TABLE 1. *Levels of tryptophanase in constitutive mutants of Escherichia coli**

| Strain† | Units of enzyme/mg of cells‡ | Uninduced/induced | Mutant uninduced/wild induced | Mutant induced/wild induced |
|---|---|---|---|---|
| | | % | % | |
| C6001 W | 24.6 | 2.3 | | |
| C6001 W (I) | 1,053.3 | | | |
| C6001 T | 319.4 | 9.3 | 30.3 | |
| C6001 T (I) | 3,425.4 | | | 3.3 |
| C6001 P | 197.5 | 4.8 | 18.8 | |
| C6001 P (I) | 4,073.2 | | | 3.9 |
| 30SO W | 163.4 | 5.1 | | |
| 30SO W (I) | 3,226.5 | | | |
| 30SO P | 1,106.7 | 8.5 | 34.3 | |
| 30SO P (I) | 12,955.9 | | | 4.0 |
| ML30 W | 3.4 | 0.4 | | |
| ML30 W (I) | 890.2 | | | |
| ML30 P22 | 368.7 | 41.7 | 41.4 | |
| ML30 P22 (I) | 884.0 | | | 0.99 |
| ML30 P19 | 450.6 | 18.9 | 50.6 | |
| ML30 P19 (I) | 2,388.9 | | | 2.7 |
| ML30 P27 | 130.5 | 3.7 | 14.7 | |
| ML30 P27 (I) | 3,561.0 | | | 4.0 |
| ML30 T6 | 92.8 | 2.1 | 10.4 | |
| ML30 T6 (I) | 4,342.5 | | | 4.9 |
| ML30 T11 | 85.1 | 2.3 | 9.6 | |
| ML30 T11 (I) | 3,696.6 | | | 4.2 |
| ML30 T12 | 53.4 | 1.8 | 6.0 | |
| ML30 T12 (I) | 2,924.0 | | | 3.3 |

* Cells were grown at 30 C in medium 56, with glycerol as the carbon source and with or without 0.05% L-tryptophan as the inducer. The cells were filtered on a membrane filter, washed with 30 ml of 0.1 M phosphate buffer (pH 7.4), and resuspended in buffer to a density of 15 and 150 μg/ml of cells (dry wt) for induced and uninduced cells, respectively.

† The letters W, P, and T following the strain numbers denote wild type, mutants isolated by pulse-feeding, and mutants selected at low temperature, respectively. The (I) indicates that the cells were induced.

‡ Tryptophanase was assayed as described by Pardee and Prestidge (Biochim. Biophys. Acta **49**:77, 1961), with the following modifications: 0.5 ml of cells (which had been treated, without shaking, for 10 min with 0.25 ml of toluene at room temperature) were incubated with 0.25 ml of substrate for 30 min, after which 3 ml of Ehrlich's reagent were added. Units of enzyme were determined by diluting a suspension of induced 30SO cells and constructing a standard curve relating optical density at 568 mμ to the dilution factor and setting the activity of the undiluted suspension arbitrarily at 100 units.

episomal gene copies is an unlikely explanation for hyperproduction of tryptophanase. Genetic analyses of the constitutive mutants are now in progress.

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## INTRACELLULAR α-AMYLASE IN *BACILLUS SUBTILIS*

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Although some bacteria release considerable amounts of exoenzymes to the extracellular environment, the activities of most exoenzymes found in the cells is barely detectable, with the exception of penicillinase of *Bacillus subtilis*, recently studied by Kushner and Pollock (J. Gen. Microbiol. **26**:255, 1961) and by Pollock (J. Gen. Microbiol. **26**:239, 1961). In the course of study of the site of the synthesis and excretion of exo-α-amylase of *B. subtilis*, we have obtained a cell preparation in which exo-α-amylase remained.

*B. subtilis* strain K was grown aerobically at 30 C in 500-ml flasks containing 100 ml of medium of the following composition, per liter of tap water: glucose, 1.0 g; citric acid·$H_2O$, 3.0 g; $Na_3$-citrate·$2H_2O$, 6.0 g; Na-glutamate, 8.0 g; $MgSO_4$, 0.5 g; KCl, 1.5 g; $CaCl_2$, 0.1 g; $(NH_4)_2HPO_4$, 10.0 g; ethyl alcohol, 10 ml; yeast extract, 2.0 g; $Zn^{2+}$ (as sulfate), 2 mg; $Fe^{3+}$ (as chloride),

0.2 mg; $Cu^{2+}$ (as sulfate), 0.1 mg; $Mn^{2+}$ (as chloride), 0.02 mg; Mo (as ammonium molybdate), 0.01 mg. $CaCl_2$ and glucose solutions were autoclaved separately.

The culture in the early stationary phase of cell growth, after exo-α-amylase activity had reached 25 to 30 units per ml, was chilled to 0 C by immersing the flask in Dry Ice-alcohol. The cells were then collected in a refrigerated centrifuge, washed four times with cold phosphate buffer (pH 7.3), and suspended in the same buffer at 2 C. The control cell preparation was obtained in the same way except for chilling the culture after leaving the flask at room temperature for 30 min. Exo-α-amylase was assayed by the method of Hagihara (Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ. **2**:35, 1954) in the supernatant liquid of the cell suspension after sedimenting the intact cells by centrifugation. Intracellular α-amylase was assayed in the same way in the cell lysate obtained by incubating the intact cell suspension at 30 C for 30 min with lysozyme (0.3 mg/ml). For preparation of the subcellular fraction, the cells were disrupted by a French pressure cell (Otake Co., Tokyo).

TABLE 1. *Intracellular α-amylase in chilled cells of Bacillus subtilis*

| Phase | Treatment | α-Amylase activity* |
|---|---|---|
| | | *units* |
| Exponential | Immediately chilled (lysate) | 0.2 |
| Stationary | Control (intact cells) | 0.9 |
| Stationary | Immediately chilled (intact cells) | 3.1–4.1† |
| Stationary | Control (lysate) | 1.3 |
| Stationary | Immediately chilled (lysate) | 7.5 |

* α-Amylase activity in 70 mg (wet wt) of cells.

† The α-amylase activity of immediately chilled cells is variable due to release of enzyme during the assay period shown in Fig. 1.

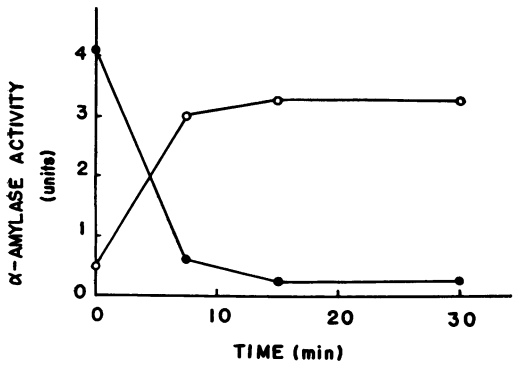


FIG. 1. *Excretion of intracellular α-amylase as a function of incubation time.* (○) *Extracellular α-amylase.* (●) *Intracellular α-amylase.*

TABLE 2. *Distribution of intracellular α-amylase among various subcellular fractions*

| Fraction | α-Amylase activity* | % of total activity |
|---|---|---|
| | *units* | |
| Pellet (10,000 × *g*, 15 min) | 0.17 | 4.4 |
| Pellet (30,000 × *g*, 30 min) | 0.09 | 2.2 |
| Pellet (105,000 × *g*, 90 min) | 0.20 | 5.2 |
| Supernatant (105,000 × *g*, 90 min) | 3.40 | 88.0 |

* α-Amylase activity in 70 mg (wet wt) of cells.

Table 1 shows the intracellular α-amylase in *B. subtilis*. A certain amount of α-amylase was detected only in the lysozyme lysate of the immediately chilled cells from a stationary-phase culture. Much less activity was observed with intact cells, or with a lysozyme lysate of the cells from an exponential-phase culture in which no α-amylase was detectable. Moreover, when the chilled cells were incubated at 30 C, most of the intracellular α-amylase was excreted (Fig. 1). The excretion was not inhibited by KCN or dinitrophenol. It was shown to be irreversible because chilling the cell suspension again after incubation did not cause any binding of released enzyme by the cells. These results suggest that intracellular α-amylase is actually a precursor of the exo-α-amylase, which was trapped inside the cells by rapid chilling.

Table 2 shows the distribution of intracellular α-amylase among the various subcellular fractions obtained by differential centrifugation of the disrupted cells. In contrast to the distribution of penicillinase, most of the intracellular α-amylase existed in a soluble state.

Studies on the mechanism of excretion of exo-α-amylase of *B. subtilis*, using this preparation, and immunochemical or enzymological comparison of the intracellular α-amylase with exo-α-amylase, are now in progress.

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