PHYSIOLOGICAL CHANGES ASSOCIATED WITH THE APPEARANCE OF SLOW VARIANTS IN CULTURES OF STREPTOCOCCUS LACTIS

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A B S T R A C T

Streptococcus lactis C10 Slow, a variant of the normal 'fast' strain of S.lactis C10, was only capable of rapid and extensive growth in skim milk if casein hydrolysates were added. It was postulated, therefore, that the slow variant is defectively proteolytic.

A sensitive assay of proteolysis, based on the release of radioactivity from iodinated casein, was developed, checked for usefulness with known proteinases, then used to assay the streptococcal enzymes.

Fractionation of the two strains, by either mechanical cell disruption and differential centrifugation or by cell-wall digestion with a muraminidase, established that most of the cell-bound proteinases of the parent strain were surface-bound. This activity was virtually absent in the slow variant.

Partial characterization of the surface-proteinase(s) showed that maximum activity was exhibited at pH 6.0-6.8, in 0.05 M phosphate buffer, at 30-32°C. It was rapidly inactivated at 37°C, both when cell-bound and when free of the cells. Examination of a second pair of strains, S.lactis H1 Fast and S.lactis H1 Slow, indicated a difference in proteinase activity and localization similar to that found between the two S.lactis C10 strains.

It was concluded, on the basis of both nutritional evidence and enzymatic analyses, that the slow variant of S.lactis C10 is limited in skim milk by the supply of amino acids and that this is due to a defective surface-bound proteolytic activity.

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PREFACE

Rapid growth in milk, with the concomitant production of lactic acid, is the primary attribute of the single-strain Cheddar cheese starter. Variations in a strain's activity may be due to changes in the culture's environment or to changes in its capabilities. A clear example of the latter is seen in those strains which segregate slow variants. The variants are stable genetic derivations of the normal 'fast' cells and, when cultured as pure isolates, exhibit limited growth in milk. Their segregation at high frequency, together with their nutritional support by the parental cells, enables accumulation to occur to the point where they contribute the majority of the cells in the culture.

It has been suggested that slow variants are proteolytically deficient in comparison to the parental type. The relationship between starter proteolysis and activity in milk is unclear, but many strains are stimulated by the addition of nitrogenous hydrolysates to the medium.

This investigation was undertaken to examine the hypothesis that slow variants are defectively proteolytic. It was anticipated that the work would also provide useful information concerning the properties and cellular location of starter proteinases in general.

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INTRODUCTION

GENERAL

A small number of strains of Streptococcus lactis and Streptococcus cremoris (the lactic streptococci) are used as 'starters' in the manufacture of Cheddar cheese. Their primary role is to produce lactic acid; they also contribute to cheese flavour (Lowrie, Lawrence, Pearce & Richards, 1972).

A clear-cut difference in activity (rate and extent of acid production in milk) between the cells in some pure cultures of S. lactis was reported by Harriman & Hammer (1931). About 2% of the cells were 'slow coagulators'. The slow characteristic was stable and the variant cultures never segregated the parental 'fast' cells. Analysis of the non-protein nitrogen fraction of 'fast' and 'slow' milk cultures indicated less proteolysis in the latter, although proteolytic activity per cell was not determined. Subsequent work has confirmed: (1) the high frequency with which slow variants appear in susceptible cultures (Garvie & Mabbitt, 1956; Citti, Sandine & Elliker, 1965; Westhoff, Cowman & Speck, 1971); (2) the one-way nature of the transition (Garvie & Mabbitt, 1956; Citti et al., 1965); and (3) the correlation between 'slowness' and relatively low proteolytic activity (Citti et al., 1965; Westhoff et al., 1971). The last point has been the least convincingly shown and its examination forms the basis of the present investigation.

CARBOHYDRATE METABOLISM

The production of lactic acid by starters is the direct

result of energy-yielding carbohydrate catabolism and is the growth function usually measured when starter 'activity' is assessed. The lactic streptococci are part of a large group of micro organisms that carry out the homolactic fermentation. This process, analagous to muscle glycolysis, has been elucidated largely through the use of radioactive tracers and has been thoroughly reviewed (Wood, 1961; Kandler, 1961; Marth, 1962; Busse, 1966). The fate of lactose is as follows:

Most lactic streptococci (including S.lactis C10) accumulate lactose by a phosphoenolpyruvate-dependent phosphotransferase system (McKay, Miller, Sandine & Elliker, 1970), analagous to that of Staphylococcus aureus. The sugar, transported as a phosphate derivative, is split by a 6-phospho-β-galactosidase into glucose and galactose-phosphate, which then enter the Embden-Meyerof-Parnas pathway. The latter is summarized as follows: (Wood, 1961)

$$\text{glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2 \text{ lactate} + 2\text{ATP} + 2\ \text{H}_2\text{O}$$

The products from one mole of lactose are, theoretically, four moles of lactate and four moles of ATP. Analysis of the products of glucose metabolism by homolactic streptococci has shown that small amounts of acetate, formate, CO_2, ethanol, acetoin, and 2,3-butanediol may be formed in addition to lactic acid, their relative proportions depending on culture conditions, particularly the pH. At the least, lactate accounted for 90% of the recovered glucose carbon (Platt & Foster, 1958).

NUTRITIONAL REQUIREMENTS

The rate and extent of acid production by a given single-strain starter reflects its overall ability to grow in the

medium, an ability determined by a complex interaction between the available nutrients, the culture conditions, and the strain's capabilities. In the absence of constraints, such as inhibitors (e.g. penicillin in milk), suboptimal temperatures, and virulent bacteriophage, the activity is probably a function of the strain's ability to obtain essential amino acids from the medium.

Niven (1944) reported that S.lactis required niacin, biotin and pantothenate for growth in a medium complete but for the B vitamins. Thiamin and riboflavin were essential for 18 and 10 strains, respectively, of the 21 examined, while pyridoxine stimulated most. Amino acid requirements were extensive. All strains required valine, leucine, isoleucine, arginine and methionine for growth, but a response comparable to that obtained in the presence of casein hydrolysates required a minimum of 16 amino acids. The only acids without effect were tyrosine, norleucine, aspartic acid, threonine, hydroxyproline and tryptophane. The absolute requirement of all strains for glutamine and asparagine was controversial, Pollack & Lindner (1944) having reported that several strains of S.lactis could use the equivalent acids if they were included at sufficient concentration. Subsequent investigations have substantiated the findings of Niven, minor discrepancies being attributed to the use of different basal media, experimental conditions, and strains. Husain & McDonald (1957) found that S.lactis and S.cremoris required histidine in addition to the acids reported by Niven; however, excellent growth was obtained in the presence of only 12 amino acids, reflecting the basal medium which was improved by the inclusion of Tween 80 (a source of oleate) and acetate. Their finding that S.cremoris was more fastidious than S.lactis for

amino acids was confirmed by Reiter & Oram (1962).

MILK AS A MEDIUM

Bovine milk, the stock substrate for starter maintenance and cheesemaking, appears to contain ample concentrations of protein, lactose, and the essential vitamins (average levels in whole milk, per litre: protein, 35 g; lactose, 49 g; niacin, 0.94 mg; biotin, 31 µg; pantothenate, 3.46 mg; thiamin, 0.44 mg; riboflavin, 1.75 mg) (Webb & Johnson, 1965; Hartman & Dryden, 1965). All the essential amino acids, except methionine, have been found in the non-protein nitrogen fraction of milk (Block, 1951; Van der Zant & Nelson, 1954; Deutsch & Samuelson, 1959; Schwartz & Pallansch, 1962), but the concentrations are generally considered to be insufficient for optimal starter development. The evidence for this contention is indirect and generally unsatisfactory but forms the basis of the "proteinase-dependency" hypothesis. There have been numerous reports of the stimulation of lactic streptococci in milk, particularly of 'slow' strains, by nitrogenous extracts (pancreas extract, yeast extract, corn steep liquor, liver fraction L; see review by Reiter & Moller-Madsen, 1963). The effect of these additives has usually been attributed to peptides but the only complex whose stimulatory component has been identified ("pancreas extract") was effective because of its content of inosine, hypoxanthine, and adenine (Koburger, Speck & Aurand, 1963). The only convincing evidence for stimulation specifically by amino acids and/or peptides was given by Garvie & Mabbitt (1956) who demonstrated that peptone, casamino acids, and acid-hydrolysed casein stimulated both S. cremoris 924 and its slow variant in milk.

STARTER PROTEINASES AND 'SLOWNESS'

There is a sound a-priori basis for the hypothesis that starter growth in milk is dependent on milk protein degredation, and that slow strains and slow variants are special cases of cultures limited by this constraint. The evidence for the importance of starter proteinases in this process is scarce and difficult to assess, due mainly to the very low proteolytic activity of even the fastest of strains.

Proteinases have been purified from the 'soluble' and 'particulate' fractions of sonicated cell suspensions of both S.lactis 3 and its slow variant (Cowman, 1966; Westhoff, Cowman & Swaisgood, 1971; Westhoff, Cowman & Speck, 1971). Quantitative differences in proteolytic activity between the two strains, whether assessed from whole cells or from the fractions, were very low (ca. 1.5-fold). The variant 'intracellular' enzyme did, however, differ in many properties from the equivalent enzyme of the parent strain, and the authors were forced to conclude that the difference in specificity of the proteinase was responsible for the inability of the variant to grow extensively in milk (Westhoff & Cowman, 1971). Much of the published work concerning S.lactis 3 is unsatisfactory, although there can be no doubt that the proteinases have been thoroughly characterized as enzymes. The proteinase assay used was not tested for its sensitivity or reproducibility and specific activities of assayed fractions are rarely stated. In addition to this lack of data for the comparison of activities, the evidence for the localization of the proteinases, essential to a meaningful interpretation of their physiological function, is insufficient, consisting merely of whether or not the activity

remains with the pellet after centrifugation of disrupted cells (Cowman, 1966). The major criticism of the work, however, concerns the lack of essential information relevant to the procedures, a deficiency which precludes both the interpretation of the results and the extension of the methods to other strains.

AIM OF THE INVESTIGATION

The aim of this study was to examine the hypothesis that slow variants are relatively non-proteolytic in comparison to normal 'fast' strains. It was evident from assessment of previous attempts to characterize the proteinase enzyme systems of S. lactis that the work would require a very sensitive assay for proteinase and subsequently a critical determination of the localization of relevant enzymes in the organisms.