Esterases and Other Soluble Proteins of Some Lactic Acid Bacteria

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SUMMARY

Electrophoresis patterns of soluble protein of 34 strains and esterases of 113 strains of lactic acid bacteria were determined. Similar protein patterns were obtained for the three species of lactic acid streptococci; with the lactobacilli most species gave constant species-specific patterns, but *Lactobacillus acidophilus* and *L. delbrueckii* strains differed markedly among themselves. Esterase patterns of lactic streptococci were generally species specific. Among the lactobacilli the thermobacteria had weak esterase activity which was only species specific for *L. lactis*, *L. leichmanii* and *L. salivarius*; in the streptobacteria, *L. casei* had a very consistent esterase pattern, whereas *L. plantarum* had very different patterns within the species; the unclassified strains were different from each other; in the betabacteria activity was weak and no consistent pattern of bands occurred. Leuconostocs grouped in patterns corresponding to their physiological groups. Esterases of a streptococcus and a lactobacillus examined were classified as α esterases. When ten strains of lactic acid bacteria were tested for substrate specificity, nine of them had a higher activity against α -naphthyl acetate than against the butyrate and caprylate. A rapid test for esterase activity of whole organisms is described.

INTRODUCTION

Lactic acid bacteria have been differentiated by physiological and serological tests into several well-defined groups or species, but a further understanding of their relationships might result if the electrophoretic patterns of their esterases and other soluble proteins were compared, as has already been shown for other groups of bacteria (Norris, 1964; Cann & Willox, 1965; Lund, 1965; Robinson, 1966). These lactic acid bacteria occur in large numbers in Cheddar cheese and the presence of esterases in different strains of these organisms might effect the breakdown of ester linkages of substances present in the cheese, and produce compounds contributing to the cheese flavour (Reiter, Fryer, Sharpe & Lawrence, 1966; Reiter *et al.* 1967). The relative activity of the different esterases might determine the quality of flavour. In experiments reported here, the multiple esterases of group N streptococci, lactobacilli and leuconostocs were studied, and the soluble protein electrophoretic patterns of some of the group N streptococci and the lactobacilli determined.

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METHODS

Organisms. The following 113 strains of lactic acid bacteria were used: *Streptococcus cremoris* HP, OP4, TEM2, RW, YP7, K, FH, HS2, UD5, AM1, TR, D9, R1, MLI, KH, 924, E8, ML2, R6, 803; *S. lactis* SC2, SC6, SC10, M2S1, ML3, 712; *S. diacetylactis* DRC1, DRC2; *Lactobacillus casei* C2, C4, C5, C6, C7, C9, C12, C20, C22, C24, C25, C42; *L. casei* var. *rhamnosus* C3, C10, C16, C34; *L. casei* var. *alactosus* C31; *L. plantarum* P1, P5, P12, AR3, A164; unclassified streptobacteria K44, A41, A101, D45, V4, V5; *L. helveticus* H4, H5, H17; *L. jugurti* J2, J4, J8; *L. bulgaricus* B4, B8, B17; *L. lactis* L1, L3, L19, AH7; *L. acidophilus* A1, A4, A15; *L. leichmannii* LE2, LE3, LE4; *L. delbrueckii* D2, D6, D10; *L. salivarius* SAL1, SAL5, SAL9, SAL10, SAL11, SAL13; *L. fermenti* F1, F15, F38; *L. buchneri* BC1, BC5, *L. brevis* X1, X2, X5; *L. cellobiosus* G1, G2, G3; *L. pastorianus* T1, T9; *Leuconostoc cremoris* NCDO 705, 828, 543; *Ln. lactis* NCDO 534; *Ln. dextranicum* NCDO 529, *Ln. mesenteroides* NCDO 803, 869, 870, 871, 516, 518, 523, 530, 551, 553.

All the test cultures were from a stock collection at the National Institute for Research in Dairying or from the National Collection of Dairy Organisms.

Culture procedures. Lactic streptococci were grown for 18 hr at 30° in 500 ml. MRS broth (de Man, Rogosa & Sharpe, 1960). With the lactobacilli, thermobacteria and *L. fermenti* were grown for 18 hr at 37° in 500 ml. MRS broth (de Man, Rogosa & Sharpe, 1960). The other betabacteria and the streptobacteria were grown for 18 hr at 30°. *Leuconostocs* were incubated for 18 hr at 30° in 500 ml. MRS broth containing 0.05 % cysteine (Dr E. I. Garvie, personal communication). Incubation periods were for 36 to 40 hr for some slow-growing strains of lactobacilli and *leuconostocs* (e.g. F15, X1, BC5, T1, 705).

Preparation of cell-free extracts. The bacteria were harvested by centrifugation, washed three times with chilled physiological saline or 0.1 M-phosphate buffer (pH 8.0) and suspended in 4 to 10 ml. saline. After the addition of Ballotini beads, the bacteria were disintegrated for 15 to 30 min. by Soniprobe type 1130 A (Dawe Instruments Ltd.) the containers being immersed in solid CO₂+methanol (−20°). The extracts were clarified by centrifugation and stored at −20° until required. The extracts contained 10 to 20 mg. protein/ml. estimated by the biuret micro-method (Itzhaki & Gill, 1964) with crystallized bovine plasma albumin as standard.

Electrophoresis of proteins and esterases. The cell-free extracts were analysed by electrophoresis in polyacrylamide gels. The gel preparation, electrophoresis, protein and esterase staining were done mainly according to the procedures described by Lund (1965). Samples were applied by absorbing 6 to 8 µml. of extract on pieces of Whatman 3 MM filter paper and inserting them into the gels. To detect very faint esterase bands as with *Lactobacillus casei* it was necessary to apply 30 to 50 µml. of extract. One % solution of α-naphthyl acetate, -butyrate, -caprylate or -laurate were used as substrates for detecting esterases. The solutions of the caprylate and laurate derivatives contained propylene glycol to give good stability (Gomori, 1953). Fast blue B salt was used as coupling dye to indicate hydrolysis.

Esterase stain. Since pH 8.0 is known to be optimal for the activity of some esterases, the optimum reaction of the staining solution was investigated. When the Fast Blue B salt was dissolved together with substrate in a buffer according to the procedure described by Lawrence, Melnick & Weimer (1960) the best result was obtained at the originally recommended value of pH 6.4, because the dye was rather unstable at the

higher pH value. It was possible and sometimes useful to separate the ester hydrolysis (at pH 6.4 to 8.0 for 1 hr at room temperature) from the following diazo-coupling (at pH 6.4). The apparent rate of hydrolysis by a majority of the esterases examined was practically the same in the range pH 6.4 to 8.0. Test cultures used were *Lactobacillus casei* C5, C9, *L. plantarum* P1, *L. helveticus* H17 and *Streptococcus cremoris* HP. Accordingly, in the present work, the pH value of the staining solution within the above-mentioned range was not so important, because the gel itself contained tris citrate buffer (pH 8.65). When compared with each other as coupling dye, Fast blue B salt gave better colour development than Fast blue BB or RR salts.

Comparison between polyacrylamide and starch gels. Lund (1965) pointed out that faint esterase bands seem to be more sensitively detected on starch gel than on polyacrylamide gel. When we compared the esterase patterns on both gels, using cell-free extracts of *Lactobacillus casei* C9, *L. plantarum* P5, *Streptococcus lactis* ML3, *S. diacetylactis* DRC1, *S. cremoris* 924, and *Leuconostoc cremoris* 828, the same or better results were obtained in the polyacrylamide gel. Polyacrylamide gels could not be used for quantitative work because it was almost impossible to extract the developed colour substance from the gel. Quantitative determinations of esterase activities of *L. casei* C9 were therefore made after starch-gel electrophoresis (Wright & Keck, 1961). The starch-gel slices were flooded separately with the staining solutions containing α -naphthyl acetate or butyrate. The reaction bands were eluted with *n*-amyl acetate+ethanol (1+1 by vol.) and determined colorimetrically at 500 m μ . Relative activities for acetate and butyrate esters were as follows: prominent esterase, 100 and 28; butyrate-specific, minor esterase, 3 and 17.

Growth media. The influence of different media upon the esterase patterns of lactobacilli were examined by using *Lactobacillus casei* C2, C9 and *L. plantarum* P1 as test organisms. Media used were as follows: APT (Evans & Niven, 1951), Rogosa (Efthymiou & Arne Hansen, 1962), MRS broth, MRS broth with glucose decreased to 0.5 % and MRS broth with Bacto peptone instead of Oxoid peptone. No significant differences were detected. Nor was any qualitative difference noted between the esterase patterns of lactic streptococci grown in glucose 1 % Lemco broth (GLB) or in GLB+0.2 % yeast extract (Oxoid). The same pattern was obtained with *Leuconostoc mesenteroides* 869 harvested from MRSB+0.05 % cysteine or from YG citrate broth (Garvie, 1960).

RESULTS

Soluble protein electrophoretic patterns of lactic acid bacteria

Streptococci. The variations in the patterns of soluble protein between the three species of lactic streptococci were small. Representative patterns of *Streptococcus cremoris*, *S. lactis* and *S. diacetylactis* were very similar, although a few protein bands in the *S. lactis* pattern were not detected in that of *S. cremoris*.

Lactobacilli. Most of the species gave consistent characteristic patterns which were different for each species. *Lactobacillus helveticus* and *L. jugurti*, which are closely related to each other, showed similar but not identical patterns and so did *L. lactis* and *L. bulgaricus*, two other closely related species. Six strains of *L. casei* showed identical patterns apart from two very weak bands; there was no difference between patterns of strains belonging to serological groups B and C.

L. plantarum strains showed a similar overall pattern with a few weak bands dif-

ferent. However, the protein patterns of *L. acidophilus* and *L. delbrueckii*, particularly the latter, showed considerable variation between strains: among the heterofermentative strains examined *L. cellobiosus* differed markedly from *L. buchneri*.

Fig. 1. Esterase patterns of lactic streptococci.

Fig. 2. Esterase patterns of thermobacteria.

Esterase electrophoretic patterns of lactic acid bacteria

Streptococci. Most of the lactic streptococci appeared to have a common esterase with a mobility of E_F 62 as shown diagrammatically in Fig. 1, only in the extracts of three strains of *Streptococcus cremoris* was this common band either not detected or

extremely weak. In general the species *S. lactis* appeared to possess a more complex esterase pattern than the species of *S. cremoris*. *Streptococcus diacetylactis*, which is physiologically nearest to *S. lactis*, had the same esterase pattern as this species. Two cultures of *S. cremoris* also had this pattern, but it was later found that these two cultures resembled *S. lactis* in ability to grow in glucose 1 % Lemco broth containing 4 % NaCl, and in fermenting maltose, but resembled *S. cremoris* in being unable to grow in milk at 37° or above. *Streptococcus cremoris* 803 also seemed to be atypical, fermenting and clotting milk at 37°.

Lactobacillus: thermobacteria. Generally speaking, thermobacteria were weak in esterase activity under our experimental conditions. For six (H4, H5, J2, J4, B8, A1) of 28 strains belonging to the eight species tested, no esterases were detected (Fig. 2). Fifteen strains possessed one to four esterases which were generally very weak in activity. *Lactobacillus delbrueckii* D6 and six strains of *L. salivarius* showed a relatively strong band of esterase.

Fig. 3. Esterase patterns of leuconostocs.

All the strains of *Lactobacillus lactis* and *L. leichmannii* showed identical esterase patterns. No species-specific pattern was observed with the following six species: *L. helveticus*, *L. jugurti*, *L. bulgaricus*, *L. acidophilus*, *L. delbrueckii*, *L. salivarius*. Different incubation temperatures, i.e. 48 hr at 25° or 18 hr at 37° had no effect upon the esterase patterns of strains *L. acidophilus* A15 or *L. bulgaricus* B4.

Lactobacillus: streptobacteria. Sixteen of 17 strains of *Lactobacillus casei* showed the same esterase pattern which was characterised by a highly active band at E_F 59. No difference in the esterase patterns was observed among their three varieties, i.e. *L. casei* var. *casei*, var. *rhamnosus* and var. *alactosus*, nor between strains belonging to the different serological groups B and C (Sharpe & Wheeler, 1957). Only one strain, C12, had a distinctive esterase pattern and this strain possessed atypical physiological characteristics, not fermenting mannitol or melezitose. In addition to the above-mentioned prominent band, one or two minor esterases were detected in the extracts of *L. casei*. For example, a very faint esterase band was usually observed at E_F 70.

Species-specific patterns of *L. plantarum* were not consistently obtained; this was in contrast to the observations with *L. casei*.

There are many strains of streptobacteria which grow at low temperature and are homofermentative, but are neither *Lactobacillus casei* nor *L. plantarum* (Naylor & Sharpe, 1958). Some of these unclassified strains were examined; strains A101, K44, D45 and V4 were found to be quite different from *L. casei* in esterase pattern. A few strains (e.g. A41, V5) of this group showed no esterase activity under our conditions.

Generally speaking, the esterase activities of streptobacteria, especially *L. casei*, were higher than those of other lactobacilli with the exception of *L. salivarius* and of lactic streptococci.

Lactobacillus betabacteria. No species-specific esterase pattern was obtained with the following organisms: *Lactobacillus fermenti*, *L. buchneri*, *L. brevis*, *L. cellobiosus*, *L. pastorianus*. The esterase activities were generally weak in these bacteria excepting *L. cellobiosus*. It is of interest that *L. cellobiosus* and *L. salivarius*, both of which were originally isolated from saliva, showed relatively high esterase activities.

Leuconostocs. All three test strains of *Leuconostoc cremoris* had an esterase at E_F 46; this was only weakly active and gave a diffuse zone (Fig. 3). Ten strains of *Ln. mesenteroides* gave two groups by the esterase analysis, four of them had a distinctive esterase pattern, the characteristic feature being a highly active esterase band at E_F 64; all these strains belong to Garvie's (1960) physiological group III. Six other strains of *Ln. mesenteroides* (Garvie's groups V and VI) were variable in esterase pattern, although the ill-defined esterase was generally detected at the same position as that for *Ln. cremoris*. One strain each of *Ln. lactis* and *Ln. dextranicum* examined gave different esterase pattern from the other leuconostocs.

Heat inactivation and inhibition of esterases

The esterases of several lactic acid bacteria (*Streptococcus cremoris* HP, *S. lactis* SC2, *Lactobacillus casei* C2, C6, C9, C10) were inactivated at 60° and 65° for 10 min. Esterases of *S. cremoris* ML3 and *L. casei* C9 were inhibited by 10^{-4} M difluorophosphate (DFP) but resistant to 10^{-4} M *c*-serine sulphate, 10^{-4} M *o*-iodosobenzoate, 10^{-4} M *p*-hydroxy mercuribenzoate and 10^{-3} M-EDTA). These results suggest that these esterases are ali esterases (Augustinsson, 1958, 1961).

Substrate specificities of esterases

Most of the esterases of lactobacilli which were tested had greater activity against α -naphthyl acetate, -butyrate and -caprylate in decreasing order. The esterases of some lactic streptococci hydrolysed α -naphthyl acetate and -butyrate to the same extent. The esterase of *Leuconostoc cremoris* 543 showed greater activity against the butyrate ester than against the acetate. However, one should be careful when estimating the substrate specificity by gel electrophoresis, because it is more difficult for a substrate of low solubility, such as α -naphthyl laurate, to diffuse to the enzyme in the polyacrylamide gel.

Esterase in the culture supernatant fluid of Lactobacillus casei

It was suggested by Stock, Uriel & Grabar (1961) that bacterial esterase and lipase Pollock, 1962; Lawrence, Fryer & Reiter, 1967) may be extracellular enzymes. To investigate this point *Lactobacillus casei* C7 was grown in MRS broth at 37° for 33 hr

and the esterase activities of the samples of the fluid supernatant examined at intervals, by adding ammonium sulphate to samples to 2/3 saturation and examining electrophoretically aqueous solutions of the precipitates in 0.1 M-phosphate buffer (pH 8.0). Esterase activity was not detected in the supernatant fluid of the *L. casei* culture during incubation up to 33 hr. This result suggested that esterase liberation was negligible in a *L. casei* culture. The change of pH value of the culture during growth is presented in Table 1. Such a pH change might have some influence upon the esterase present. When the cell-free extract was adjusted to pH 4.1 with HCl and incubated for 2 hr at 37° the greater part of the esterase was precipitated. This result does not mean that the isoelectric point of the esterase is pH 4.1; co-precipitation with other proteins in the cell-free extract, which contained about 20 mg. protein/ml., might be possible; also, the inactivation of enzymes liberated might not be unexpected at pH 3.8. To eliminate effects of low pH value, a culture of the same organism (*L. casei* C7) was held at pH 6.5 by adding NaOH. Esterase activity was then observed in the supernatant fluid after 17 hr of incubation. The electrophoretic mobility of the liberated esterase was the same as that of the main esterase (E_r 59) found after disrupting the bacteria. The proportion of the esterase activity which appeared in the supernatant fluid was estimated to be only 1 to 3 % of the cell-bound enzyme.

Table 1. *Detection of esterase activity in the supernatant fluid of cultures of Lactobacillus casei strain C7*

		Time of incubation (hr) at 37°			
		8	17	26	33
MRS broth, pH unadjusted	Esterase activity	—	—	—	—
	pH	5.02	4.05	3.83	3.75
MRS broth, pH adjusted	Esterase activity	±	+	+	+
	pH	6.44	6.43	6.53	6.50

* Less than 3 % of the activity of cell-bound esterase.

Effect of trypsin on esterases of Lactobacillus casei

Trypsin destroyed the esterases in the cell-free extract of *Lactobacillus casei* C7. However, when washed whole bacteria were suspended in 0.1 M-phosphate buffer (pH 8.0) containing 2 mg. Armour crystallized trypsin/ml. and incubated at 37° for 4 hr, no change occurred in the esterase activity. Accordingly the esterase in the whole cells seems to be protected from trypsin action.

Esterase activity of whole bacteria

The intensity of the colour reaction produced by a suspension of whole bacteria on paper impregnated with α -naphthyl acetate and 0.02 % of Fast blue salt correlated approximately with the esterase activities in cell-free extracts. About 60 strains of lactobacilli, streptococci and leuconostocs were compared by these two methods. *Lactobacillus casei*, *L. salivarius* and *L. cellobiosus* rapidly produced strong pink to red colours, while the weakly esterase positive or apparently negative strains such as H5, J2, B8, F1, A41 produced little or no colour. These results were further confirmed by using a 0.02 % solution of indoxyl acetate as substrate (Barnett & Seligman, 1951; Clarke & Steel, 1966). Esterase-active organisms such as *L. casei* and *L. salivarius*

produced in 2–3 min. the deep blue colour of indican. Since control suspensions heated to 100° for 2 min. did not produce any colour, it was concluded that the reaction was caused by esterase activity.

DISCUSSION

Similarities in the electrophoretic patterns of soluble proteins such as those observed with the lactic streptococci may indicate similarity in the metabolism of the organisms, which is likely with these three species. The same applies to species of lactobacillus, where similar protein patterns were found within a species. Where there are marked differences of protein pattern within a species, as with *Lactobacillus delbrueckii*, perhaps further differentiation could be made by physiological and biochemical tests.

The esterase patterns confirm that esterase typing can help in the identification of some species of lactic acid bacteria. It seems possible to distinguish between *Streptococcus cremoris* and *S. lactis* by their characteristic esterase patterns. This is very similar to Lund's (1965) finding with *S. faecalis* and *S. faecium*. *Lactobacillus casei* strains showed a distinctive esterase pattern which may be helpful in identifying this species.

Most of the thermobacteria were weak in esterase activity under our conditions and few species-specific patterns were observed. Similarly, in the betabacteria the esterase activities were generally weak and the pattern variable, and would therefore be of little taxonomic value. Strains of *Lactobacillus plantarum* showed two to four bands, with great variation in pattern; Rogosa *et al.* (1953) has already suggested that *L. plantarum* as presently constituted, may not be homogenous.

In the study of *Bacillus thuringiensis*, Norris (1964) found a close correlation between esterase types and groups defined by the possession of H antigen. Although the *Streptococcus cremoris* strains used here could be subdivided into four groups based on their phage relationships and on agglutination with immune whey (Whitehead & Bush, 1957; Reiter, Di Biase & Newbould, 1964), there was no difference in esterase pattern among these subgroups. *Leuconostoc mesenteroides* could be divided by esterase pattern into two main groups which closely correlated to the grouping based on physiological tests proposed by Garvie (1960).

The present results indicate that many lactic acid bacteria possess active esterase. It is difficult to verify the 'true esterase', because non-enzymic proteins of milk and of serum (Downey & Andrews, 1965) and some proteolytic enzymes also show esterase activity (Pickering & Reiter, 1962). However, the esterase activities of lactic streptococci and streptobacteria were destroyed by conventional heat tests for enzymes. Inhibition experiment suggests that the activities of *Streptococcus lactis* ML3 and *Lactobacillus casei* C9 might be due to ali esterases.

The main esterase (E_F 59) of *Lactobacillus casei* C7 was found to be a cell-bound enzyme. Liberation of esterase into the medium was negligible with growth in the ordinary culture medium unless the pH was maintained at 6.15 when a weak activity was detected in the supernatant fluid. However, the cell-bound esterase of *L. casei* appeared to be stable, irrespective of the environmental low pH value, because it remained highly active in cells after prolonged incubation, such as 3 to 5 days at 37°.

The rapid decomposition of α -naphthyl acetate by whole organisms of *Lactobacillus casei* as indicated by the immediate development of colour on addition of α -naphthyl

acetate + staining solution suggests that the esterases of *L. casei* might be situated on or near to the cell surface, because it is unlikely that these substances are incorporated rapidly into the organism. The location of the esterase may be just within the cell wall.

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