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Note

Alkaloid Production during the Cultivation with Shaking of *Claviceps* sp.: Effects of Asparagine

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Among the nitrogen sources tested, asparagine stimulated alkaloid production maximally. Ammonium salts supported alkaloid production poorly. During the cultivation with shaking of *Claviceps* sp. strain SD-58 in asparagine containing medium, the activity of asparaginase increased during the exponential growth (up to 8 days) with the intracellular accumulation of ammonium ions. Among the ammonia-assimilating enzymes we studied, $NADP^+$-glutamate dehydrogenase (GDH) had a higher activity in the growth phase (up to 6 days), while in the intensive alkaloid producing phase (after 6 days) the activity of glutamine synthetase was higher. The latter was associated with increases in the intracellular level of tryptophan and alkaloid production. The levels of $NADP^+$- and NAD^+-alanine dehydrogenases and glutamate synthase were negligible.

The effects of various nitrogen sources on the yield of ergot alkaloids have been investigated.[1–3] However, we still do not know all of the nitrogen effects at the cellular level on alkaloid biosynthesis. In this manuscript, which is an extention of our work on the physiology of alkaloid biosynthesis,[4–9] we report on the effects of nitrogen sources and the role of asparagine on alkaloid production during the cultivation with shaking of *Claviceps* sp. strain SD-58.

Materials and Methods

Organism and cultivation *Claviceps* species, strain SD-58 (ATCC 26019) from the American Type Culture Collection, Md., USA, was maintained on potato dextrose agar slopes by subculturing every two weeks at 25°C for 5 days and storing at 5°C.

Inoculum preparation, NL-406 medium composition, and shake cultivation conditions (200 rpm) were the same as described earlier.[7–9] To study the effects of nitrogen source on alkaloid production, nitrogen sources were omitted from the medium (NL-406) and the indicated nitrogen source was added to give a nitrogen content of 4.24 g/*l*. The amino acids added were L-isomers.

Cell-free extracts Cell-free extracts were prepared as described earlier.[4–6] Mycelia were harvested on sintered glass funnels by vaccum filtration. Mycelial pads were washed with distilled water, frozen to −10°C, and disrupted in a pre-cooled X-press (Biotec, Sweden) at 100 MPa pressure. A 20% (w/v) extract was prepared in 50 Mm Tris-HCl buffer (pH 7.2) and was centrifuged at 20,000 × *g* for 30 min. The supernatant was used for enzyme assays.

Enzyme assays The spectrophotometric measurements were made on a Shimadzu double beam spectrophotometer, UV-150-02 (Japan). Unless otherwise specified, the initial rates of enzyme reactions were measured at 30°C and one unit represents the amount of enzyme which brought about the formation of 1 μmol of product per minute under the given experimental conditions. $NADP^+$ glutamate dehydrogenase (EC 1.4.1.4), NAD^+-glutamate dehydrogenase (EC 1.4.1.3) and glutamate synthase (EC 2.6.1.53) were measured by the methods of Desai and Modi[11] and Roon *et al.*,[12] respectively. The method of Elliot[13] was followed for the measurement of glutamine synthetase (EC 6.3.1.2) and asparagine synthetase (EC 6.3.1.1.). The methods described by Schwartz *et al.*,[14] and Thomulka and Moat[15] were used to assay asparaginase (EC 3.5.1.1) and NAD^+- and $NADP^+$-alanine dehydrogenases (EC 1.4.1.1. and

EC 1.4.1.2, respectively).

Analytical methods The method of Allport and Cocking[16] was used for the measurement of alkaloids, using elymocalvine as a standard. Intracellular free tryptophan and ammonium ions were measured by the methods of Spiess and Chambers[17] and Fawcett and Scott,[18] respectively. Protein was estimated by the method of Lowry *et al.*,[19] using bovine serum albumin as a standard. The results listed here are the average values from at least three independent experiments.

Results and Discussion

Figure 1 illustrates the effects of nitrogen source on alkaloid production and growth of *Claviceps* sp. strain SD-58. Asparagine stimulated alkaloid production the most among the nitrogen sources tested. Ammonium salts were a poor nitrogen source. When an inorganic nitrogen source (ammonium salts) were partially or totally replaced by other organic nitrogen sources, *i.e.* asparagine, aspartate, or glutamate, about a 1.5- to 3-fold increase in alkaloid yield and a substantial increase in mycelial dry weight were observed. Although different nitrogen sources gave different alkaloid yields, the fermentation cycle and growth cycle were not affected and maximum alkaloid production was attained with 12 days of fermentation. Thus, it is reasonable to believe that the nitrogen source affects the rate of alkaloid synthesis. Our results are in line with those reported for production of antibiotics.[20,21]

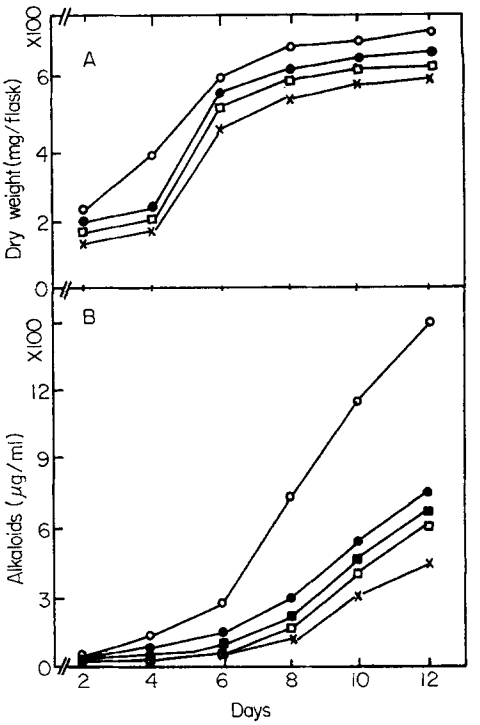

Fig. 1. Effects of nitrogen sources on growth (A) and alkaloid production (B) by *Claviceps* sp. strain SD-58. Cells were grown in NL-406 medium. (□) and NL-406 medium from which the nitrogen source was omitted and with the incorporation of asparagine (○), aspartic acid (●), ammonium nitrate (×), and aspartic acid+ammonium nitrate as 1 : 1 ratio (■). The nitrogen content of the medium was kept constant 4.24 g N/*l*.

The activity of asparaginase, alkaloid production, and intracellular ammonium ion concentration during the submerged cultivation of *Claviceps* sp. strain SD-58 are illustrated in Fig. 2. The activity of asparaginase increased up to the end of exponential growth (8 days). The activity of asparaginase decreased after 8 days of fermentation and was associated with intensive alkaloid production. The intracellular level of ammonium ions was parallel to the activity of asparaginase and accumulated during the exponential phase of growth. The rapid use of intracellular ammonium ions during the intensive alkaloid producing phase of the growth was observed. The level of asparagine synthetase remained much lower

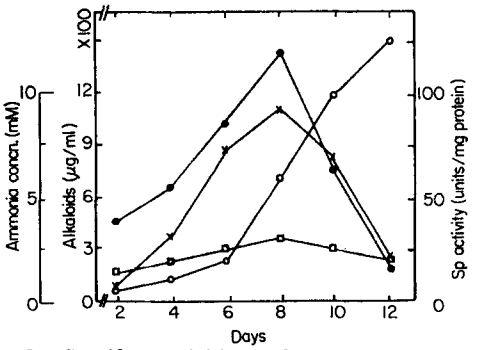

Fig. 2. Specific activities of asparaginase (●), asparagine synthetase (□), intracellular ammonium ion concentration (×), and alkaloid content (○) during the submerged cultivation of *Claviceps* sp. strain SD-58 in asparagine medium.

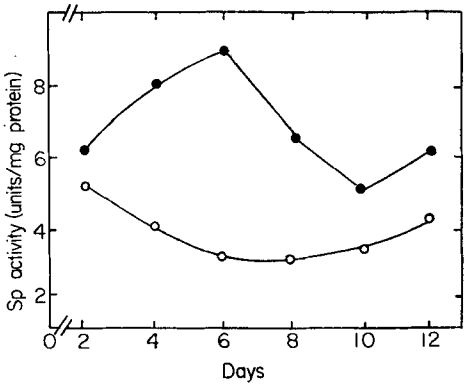

Fig. 3. The levels of NADP+-glutamate dehydrogenase (●) and NAD+-glutamate dehydrogenase (○) during the submerged cultivation of *Claviceps* sp. strain SD-58 in asparagine medium.

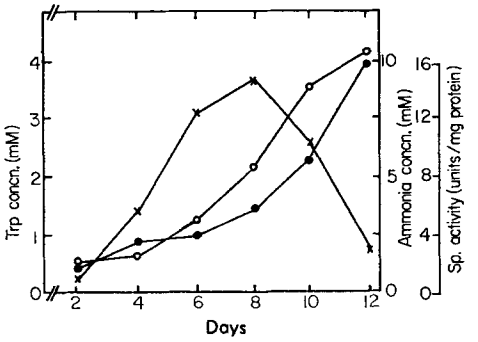

Fig. 4. Specific activity of glutamine synthetase (○) and intracellular concentration of tryptophan (●) and ammonia (×) during the cultivation with shaking of *Claviceps* sp. strain SD-58 in asparagine medium.

than asparaginase and almost constant throughout the fermentation cycle (Fig. 2).

Since ammonium ions have strong effects on the biogenesis of many secondary metabolites[22-24] and a drastic fall in intracellular level of ammonium ions was associated with the intensive alkaloid production (Fig. 2), the activity of enzymes concerned with ammonia assimilation was investigated during alkaloid production by *Claviceps* sp. strain SD-58. As depicted in Fig. 3, the level of NAD+–GDH was lower than that of NADP+–GDH. The activity of NADP+–GDH increased up to 6 days of fermentation and declined later. The activities of NADP+–alanine dehydrogenase (1–2 units/mg prot.), NAD+-alanine dehydrogenase (0.3–0.8 units/mg prot.) and glutamate synthase (1–2 units/mg prot.) were very low and not much altered during the entire fermentation cycle. The parallel relationship between the activities of NADP+–GDH and asparaginase up to 6 days of growth suggested that during the growth phase the major assimilatory route of ammonia, a product of asparaginase, might be through NADP+–GDH. Several investigators have failed to detect glutamate synthase in a variety of eukaryotes including fungi.[25-27] We demonstrated the absence of NAD+-alanine dehydrogenase in *Claviceps purpurea* (Fr.) Tul.[10]

The fall in the intracellular level of ammonium ions durnig intensive alkaloid production and the rise in glutamine synthetase activity can be correlated (Fig. 4). Ammonium ion is one of the substrates for glutamine synthetase and the enzyme is involved in the regulation of macromolecules in bacteria[28] and fungi.[29] Glutamine has been reported as an amino donor for the synthesis of other compounds including tryptophan,[5,30] as an ergoline ring precursor and inducer for alkaloid synthesis.[31-34] The level of tryptophan (Fig. 4) in the cell during cultivation with shaking of *Claviceps* sp. SD-58 showed dynamics parallel to the activity of glutamine synthetase.

Thus, the results indicated that asparagine is prominant in expression of the *Claviceps* species, strain SD-58 genotype. The data also suggested that the metabolism of asparagine *via* asparaginase resulted into the accumulation of ammonium ions in the early phase of fermentation. Ammonium ions might be assimilated through NADP+–GDH for cellular growth and metabolized through glutamine synthetase during the intensive alkaloid producing phase resulting in higher tryptophan synthesis. The accumulation of tryptophan in turn may be one of the factors for overproduction of alkaloids.

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References

1) Banks, G. T., Mantle, P. G., Szezyrbak, C. A.: *J. Gen. Microbiol.*, **82**, 345 (1974).
2) Robbers, J. E., Eggert, W. F., Floss, H. G.: *J. Nat. Prod.*, **41**, 120 (1978).
3) Formal, C., Wojcek, W. J., Radvlovacki, M., Schlossberger, H. G.: *Pharma. Biochem. Behav.*, **11**, 319 (1979).
4) Desai, J. D., Desai, A., Shah, S.R.: *Folia Microbiol.*, **27**, 245 (1982).
5) Vaidya, H. C., Desai, J. D.: *Folia Microbiol.*, **28**, 12 (1983).
6) Vaidya, H. C., Desai, J. D.: *Ind. J. Expt. Biol.*, **20**, 475 (1982).
7) Vaidya, H. C., Desai, J. D.: *Ind. J. Biochem. Biophy.*, **20**, 222 (1983).
8) Desai, J. D., Desai, A. J., Patel, H. C.: *Appl. Environ. Microbiol.*, **45**, 1694 (1983).
9) Patel, H. C., Desai, J. D.: *Experientia.*, **41**, 96 (1985).
10) Rehacek, Z., Desai, J.D., Pazoutova, S.: *Can. J. Microbiol.*, **23**, 596 (1977).
11) Desai, J. D., Modi, V. V.: *Experientia.*, **32**, 423 (1976).
12) Roon, R. J., Even, H. L., Larimore, I.: *J. Bacteriol.*, **118**, 89 (1974).
13) Elliot, W. H.: *Methods Enzymol.*, **2**, 337 (1955).
14) Schwartz, J. H., Reeves, J. Y., Broomf, J. D.: *Proc. Natl. Acad. Sci., (USA)*, **56**, 1516 (1966).
15) Thomulka, K. W., Moat, A. G.: *J. Bacteriol.*, **109**, 25 (1972).
16) Allport, N. L., Cocking, T. T.: *Q.J. Pharm. Pharmacol.*, **5**, 341 (1932).
17) Spiess, J. R., Chambers, D. C.: *Anal. Chem.*, **20**, 30 (1948).
18) Fawcett, J. F., Scott, J. E.: *J. Clin. Pathol.*, **13**, 156 (1960).
19) Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: *J. Biol. Chem.*, **193**, 265 (1951).
20) Sanchez, S., Paniagva, L., Mateos, R. C., Lara, F., Mora, J.: *Adv. Biotechnol.*, **3**, 147 (1981).
21) Inoue, S., Nishizawa, Y., Nagai, S.: *J. Ferment. Technol.*, **61**, 7 (1983).
22) Poirier, A., Demain, A. L.: *Antimicrob. Agents. Chemother.*, **20**, 108 (1981).
23) Maier, W., Erge, D., Schimann, B., Groger, D.: *Biochem. Biophy. Res. Commun.*, **99**, 155 (1981).
24) Nimi, O., Kobata, H., Sugiyama, M.: *J. Antibiotic.*, **35**, 615 (1982).
25) Branchley, J. E., Prival, M. J., Magasanik, B.: *J. Biol. Chem.*, **248**, 6122 (1973).
26) Desai, J. D., Modi, V. V.: *Experentia.*, **33**, 1952 (1977).
27) Tempest, D. W., Meerj, J. L., Brown, C. M.: *Enzymes of Glutamine Metabolism*, (Prvsiner, S., Stadtman, E. R.), p. 167, Academic Press, New York (1978).
28) Tyler, B., Beleo, A., Magasani, B.: *Proc. Natl. Acad. Sci. (USA)*, **71**, 225 (1974).
29) Dunn Coleman, N. S., Garrett, R. H.: *Mol. Gen. Genet.*, **179**, 25 (1980).
30) Zalkin, H., Murphy, T.: *Biochem. Biophys. Res. Commun.*, **67**, 1370 (1975).
31) Taber, W. A., Vinning, L. C.: *Can. J. Microbiol.*, **4**, 611 (1958).
32) Arcamone, F., Chain, F. B., Ferretti, A., Minghetti, A., Panella, P., Tonolo, A.: *Biochim. Biophys. Acta*, **57**, 174 (1962).
33) Floss, H. G., Mothes, U.: *Arch. Microbiol.*, **48**, 213 (1962).
34) Krupinski, V. M., Robbers, J. E., Floss, H. G.: *J. Bacteriol.*, **125**, 158 (1976).

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