Stimulation of Proteinase Biosynthesis by Canavanine in *Streptococcus faecalis* var. *liquefaciens*¹

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**ABSTRACT**

HAMMEL, JAY M. (The Pennsylvania State University, University Park), AND LEONARD N. ZIMMERMANN. Stimulation of proteinase biosynthesis by canavanine in *Streptococcus faecalis* var. *liquefaciens*. Appl. Microbiol. 14:337–339. 1966.—L-Canavanine, an analogue of arginine, was found to stimulate the synthesis of an extracellular proteinase in *Streptococcus faecalis* var. *liquefaciens*. Cells grown in a synthetic medium containing $10^{-4}$ M arginine and $10^{-4}$ M canavanine produced almost twice as much proteinase as cells grown in $2 \times 10^{-3}$ M arginine alone; total growth was the same in both media. Hydrolyzed proteinase samples were analyzed for arginine and canavanine by means of paper chromatography and electrophoresis. Arginine, but not canavanine, was detected in the purified enzyme sample.

L-Canavanine, a competitive inhibitor of arginine, was found by Hammel and Zimmerman (3) to stimulate the growth of *Streptococcus faecalis* var. *liquefaciens* in a synthetic medium containing arginine. This stimulatory effect was unique, since canavanine was previously found to inhibit the growth of organisms which require arginine. Hammel and Zimmerman suggested that the mechanism of canavanine stimulation involved an inhibition of the arginine desimilase (5), which in turn left available more arginine for protein synthesis.

The data presented in this paper are directed toward two points: (i) can canavanine increase the synthesis of an enzyme; and (ii) is canavanine incorporated into this enzyme?

**MATERIALS AND METHODS**

Cultures. *S. faecalis* var. *liquefaciens*, strain 31, was the experimental organism. Stock cultures were maintained at $-17^\circ$ in litmus milk, whereas the incubation temperature for all experiments was $37^\circ$. Inocula were twice-washed cells; a 1% inoculum of cells grown in A C broth (6) was used for the time-course study, whereas a 2.5% inoculum of cells grown in CSM [casein semisynthetic medium (6)] was employed for data obtained in Table 1.

Enzyme induction and assay. The extracellular proteinase of *S. faecalis* var. *liquefaciens* was the enzyme chosen for these studies. Proteinase biosynthesis was carried out in Erlenmeyer flasks containing 250 ml or tubes containing 10 ml of synthetic media (6), to which the desired amounts of L-arginine or L-canavanine, or both, were added. Samples (10 ml) were removed from the flasks at intervals over a 24-hr incubation period, and enzyme synthesis was stopped with 0.1 ml of 1.0 M NaF; enzyme synthesis in the test tubes was allowed to go to completion before adding the NaF. Cells were removed by centrifugation at 14,500 × g for 5 min, and the supernatant fluids were retained for proteinase assay.

Where large batches of enzyme were necessary for the amino acid incorporation studies, cells were incubated for 24 hr in 4 liters of synthetic media. These cells also were removed (and saved for analysis when indicated) by centrifugation, and the supernatant fluids were saved for proteinase purification as described by Bleiweis and Zimmerman (2).

Enzymatic activity was measured by the method of Anson (1) with a 1% vitamin-free casein solution as the substrate. The mechanics of the assay are reported in detail by Bleiweis and Zimmerman (2).

Hydrolysis of protein. Purified proteinase was transferred to an ampoule and evaporated almost to dryness on a steam bath in a stream of air. A 5-ml amount of 6 N HCl was added, and the proteinase was hydrolyzed as prescribed by Richmond (7). An identical hydrolysis was carried out on the cell cytoplasm. Cells were disrupted in an MSE sonic oscillator, and the cell debris was removed by differential centrifugation.

Footnote:

¹ This research was authorized for publication as paper no. 3068 in the journal series of the Pennsylvania Agricultural Experiment Station, 27 September 1965. The data reported in this paper are taken from a Ph.D. dissertation submitted by Jay M. Hammel to the Graduate School of The Pennsylvania State University.
This supernatant fluid was dialyzed against tap water for 36 hr.

**Chromatography.** Descending chromatography of the hydrolyzed and unhydrolyzed protein was carried out on samples (1.0 to 1.5 mg) in an aqueous mixture of 77% ethyl alcohol and 1% diethylamine for 6 hr. Whatman no. 4 paper was used, and the amino acids were detected by spraying with ninhydrin (0.25% in acetone).

The arginine and canavanine moved as one spot and were eluted with 30% ethyl alcohol until 5 ml was collected. This solution was concentrated to 0.1 ml on a steam bath in a stream of air and saved for paper electrophoresis.

**Paper electrophoresis.** Arginine and canavanine were separated by electrophoresis in 2% ammonium carbonate buffer (pH 9.1; 7). The amino acids were detected with ninhydrin.

**RESULTS**

**Effect of canavanine on proteinase biosynthesis.** Hammel and Zimmerman (3) demonstrated the sparing action of canavanine on the arginine-requiring cells of *S. faecalis* var. *liquefaciens*. In Fig. 1 are found data that apparently show canavanine exercising a similar effect on enzyme synthesis. Curve A depicts the time-course study of proteinase biosynthesis by the growing organism in a synthetic medium containing $2 \times 10^{-2} \text{ M}$ arginine. Curve B represents the values obtained from a similar experiment in which $10^{-4} \text{ M}$ arginine and $10^{-4} \text{ M}$ canavanine replace the higher arginine concentration of the control flask. Although both the control and the experimental flasks have the same number of cells at 24 hr (3), almost twice as much enzyme is synthesized at the lower arginine concentration (with canavanine added).

When equivalent growth is removed as a requirement of the experimental design, data as represented in Table 1 are obtained. Note that canavanine alone neither stimulates growth nor enzyme synthesis. The high arginine concentration is used to demonstrate that this amino acid is involved in neither repression nor feedback inhibition.

**Amino acid incorporation.** Purified enzyme samples from cells grown in the presence of either $2 \times 10^{-3} \text{ M}$ arginine or $10^{-4} \text{ M}$ arginine and $10^{-4} \text{ M}$ canavanine were hydrolyzed and chromatographed. Known samples of arginine and canavanine were always chromatographed with the experimental samples. Arginine moved about 4 cm toward the cathode, whereas canavanine moved about the same distance in the opposite direction.

Hydrolysates of enzyme and cell protein formed in the presence of $2 \times 10^{-3} \text{ M}$ arginine gave distinct spots for arginine after electrophoresis; samples tested before hydrolysis gave no such spots.

Hydrolysates of protein formed in the presence of $10^{-4} \text{ M}$ arginine and $10^{-4} \text{ M}$ canavanine were tested in a similar manner. Only a distinct spot for arginine was observed; canavanine could not be detected.

**TABLE 1. Effect on arginine and canavanine of growth and enzyme biosynthesis**

<table>
<thead>
<tr>
<th>Arginine concn (µmoles/10 ml)</th>
<th>Canavanine concn (µmoles/10 ml)</th>
<th>Growth*</th>
<th>Proteinase†</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>—</td>
<td>.063</td>
<td>130</td>
</tr>
<tr>
<td>—</td>
<td>12</td>
<td>.016</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>.078</td>
<td>375</td>
</tr>
<tr>
<td>92</td>
<td>—</td>
<td>.118</td>
<td>795</td>
</tr>
</tbody>
</table>

* Growth is expressed as Δ optical density units as measured in an Evelyn colorimeter at 620 mµ. † Proteinase activity is reported as micrograms of tyrosine solubilized per 6 ml.

![Fig. 1. Effect of canavanine on proteinase biosynthesis. Curve A = $2 \times 10^{-2} \text{ M}$ arginine; curve B = $10^{-4} \text{ M}$ arginine and $10^{-4} \text{ M}$ canavanine.](image-url)

**DISCUSSION**

Hartman and Zimmerman (4) showed that proteinase biosynthesis of *S. faecalis* var. *liquefaciens* varies as a function of the arginine con-
centration; they report that the amount of proteinase, which is zero when no arginine is present in the medium, increases until the arginine concentration reaches 36 mm. Hammel and Zimmerman (3), investigating the effect of arginine and canavanine on growth, showed that growth is also arginine-dependent, but stimulated by canavanine. In this paper the data seem to be quite clear in demonstrating the ability of canavanine to stimulate increased proteinase biosynthesis; however, the amino acid incorporation experiments are not as unambiguous. The sole use of these data would be inconclusive, because of the non-crystalline nature of the enzyme and a lower detection limit of 10 μg for the amino acid assay. But other lines of evidence tend to substantiate the absence of canavanine from the enzyme molecule. For instance, previous work (4) indicated that it is arginine and not any of its metabolic products that is absolutely necessary for enzyme synthesis. The data presented now show that canavanine cannot substitute for arginine in enzyme synthesis; canavanine affects enzyme synthesis only when arginine is also present, and then this effect is not on a stoichiometric basis. A concentration of 10^{-4} M canavanine and 10^{-4} M arginine gives twice as much enzyme as 2 \times 10^{-3} M arginine. In addition, the canavanine incorporation test is run on cells that have been grown for 24 hr in the presence of canavanine. Certainly the proteinase, which is formed entirely during the course of this experiment, should contain detectable quantities of canavanine if it were substituting for arginine during protein synthesis. It appears likely, then, that canavanine is not incorporated into the enzyme molecule, at least, certainly not at the same rate nor to the same extent as arginine.

The most plausible model to explain the role of the amino acid analogue is the one proposed by Hammel and Zimmerman (3) with respect to the stimulation of growth by canavanine, that is, that canavanine inhibits the arginine desimidase. This inhibition in turn allows for an increased availability of arginine for synthetic purposes.

The increased lag in proteinase biosynthesis that occurs in the presence of canavanine probably is a reflection of the increased lag in growth that was reported by Hammel and Zimmerman (3). In any event, the lag may be explained by assuming a competition between arginine and its analogue for a common amino acid transport enzyme or aminoacyl synthetase. For protein synthesis, however, only arginine and not canavanine, or canavanine at a much slower rate, would be picked up by the arginine soluble ribonucleic acid (sRNA). Such an explanation would require a higher order of specificity from sRNA than from the active sites of enzymes. Those bacteria (7) capable of incorporating canavanine rapidly might also be capable of synthesizing a canavanine sRNA.

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LITERATURE CITED