglycerides in the same homologous series (C_8 through C_{16}) or milk fat were used as substrates, complete clearing of the emulsion did not take place, although faint zones indicating weak or partial hydrolysis were frequently observed. These findings are in agreement with the conclusions of J. A. Alford and E. Steinle (Bacteriol Proc., p. A80, 1966) that microbial lipases preferentially hydrolyze tributyrin.

No activity was found in the growth medium after cells were harvested. Failure to demonstrate lipolytic activity in the growth medium suggested that the lipase produced by these organisms was not extracellular. Conversely, if the lipase were intracellular, it would be recoverable from cell-free extract. Accordingly, whole cells, cell-free extract, and cell debris from the relatively active *Lactobacillus brevis* X2 were incubated for 24 hr at 35 C in a substrate mixture containing 2 ml of 5% tributyrin emulsion in 10% gum arabic, 0.5 ml of 0.5 M $CaCl_2$, and 2.5 ml of distilled

TABLE 1. *Lipase activity of some lactic acid bacteria as determined by the agar-well assay technique*

Organism	Source of acquisition	Zone size (mm × 10^2)	
		Assay 1[a]	Assay 2[b]
Lactobacillus bulgaricus H1	CHL[c]	73	23
L. helveticus H2	CHL	70	31
L. lactis H3	CHL	80	10
L. acidophilus M1	MDL	89	30
L. casei 13065	ATCC	66	14
L. casei C2	NIRD	73	21
L. casei C9	NIRD	75	13
L. plantarum AR3	NIRD	62	20
L. plantarum P1	NIRD	73	14
L. plantarum P6	NIRD	69	13
L. brevis X2	NIRD	91	22
L. brevis X16	NIRD	98	21
Streptococcus diacetylactis 188	CHL	59	—
Lactic starter culture M11	CHL	84	32
Leuconostoc citrovorum CAF	CHL	64	—
Leuconostoc mesenteroides 545	URL	66	10
Pediococcus cerevisiae 585	URL	75	15

[a] Assayed on tributyrin (MRS-agar) plates.
[b] Assayed on tricaproin (MRS-agar) plates.
[c] For abbreviations, see Materials and Methods.

water. After incubation, the acid produced was determined by back titration to the initial *p*H 8 with the *p*H-stat. The free fatty acids produced by whole cells were compared to the cell-free extract and the cell debris fraction obtained from an equivalent amount of whole cells. After correcting for blank titrations, the microequivalents of acid produced were: whole cells, 13.1; cell-free extract, 13.2; and cell debris, 1.0. These results are in agreement with the suggestion that the lipase of lactic acid bacteria is intracellular rather than extracellular, and that cell-free extract therefore gave a good basis for evaluation of lipolytic activity of these microorganisms.

Cell-free extracts of eight identified species of lactic acid bacteria were tested for lipase activity by using the same assay procedure and substrate mixture as described above (Table 2). All the cell-free extracts possessed lipolytic activity; and the activities of the cell-free extracts from *Leuconostoc citrovorum* CAF, the lactic starter culture M11, and *Streptococcus diacetylactis* 188 were several-fold greater than those of the six species of the genus *Lactobacillus*.

TABLE 2. *Glycerol ester hydrolase (lipase and esterase) activity of cell-free extracts of some lactic acid bacteria*[a]

Organism	Glycerol ester hydrolase activity (units/mg of protein)	
	Lipase[b]	Esterase[c]
Lactobacillus bulgaricus H1	0.37	Traces
L. helveticus H2	0.72	Traces
L. lactis H3	0.68	Traces
L. casei C9	0.66	8.0
L. plantarum P6	0.34	38.5
L. brevis X2	0.48	27.9
Streptococcus diacetylactis 188	1.29	1.1
Leuconostoc citrovorum CAF	1.94	9.1
Lactic starter culture M11	1.45	1.3

[a] All cultures were grown in MRS broth containing 1% of 10% cream at the approximate optimal growth temperatures. The average of duplicate tests corrected for titration of the blanks was used to obtain the results given.

[b] The reaction mixture contained 58 mmoles of tributyrin sonically dispersed in 10% gum arabic, water, and cell-free extract in a total volume of 6 ml. Hydrolysis was determined by titration of the liberated acids after 10 hr of incubation at 35 C against boiled enzyme blanks.

[c] The reaction mixture contained 0.2 M aqueous triacetin and cell-free extract in a total volume of 7 ml. Hydrolysis was determined at *p*H 7.2 and 35 C by continuous titration of the liberated acids with the *p*H-stat.

Esterase activity. An expression of the esterase activity of glycerol ester hydrolases was obtained when an aqueous solution of triacetin was used as the substrate (16). Since no activity was found in the growth medium after the cells were harvested, the recoverability of esterase activity in cell-free extracts of lactic acid bacteria was determined. The activity of whole cells was compared to that of a cell-free extract and a cell-debris fraction prepared from an equivalent amount of whole cells with the following results: 0.14, 0.13, and 0.06 μeq of acid per min, respectively. Although the larger portion of the activity of whole cells was recovered in the cell-free extract, a considerable amount of activity remained in the debris fraction obtained after centrifugation (25,000 × *g* for 20 min) of the disrupted cells. The esterase, therefore, appeared to be more strongly associated with the cell particulate matter than did the lipase. It is noted that the combined activity of the cell-free extract and cell-debris fractions was greater than that of the whole cells from which they were obtained. This apparent discrepancy is probably due to variation in the hydrolyzing capacity of the enzyme before and after it is removed from its natural environment, the intact cell.

Several attempts were made to solubilize the portion of the esterase associated with the cell-debris fraction after disintegrating the cells. The alkaline incubation procedure of Aspen and Wolin (1) was destructive to the enzyme and little or no activity was recovered. Incubation with trypsin was hampered by the impure commercial preparation, which apparently contained relatively large amounts of glycerol ester hydrolase activity. Treatment of the cell debris with lysozyme, with a 3× crystallized commercial preparation, brought appreciable amounts of the enzyme from the debris to the extract fraction, but the results were somewhat variable. Finally, most of the activity was found to be recovered in the cell-free extract by repeated passage (1) of the mixture through the French press.

The cell-free extracts from the eight lactic acid bacteria and one culture tested for lipase activity were also tested for esterase activity (Table 2). Cell-free extracts of all the lactic acid bacteria tested showed esterase activity, although the activity for some was slight. The lactobacilli showing only slight esterase activity were all from the subgenus *Thermobacterium*, according to Orla-Jensen's classification. *L. plantarum* and *L. brevis* had much greater capacity to hydrolyze triacetin than any of the other microorganisms tested.

DISCUSSION

The evidence presented in this paper points to the fact that lactic acid bacteria do contain glycerol ester hydrolase activity, and that such activity is associated with two enzymes, or groups of enzyme, lipase and esterase.

The presence of both enzymatic activities in cell-free extracts, while neither activity was found in the growth medium after harvesting the cells, supports the conclusion that the lipase and esterase are both intracellular enzymes. This is in contrast to the recent suggestion of Fryer, Reiter, and Lawrence (5) that the lipase of lactic acid bacteria is extracellular. However, Pollock (14) has pointed out that, in order to prove the extracellular nature of an enzyme, the following must be established: (i) the enzyme appears early in the growth of the culture when cells are not prone to lysis; (ii) the enzyme is not found inside the cell; and (iii) when the enzyme appears in the culture medium, an intracellular marker does not simultaneously appear. Neither of these requirements was investigated by the above workers, and their conclusion should therefore be viewed with caution.

When the cells were disrupted by the French press, the lipase activity was found to be in the fraction not sedimented by centrifugation at 25,000 $\times$ *g* for 20 min. In contrast, an appreciable amount of the esterase activity appeared in the sedimented fraction after the same treatment. This indicated that the two enzymes had different locations within the cell, the lipase resembling an endoenzyme, the esterase an ectoenzyme. For the latter enzyme, this assumption was further substantiated by the ability of lysozyme to solubilize the esterase activity associated with the debris fraction. The results shown in Table 2 also indicate that the lipase and esterase were different with respect to distribution and level of activity.

The role of lipases and esterases of lactic acid bacteria in fat hydrolysis and flavor development in cheese ripening is yet to be elucidated. The fact that glycerol ester hydrolases are present in these organisms, which account for the predominant bacterial flora in a hard rennet cheese like cheddar, suggests, however, that lactic acid bacteria are important in cheese ripening, not only in fermentation and proteolysis, but also in lipolysis.

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