Threonine Aldolase Activity in Yogurt Bacteria as Determined by Headspace Gas Chromatography

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The measurement of threonine aldolase activity in whole cell suspensions and cell-free extracts of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, which involves acetaldehyde measurement using headspace gas chromatography (HSGC), is described. Mean relative standard deviations for assays of whole cell suspensions and of cell-free extracts were 14.6% and 7.9%, respectively. The enzyme reached saturation with L-threonine substrate levels of 75 μmol and above. Enzyme activity was linear with increased levels either of whole cell suspension or of cell-free extract in the assay mixture. Significantly (p 0.05) higher threonine aldolase activity was observed in whole cell suspensions of *L. bulgaricus* MR1 compared to that of *S. thermophilus* MS1. Cell-free extracts of the *Streptococcus*, however, had higher activity than those of the *Lactobacillus*. Glycine levels of 20 and 50 μmol in the assay mixture resulted in 85% and 90% inhibition of threonine aldolase activity, respectively, for *S. thermophilus* MS1 but did not inhibit the enzyme activity in *L. bulgaricus* MR1. Addition of 100 μmol of glycine to the *Lactobacillus* resulted in approximately 25% inhibition.

INTRODUCTION

In the typical fermentation of yogurt, the lactic acid starter bacteria *Steptococcus* thermophilus and *Lactobacillus bulgaricus* grow symbiotically, producing lactic acid and acetaldehyde, which is responsible for typical yogurt flavor (Keenan and Bills, 1968; Law, 1981; Lindsay et al., 1965; Tamime and Deeth, 1980; Sandine and Elliker, 1970). An important metabolic precursor for acetaldehyde synthesis by lactic acid bacteria is glucose through the pyuvate and acetyl CoA intermediates of glycolysis (Lees and Jago, 1976a). Amino acids and other metabolites that are converted to pyruvate also may contribute to acetaldehyde synthesis. Certain amino acids such as threonine and methionine may be direct precursors of acetaldehyde in lactic acid bacteria. Threonine is directly converted into acetaldehyde and glycine through the activity of threonine aldolase (E.C. 2.1.2.1) while methionine is metabolically converted to threonine (Law, 1981; Lees and Jago, 1976b, 1978a,b; Shanker, 1977a,b). While it has been reported that lactic acid bacteria, with few exceptions, possess threonine aldolase activity, this enzyme has not been sufficiently characterized. The characteristics of threonine aldolase activity in yogurt microorganisms may be important to flavor biogenesis by these bacteria in single and in mixed culture. A better understanding of the characteristics of this enzyme could provide important information into the control of flavor production during yogurt manufacture.

Characterization of threonine aldolase has been partially hampered by the lack of an assay procedure that provides sufficient sensitivity and convenience for multiple use. Assays for threonine aldolase activity have been primarily performed through estimation of acetaldehyde production from assay mixtures containing threonine and microbial cell extracts. The assay procedure used by Lees and Jago (1976b) involved separation and trapping of acetaldehyde by Conway microdiffusion and formation of a semicarbazone derivative followed by gas chromatographic analysis. Other methods (Veringa and Schrijver-Davelaar, 1970) involved indirect estimation of acetaldehyde by a coupled enzyme method using alcohol dehydrogenase and reduced cofactor nicotinamide adenine dinucleotide (NADH). These techniques are generally tedious and have potential problems associated with losses during the assay due to the volatility of acetaldehyde and the possibility of competing side reactions (Schmidt et al., 1983).

The use of headspace gas chromatography (HSGC) for the estimation of acetaldehyde in cultured dairy products has been reported (Marsili, 1981; Shankar, 1977a). In this technique, volatile components from a sealed, equilibrated vial are directly swept onto a gas chromatography column for analysis, thus avoiding some of the potential problems with sample handling, derivatization, and side reactions. While this technique has not been used in the assay of threonine aldolase, it may have additional advantages in this application. If assay mixtures could be incubated in the sealed vials, potentially all of the acetaldehyde produced by the enzyme reaction would be trapped in the vial. Thus, potential losses, especially with higher enzyme assay temperatures, and the need for trapping and formation of less volatile derivatives of acetaldehyde are avoided. Since HSGC is a direct, comparatively rapid technique and since the advent of precise headspace sampling instrumentation, it may be amenable to multiple, repetitive sampling for enzyme assays under varied conditions. The objectives of this study were, therefore, to evaluate the application of HSGC for the assay of threonine aldolase activity by analysis of acetaldehyde produced by assay mixtures containing threonine substrate and extracts of lactic acid bacteria that were incubated directly in sealed vials.

MATERIALS AND METHODS

Organisms and Culture Conditions. *S. thermophilus* MS1 and *L. bulgaricus* MR1 (Microlife Technics Inc., Sarasota, FL) were grown and maintained in sterile 11% reconstituted nonfat dry milk. A 1.0% inoculum of stock culture was made into Elliker broth (Difco) and incubated at 42 °C for 8 h. After three successive subculturings, a 1.0% inoculum was added to 250 mL of Elliker broth, which was used for preparation of cells for enzyme assay.

Harvesting and Disintegration of Cells. Log phase cells were harvested by centrifugation at 4000g for 10 min. The cell pellet was suspended and washed three times in 0.15 M sodium chloride (pH 7). The cell paste was diluted 1:5 with sodium chloride and used as whole cell suspensions for enzyme assays. Dry cell weight was determined gravimetrically on an aliquot of cell suspension. For experiments involving disintegrated cells, the diluted cell

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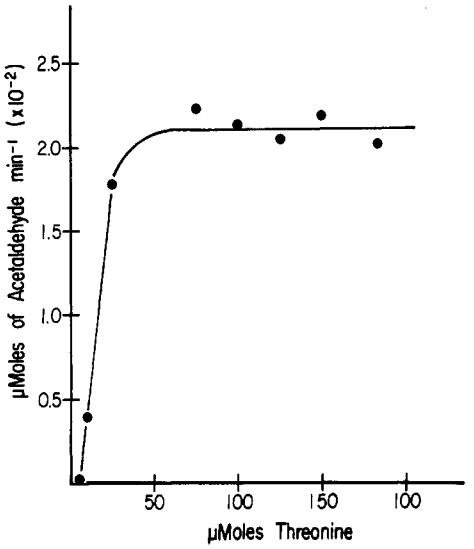

Figure 1. Effect of L-threonine substrate level on threonine aldolase activity of *S. thermophilus* measured by headspace gas chromatography.

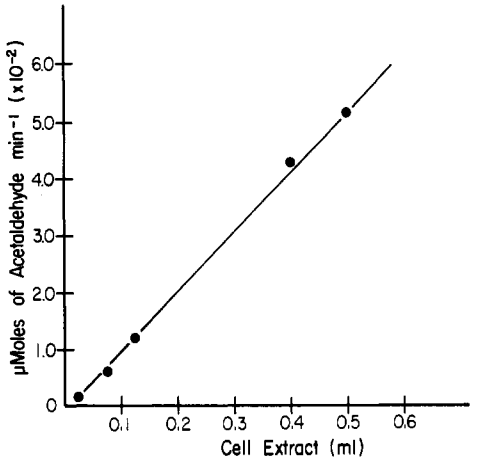

Figure 2. Effect of volume (mL) of *S. thermophilus* cell-free extract on threonine aldolase activity measured by headspace gas chromatography.

suspension was transferred to a dry ice chilled Eaton pressure cell (Eaton, 1962) and stored under dry ice for 3 h. The frozen microbial cells were disintegrated by extrusion through the Eaton cell at a constant pressure of 7.03×10^6 kg/m^2 on a Carver hydraulic laboratory press. Remaining whole cells and cellular debris were removed by centrifugation at 6000*g* for 20 min at 4 °C. The supernatant, cell-free extract was used in enzyme assays. Protein concentration of cell-free extract was determined according to the procedure of Lowry et al. (1951).

Threonine Aldolase Assay. Assay reaction mixtures contained an appropriate level of L-threonine and 23 μmol of anhydrous dibasic sodium phosphate (pH 7.0) in a HSGC sample vial. A 250-μL portion of the enzyme source (cell-free extract or whole cell suspension) was added, the assay mixture was brought to a total volume of 2.0 mL with distilled water, and the vials were sealed.

Assay mixtures, in the HSGC vials, were incubated at 42 °C in a water bath. For assays involving cell-free extracts, a 30-min incubation time was used while 60-min incubation was used for whole cell suspension assays. At appropriate incubation times, sample vials were immediately withdrawn and placed in an ice bath. Blanks contained the reaction mixture without enzyme or contained the enzyme without the L-threonine substrate.

Acetaldehyde was determined by HSGC on a Perkin-Elmer Sigma 3B gas chromatograph equipped with an HS-6 headspace sampling attachment (Perkin-Elmer Corp., Norwalk, CT). Samples were tempered at 70 °C for 30 min, followed by a 30-s pressurization and a 5-s injection. Chromatographic separation of volatile components was done on a glass column (6 ft × $^1/_8$ in.) packed with Porapak S (Supelco Inc., Bellefonte, PA). Analysis was performed isothermally at 130 °C at a flow rate of 30 mL/min. The injector temperature was 200 °C, and the detector temperature was 250 °C. Acetaldehyde estimation was through the use of external standard curves. For comparison purposes, enzyme activity for whole cell suspensions is expressed as nmol of acetaldehyde produced/min per mg of dry cell weight. For cell-free extracts, specific activity is reported as nmol of acetaldehyde/min per mg of protein in extract. Under the assay conditions, a linear relationship (r^2 0.989) between enzyme activity and incubation time to 60 min was observed for assays involving both whole cell suspensions and cell-free extracts.

Table I. Comparison of Threonine Aldolase Activity of *Streptococcus Thermophilus* MS1 and *Lactobacillus bulgaricus* MR1 as Measured in Whole Cell Suspensions and in Cell-Free Extracts

	threonine aldolase act.	
microorganisms	whole cells[a]	cell-free extr[b]
S. thermophilus MS1	2.94 a	4.80 c
L. bulgaricus MR1	5.75 c	2.35 a

[a] Units: nmol of acetaldehyde/min per mg of dry cell weight. Means from triplicate assays from triplicate trials. [b] Units: nmol of acetaldehyde/min per mg of protein. Means from triplicate assays from triplicate trials. Means followed by the same letter are not different (p 0.05).

Statistical Analyses. The degree of replication of tabulated data is given on the tables where appropriate. Data were analyzed by analysis of variance (ANOVA). Calculated mean relative standard deviations (RSD) for nine replicate assays involving whole cell suspensions and cell-free extracts were 14.6% and 7.9%, respectively.

RESULTS AND DISCUSSION

Effect of Substrate and Enzyme Level on Threonine Aldolase Activity. As data for *S. thermophilus* show (Figure 1), enzyme activity followed typical Michealis–Menton behavior with respect to increasing levels of substrate from 5 to 200 μmol of threonine in the assay mixture. Similar results were obtained with whole cell suspensions and for *L. bulgaricus*. Since threonine levels above 75 μmol were sufficient to saturate the enzyme, a substrate level of 125 μmol was used in subsequent assays.

A linear relationship was observed for plots of enzyme activity against milliliters of whole cell suspension or of cell-free extract for the organisms examined. Typical data for *S. thermophilus* cell extracts are presented in Figure 2.

Comparison of Threonine Aldolase Activity for *S. thermophilus* and *L. bulgaricus*. Threonine aldolase activity data for whole cell suspensions and cell-free extracts of *S. thermophilus* MS1 and *L. bulgaricus* MR1 are summarized in Table I. The enzyme activity of whole cells of the *L. bulgaricus* strain was significantly (p 0.05) higher than that of *S. thermophilus* strain used. A reverse observation was made when comparing cell-free extracts of the two organisms where the specific activity of the *Streptococcus* was higher than that of the *Lactobacillus*. It is not possible to speculate as to reasons for these differences in data trends without further research. Since threonine aldolase activity of *L. bulgaricus* whole cells is

Table II. Glycine Effect on Threonine Aldolase Activity of *Streptococcus thermophilus* MS1 and *Lactobacillus bulgaricus* MR1 Cell Suspensions

microorganisms	glycine level, μmol	enzyme act.[a]
S. thermophilus MS1	0	2.94 c
	20	0.35 d
	50	0.20 d
L. bulgaricus MR1	0	5.75 a
	25	6.30 a
	50	5.20 ab
	100	4.10 b

[a] Units: nmol of acetaldehyde/min per mg of dry cell weight. Means from triplicate assays. Means followed by the same letter are not different (p 0.05).

enhanced by addition of glucose, it has been suggested (Lees and Jago, 1976b) that threonine transport into the cell is the rate-limiting step in the metabolic conversion of threonine to acetaldehyde and glycine. Activity data for whole cell suspensions of *S. thermophilus*, however, were not included in that investigation. It is conceivable that differences observed in activity for whole cell suspensions reflect differences in threonine transport rather than differences in enzyme activity between the two organisms.

Effect of Glycine on Threonine Aldolase Activity. On the basis of strong inhibition by glycine of threonine aldolase activity for *S. lactis* subsp. *diacetylactis* DRC3, Lees and Jago (1976b) have suggested that this enzyme has an allosteric function in amino acid metabolism in lactic acid bacteria. Other investigators (Shankar, 1977a) have observed that glycine addition to the growth medium markedly reduced acetaldehyde synthesis by *S. thermophilus* cultures. To investigate glycine effects on threonine aldolase activity for the strains of *S. thermophilus* and *L. bulgaricus* used, various levels of glycine were added to the assay mixture. These data are summarized in Table II. The addition of 20 and 50 μmol of glycine resulted in approximately 85% and 90% inhibition of threonine aldolase activity, respectively, for *S. thermophilus* MS1 cell extracts. Addition of alanine, the next higher homologue of glycine, at a level of 50 μmol did not inhibit MS1 threonine aldolase activity. The threonine aldolase activity of *L. bulgaricus* MR1 was apparently not as affected by glycine addition to the assay mixture. In fact, addition of 100 μmol of glycine was required to significantly affect measurable threonine aldolase activity of bulgaricus. At this glycine level, approximately 25% inhibition of activity was observed. These data may suggest that *S. thermophilus* and *L. bulgaricus* have differing metabolism with respect to threonine aldolase activity, which may account for differences in acetaldehyde synthesis in different growth media by yogurt organisms. However, more definitive research will be needed to determine whether the differences noted are species or strain related.

Data presented suggest that the use of HSGC is amenable to the assay of threonine aldolase activity in yogurt cultures. This technique is a sensitive, rapid alternative to more tedious techniques previously used. We are presently studying effects of various growth parameters on threonine aldolase activity and acetaldehyde synthesis in lactic acid bacteria.

Registry No. Threonine aldolase, 9029-83-8; glycine, 56-40-6.

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