

EFFECT OF ARGININE ON GRAMICIDIN S BIOSYNTHESIS
BY *BACILLUS BREVIS*

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Effects of arginine on gramicidin S (GS) biosynthesis were investigated by growing *Bacillus brevis* ATCC 9999 in a synthetic medium consisting of 10 g fructose, 0.15 g L-proline, 1.3 g L-histidine, 1.3 g L-glutamine, 0.5 g L-methionine, 1 g L-phenylalanine and six mineral salts per liter.

Supplement of 3 g/liter L-arginine to the medium, especially at the logarithmic phase of growth, enhanced the cell growth and GS production. Twice supplement of 3 g/liter arginine at the beginning and middle logarithmic phase of growth gave much more GS production than any once supplement, but the soluble GS synthetase extractable by lysozyme digestion was remarkably decreased. However, the decrease of enzyme by arginine seemed to be merely an apparent phenomenon, because GS-synthesizing ability of the cell was strongly enhanced by arginine and the enzyme which was not extracted by lysozyme digestion could efficiently be solubilized by ultrasonic homogenization.

In the soluble fraction of cells grown in an arginine-added synthetic medium, no arginine was detected, but a large amount of ornithine was accumulated. When L-ornithine, instead of L-arginine, was added to the synthetic medium, cell growth and GS production were stimulated with increase of its concentration without decrease in the soluble enzyme activity.

For gramicidin S (GS) fermentation, MATTEO *et al.*¹⁾ and VANDAMME and DEMAÏN²⁾ used a chemically defined medium containing amino acids as nitrogen source to study the correlation between the growth of *Bacillus brevis* ATCC 9999 and GS formation. They revealed that six amino acids (L-proline, L-histidine, L-methionine, L-arginine, L-glutamine and L-phenylalanine) had positive effect on growth and GS production and L-phenylalanine acted as a precursor of GS.

NIMI and DEMAÏN³⁾ assessed the relative importance of the individual amino acids in the above synthetic medium and found L-arginine to give notable and paradoxical effects in GS biosynthesis; that is, growth in L-arginine stimulated the antibiotic production but decreased GS synthetase activity in the soluble fraction prepared by lysozyme digestion of the cells.

In the present investigation, the effect of arginine was further examined by changing the concentration and time of addition. While the addition of arginine enhanced the GS productivity of cells, it seemed to change the intracellular location or/and chemical nature of GS synthetase, because the enzyme which was not extracted by lysozyme digestion of the cells could efficiently be solubilized by ultrasonic treatment.

Though L-arginine taken up in the cells was completely deaminated to L-ornithine, L-ornithine added to the medium gave no paradoxical effects.

Materials and Methods

Microorganisms and Cultivation

Bacillus brevis ATCC 9999, a GS producer, was maintained at 4°C as a spore suspension (5.8×10^{10}

spores/ml). *Bacillus subtilis* IFO 13719 was used as a GS bioassay strain.

Medium and Growth Conditions

The basal medium employed in the present study consisted of 10 g of D-fructose, 6.5 g of K_2HPO_4 , 1.7 g of KH_2PO_4 , 203 mg of $MgCl_2 \cdot 6H_2O$, 103 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, 0.27 mg of $FeCl_3 \cdot 6H_2O$, 0.15 g of L-proline, 1.3 g of L-histidine, 1.3 g of L-glutamine, 0.5 g of L-methionine and 1 g of L-phenylalanine per liter of distilled water, pH 7.4.

To stimulate the germination of spores, a 0.1 ml portion of the stock spore suspension was introduced into 100 ml of the 3 g/liter L-arginine-added basal medium in a 300-ml Erlenmeyer flask and heated at 65°C for 15 minutes. After cooling, the flask was shaken on a rotary shaker (220 rpm, 24.5mm diameter orbit) at 37°C until the cell growth reached to 500 KLETT units (about 18 hours). One hundred ml of the broth was inoculated in 1.5 liters of the indicated medium in a 2.5-liter jar fermentor (13.3 cm diameter, 23.3 cm height, equipped with a paddle type impeller). Cultivation was carried out at 37°C with 1 liter/minute aeration and 490 rev/minute agitation. At indicated times, a small amount of broth was taken from the fermentor for determination of growth, pH, GS production and GS synthetase activity.

Analytical Methods

Measurement of growth, GS assay, determination of protein and preparation of crude cell-free extract were as described previously³⁾. For assay of the activity of GS synthetase complex, the previous procedure³⁾ was modified to use L-[¹⁴C]leucine in place of L-[¹⁴C]ornithine, because *B. brevis* cells grown in an arginine-containing medium contained a significant amount of L-ornithine. L-[¹⁴C]leucine was not incorporated into protein fraction during the enzyme assay as reported by KAMBE *et al.*⁴⁾ D-Fructose, L-arginine and L-ornithine were determined according to the methods of SOMOGYI⁵⁾, TOMLISON and VISWANATHA⁶⁾ and CHINARD⁷⁾, respectively.

GS-Synthesizing Ability of Growing Cells

The GS-synthesizing ability of intact cells were determined as follows. Five ml of culture broth at an indicated time was introduced into a large test tube together with 2.5×10^{-6} M chloramphenicol (final concentration). After incubation on a shaker for 5 minutes at 37°C, a half ml of L-[¹⁴C]leucine (7.28 μ Ci/ml, 36.4 μ Ci/ μ mole) was added and shaken for 30 minutes. A half ml of cold 50% trichloroacetic acid (TCA) solution was then added the test tube to stop GS formation and the cells were harvested by centrifugation. The radioactive GS synthesized during 30 minutes shaking was extracted from the cell debris according to the procedures described by KAMBE *et al.*⁴⁾ The 200 μ l of the GS solution obtained was spotted on glass fiber filter (Whatmann GF/F, 25 mm diameter) and was dried up under an infrared lamp. The radioactivity was determined by liquid-scintillation spectrometry.

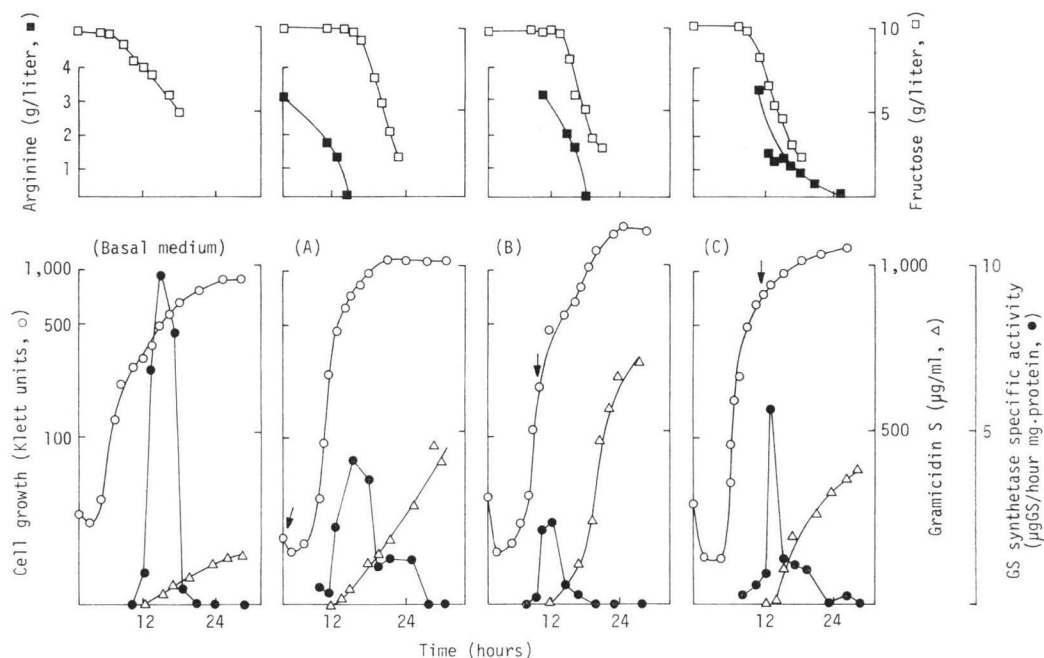
Results

Effect of Arginine on GS Formation and Apparent GS Synthetase Activity

The stimulative effect of arginine on cell growth and GS production were examined by adding the amino acid to the basal medium from 0 g/liter to 10 g/liter. The highest specific productivity of GS was at 6 g/liter of L-arginine (data not shown), but the specific activity of GS synthetase was very low as expected from the previous study³⁾. For further investigation of the stimulative effects, 3 g/liter L-arginine was added to a culture in basal medium at different stages of culture, that is, at the start of culture, the middle logarithmic or late logarithmic stage. The results are shown in Fig. 1. When arginine was added at the logarithmic stage before significant consumption of the fructose occurred. GS formation considerably increased, but GS synthetase activity notably decreased. On the other hand, the addition of arginine at the late logarithmic stage when fructose consumption had started, had no marked effect on GS production.

Fig. 1. Influence of arginine fed at different growth phases on cell growth, GS production and GS synthetase activity by *Bacillus brevis* ATCC 9999.

The culture grown in the basal medium was fed 3 g/liter L-arginine at the arrow point. ○: cell growth, ●: GS synthetase specific activity, △: GS production, □: fructose in the cell-free supernatant of culture and ■: arginine in the cell-free supernatant of culture.



Fate of Arginine Taken up in the Cell

It is important to know whether the arginine effect is caused by arginine itself or a metabolite. The free amino acids in the cells were compared between a culture in basal medium and that in 6 g/liter arginine-added basal medium. The cells were harvested from 8 hours culture broths which usually gave the highest GS synthetase specific activity. After extracting the cells with boiling water, the extract was treated with TCA in 6% final concentration and centrifuged at $4000 \times g$ for 15 minutes. After removal of TCA by extraction with ethyl ether, the supernatant was adjusted to pH 7.0 and subjected to amino acid analysis. As shown in Fig. 2, five amino acids were detected in both kinds of cell, but, remarkably, only the cells grown in the L-arginine-supplemented medium contained a significant amount of L-ornithine. Unexpectedly, no L-arginine was detected in either type of cell. These results suggest that L-arginine in the soluble fraction is easily deaminated.

Fig. 2. Amino acid profile in cell extracts of *B. brevis* grown in basal medium or 6 g/liter L-arginine-added basal medium.

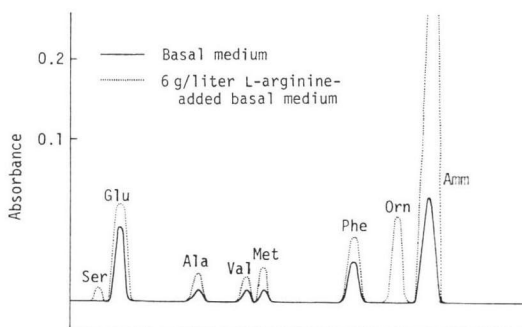


Fig. 3. Effects of L-ornithine on cell growth, GS production and GS synthetase activity. 3 g/liter (A) or 9 g/liter (B) L-ornithine was added to the basal medium. ○: cell growth, ●: GS synthetase specific activity and △: GS production.

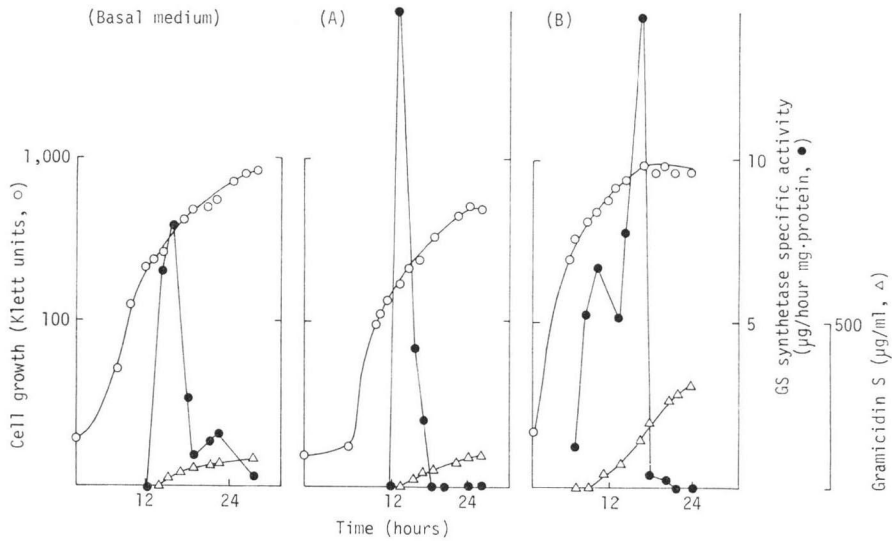
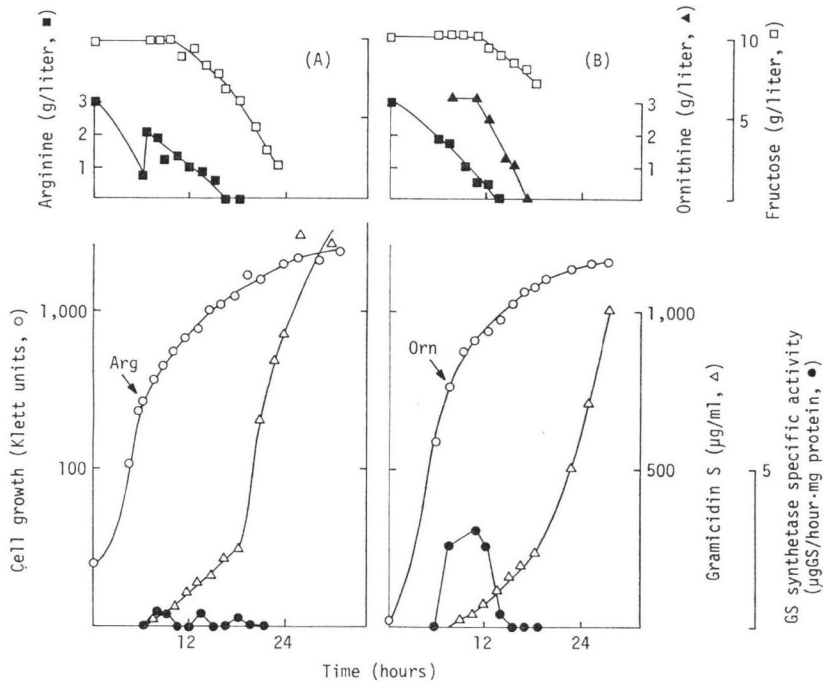


Fig. 4. Feeding effects of L-arginine and L-ornithine on cell growth, GS production and GS synthetase activity.

The culture in 3 g/liter L-arginine-added basal medium was fed another 3 g/liter L-arginine (A) or 3 g/liter L-ornithine (B) at the arrow point.

▲: ornithine in the cell-free supernatant of culture and the other symbols are the same as in Fig. 1.



Effect of L-Ornithine on GS Fermentation

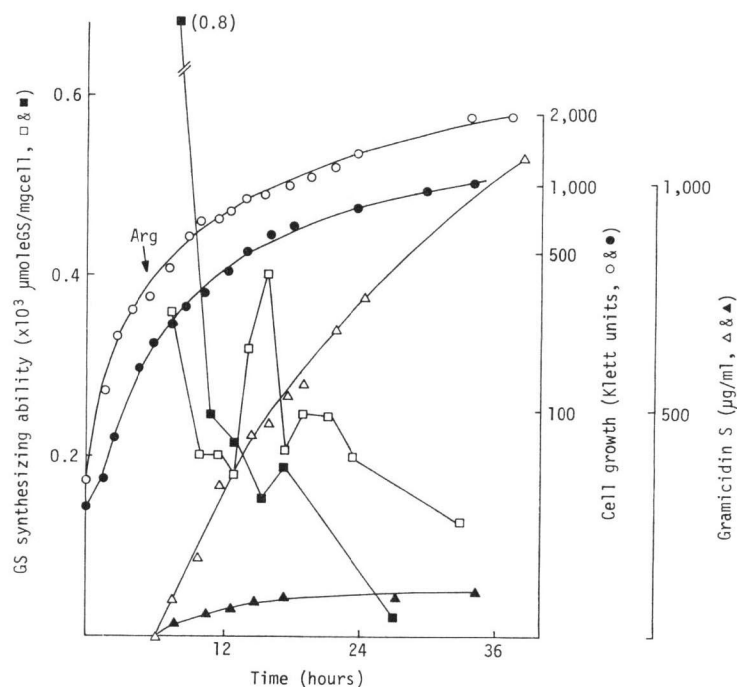
Since L-arginine is deaminated to L-ornithine in the cell, investigations were performed to test the effect of L-ornithine on cell growth, GS production and GS synthetase activity. The seed culture was prepared with a 3 g/liter L-ornithine-added basal medium. The fermentation was carried out by using the basal medium and 3 g/liter or 9 g/liter L-ornithine-added basal medium. As shown in Fig. 3, the cell growth in the 3 g/liter L-ornithine medium (A) was a little poorer than that in the basal medium. But in 9 g/liter L-ornithine medium (B), the growth and production were stimulated to some extent. On the other hand, the specific activity of soluble GS synthetase was remarkably high in the ornithine medium as compared with the culture using the basal medium (Fig. 3) and L-arginine (Fig. 1-A), suggesting that the apparent suppression of the enzyme activity in an arginine-added medium was not ascribable to the ornithine accumulated in the cells.

Another comparison was made by feeding additional 3 g/liter L-arginine or 3 g/liter L-ornithine to the culture grown in a 3 g/liter L-arginine-added basal medium at 6 to 7 hours after inoculation. The results are shown in Fig. 4. Feeding of L-arginine and L-ornithine both gave similar cell growth and GS production. However, feeding of L-arginine brought a strikingly low activity of soluble GS synthetase in contrast with feeding of L-ornithine. From Fig. 1 and 4, it seems likely that the paradoxical effect of arginine is induced by a high concentration of extracellular arginine before the beginning of fructose consumption.

Effect of Arginine on GS-synthesizing Ability of the Cells

The suppressive effect of arginine on the soluble GS synthetase activity can be attributed to neither

Fig. 5. Effect of L-arginine on GS synthesizing ability of the growing cells. Closed marks show the ability of the cells grown in a basal medium. Open marks show the ability of the cells grown in a 3 g/liter L-arginine-added medium which was fed another 3 g/liter L-arginine at 6 hours after inoculation.



inhibition of enzyme activity nor the repression of enzyme formation, because GS can be produced even by the cells with a very weak apparent activity of the enzyme. Since GS synthetase is indispensable for GS production, the true enzyme activity in the cells may be estimated from the amount of GS which is synthesized when the five amino acids composing GS are administered extracellularly. The five amino acids were given to the resting cells harvested from the logarithmic phase. Only a small amount of the amino acids was taken up into the cells during 30 minutes. On the other hand, L-leucine and L-valine among these amino acids were taken up by the growing cells in 3 g/liter L-arginine-added basal medium (data not shown). Then, by using L-[¹⁴C]leucine as a tracer, the ability of the growing cells to synthesize GS from the intracellular precursors was determined at the various stages of growth by the procedure described in "Materials and Methods".

GS-synthesizing ability was determined by using two kinds of cells. One was grown in the basal medium. The other was grown in a 3 g/liter L-arginine-added basal medium and the culture was fed another 3 g/liter L-arginine at 6 hours after inoculation. The results are shown in Fig. 5. The higher GS-synthesizing ability appeared after middle logarithmic phase in the case of addition of L-arginine and was maintained for a longer period during fermentation as compared with the arginine-non-containing culture. This leads us to the conclusion that the cells grown in a high concentration of arginine surely contain a significant amount of GS synthetase as long as they produce GS.

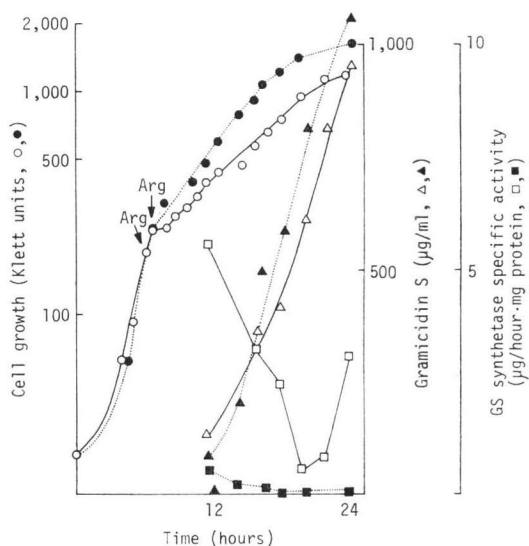
GS Synthetase Extractable by Sonication

It was inferred from the above results that arginine in the medium acted to modify GS synthetase to a form non-extractable by lysozyme digestion. Then, ultrasonic treatment, instead of lysozyme digestion, was applied for preparation of the cell free extract. The cells were grown in a 3 g/liter L-arginine-added basal medium and another 3 g/liter L-arginine was fed at 6 hours after inoculation. At indicated times, 50 ml of the culture broth was taken for determination of cell growth, GS production and GS synthetase activity. The cells were treated by ultrasonic homogenizer for 3 minutes at pH 7.7, followed by centrifugation at $3000 \times g$ for 15 minutes in cold. The enzyme activity in the supernatant was measured. The result is shown in Fig. 6 together with data obtained by lysozyme digestion. A significant enzyme activity was observed in the logarithmic phase cells although only a little enzyme activity was found when lysozyme digestion was applied. But it should be pointed out in Fig. 6 that there was still an inconsistency between the detected enzyme activity and GS production.

Fig. 6. Extraction of GS synthetase by ultrasonic treatment from the cells grown in a 3 g/liter L-arginine-added basal medium and one which was fed another 3 g/liter L-arginine after 6-hour cultivation.

Closed marks and dotted lines show the culture in 3 g/liter L-arginine-added basal medium. Open marks and straight lines show the culture in 3 g/liter L-arginine-added basal medium which was fed another 3 g/liter L-arginine after 6-hour cultivation.

○ or ●: cell growth, △ or ▲: GS production and □ or ■: GS synthetase specific activity.



Discussion

Arginine stimulated the growth and GS production in the GS fermentation by *B. brevis*. But, since no arginine was detected in the cell extract while ornithine was accumulated, the arginine was probably deamidated to ornithine in the cells. Although L-ornithine was less favorable for cell growth than L-arginine (Fig. 1-A and Fig. 3-A), it could stimulate GS production to a similar extent as arginine when it was added to the cultures grown in an arginine medium at logarithmic phase (Fig. 4). These results suggest that stimulation of GS production in an arginine medium is caused by ornithine and/or its metabolites in the cells, but not by arginine.

Arginine in the medium acted to decrease the apparent activity of GS synthetase extractable by lysozyme digestion, whereas ornithine did not. Since arginine is immediately deamidated to ornithine in cell, the apparent suppression of the enzyme activity is considered to be brought about by the extracellular arginine. This suppressing mechanism remains to be proven. When ultrasonic treatment was employed for preparation of cell-free extract, much more GS synthetase activity was detected in the cells as compared with lysozyme digestion. This phenomenon seems to indicate that the GS synthetase bound to particle fraction of the cell was liberated to soluble fraction by this treatment. DEMAIN *et al.*⁸⁾ and POIRIER and DEMAIN⁹⁾ reported recently that arginine stimulated specific GS formation by increasing GS synthetase-specific activity of the particle fraction of the cell, and that although the activity specific to soluble fraction decreased when cells were grown with arginine, the specific activity of the pellet fraction almost tripled and treatment of the cell pellets with Triton X-100 resulted in extraction of the insoluble heavy GS synthetase activity. These results are well supported by data in this paper. It is noteworthy in the present investigation that a paradoxical effect of arginine was observed when the concentration of arginine in the culture was high before the consumption of fructose started.

Though GS synthetase was extracted by ultrasonic treatment more effectively than lysozyme digestion, there was still an inconsistency between the detected enzyme activity and GS production (Fig. 5). It means that there is the enzyme non-extractable even by ultrasonic treatment. Further study is required for elucidation of this unique behavior of the enzyme.

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