Formation of L-Asparaginase by *Fusarium* Species

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(Received 14 September 1972)

SUMMARY

L-Asparaginase was formed in the culture filtrates of a number of *Fusarium* species, as well as in those of ascomycetous fungi having a *Fusarium* imperfect state, such as species of *Hypomyces* and *Nectria*. Species of *Gibberella*, though having a *Fusarium* state, formed little L-asparaginase. The distribution of the ability to form the enzyme was related to taxonomic position.

INTRODUCTION

Occurrence of L-asparaginases (L-asparagine amidohydrolase, EC. 3.5.1.1) in fungi, yeasts, bacteria and animal cells, and their antitumour effects were reviewed by Adamson & Fabro (1968) and Wriston (1971). With respect to the occurrence of L-asparaginase in fungi, Dox as early as 1909 found the activity in *Penicillium camemberti*. In 1928 and 1930, L-asparaginase of *Aspergillus niger* was studied by Bach and Schmalfuss & Mothes. Recently, De-Angeli *et al.* (1970) reported on L-asparaginase of *A. terreus*; the enzyme suppressed the growth of Walker 256 ascites sarcoma in rats. Scheetz, Whelan & Wriston (1971) studied the properties of L-asparaginase purified from mycelia of *Fusarium tricinctum*, and pointed out the inability of the enzyme to suppress the growth of 6C3HED lymphosarcoma in mice. Arima, Sakamoto, Araki & Tamura (1972) examined the extracellular formation of L-asparaginase by a variety of micro-organisms and indicated that the enzyme was produced by several species of *Penicillium*, *Aspergillus*, and *Nectria*. We have studied the distribution of L-asparaginases among micro-organisms and have found them in Ascomycetes and Fungi Imperfecti as well as in bacteria and yeasts (Imada, Nakahama, Igarasi & Isono, 1973). In that study we noticed that a large proportion of *Fusarium* species produced extracellular L-asparaginase. In the present report, studies on the distribution of L-asparaginase in species of the genus *Fusarium* and in related genera have been undertaken to investigate further the correlation between the distribution of enzyme activities and the classification of micro-organisms.

METHODS

Organisms. All the fungal strains used in the present experiments were cultures held at the Institute for Fermentation, Osaka, Japan.

Cultivation. Fungal strains were grown on agar slants of M medium (pH 6.0) which contained (g/l distilled water): sucrose, 30; Polypepton (Daigo Nutritive Chemicals, Osaka, Japan), 5; beef extract (Wako Pure Chemicals, Osaka, Japan), 2; yeast extract (Difco

Table 1. *L-Asparaginase formation by Fusarium species after 3 and 5 days of culture*

Strain	Activity (i.u./ml)	
	3-day	5-day
<i>Fusarium anguioides</i> IFO4467	0.46	0.15
<i>F. caucasicum</i> IFO5979	0.65	2.03
<i>F. culmorum</i> IFO5902	0.55	0.62
<i>F. culmorum</i> IFO6814	0.09	0.08
<i>F. oxysporum</i> IFO5942	0.14	0.31
<i>F. oxysporum</i> IFO7152	0	0.23
<i>F. oxysporum</i> IFO7155	0.37	0.08
<i>F. oxysporum</i> IFO7156	0.51	0.23
<i>F. oxysporum</i> IFO7157	0.19	0.08
<i>F. oxysporum</i> IFO7190	0	0.31
<i>F. oxysporum</i> IFO5264	2.59	2.95
<i>F. oxysporum</i> IFO9331	1.94	2.50
<i>F. oxysporum</i> f. <i>batatas</i> IFO4468	0.14	0.23
<i>F. oxysporum</i> f. <i>conglutinans</i> IFO6383	0.19	0.08
<i>F. oxysporum</i> f. <i>cucumerinum</i> IFO6384	0.19	0.08
<i>F. oxysporum</i> f. <i>gladioli</i> IFO5894	0	0.39
<i>F. oxysporum</i> f. <i>gladioli</i> IFO7166	0.23	0.15
<i>F. oxysporum</i> f. <i>lini</i> IFO5880	0.19	2.85
<i>F. oxysporum</i> f. <i>lini</i> IFO5885	0	0.23
<i>F. oxysporum</i> f. <i>lycopersici</i> IFO5901	0.37	0.85
<i>F. oxysporum</i> f. <i>lycopersici</i> IFO6531	0.32	0.23
<i>F. oxysporum</i> f. <i>melongenae</i> IFO7706	0	0.08
<i>F. oxysporum</i> f. <i>melonis</i> IFO6385	0	0.08
<i>F. oxysporum</i> f. <i>narcissi</i> IFO5265	2.77	0.31
<i>F. oxysporum</i> f. <i>nicotianae</i> IFO6386	0.14	0.62
<i>F. oxysporum</i> f. <i>niveum</i> IFO4471	1.15	0.08
<i>F. oxysporum</i> f. <i>vasinfectum</i> IFO4472	1.11	0.15
<i>F. oxysporum</i> f. <i>vasinfectum</i> IFO5903	0.23	0.23
<i>F. roseum</i> IFO8501	0.74	0.15
<i>F. roseum</i> IFO8502	0	0.39
<i>F. roseum</i> IFO8503	0.23	0.08
<i>F. roseum</i> IFO5421	3.23	3.14
<i>F. solani</i> IFO5232	0	1.54
<i>F. solani</i> IFO5890	0.32	0.62
<i>F. solani</i> IFO5891	0	0.54
<i>F. solani</i> IFO5892	1.89	0.46
<i>F. solani</i> IFO5893	0.72	1.30
<i>F. solani</i> IFO5899	1.52	1.69
<i>F. solani</i> IFO5977	0.05	2.39
<i>F. solani</i> IFO7153	1.01	0.62
<i>F. solani</i> IFO7154	2.21	3.14
<i>F. solani</i> IFO7163	1.48	2.46
<i>F. solani</i> IFO7164	1.20	1.15
<i>F. solani</i> IFO8505	1.11	1.31
<i>F. solani</i> IFO8506	0.61	0.54
<i>F. solani</i> IFO8508	0	1.77
<i>F. solani</i> IFO8509	0.19	0.15
<i>F. solani</i> IFO8510	2.91	3.14
<i>F. solani</i> f. <i>phaseoli</i> IFO5978	0.32	0.08
<i>F. solani</i> var. <i>martii</i> IFO5900	0.51	1.62

Table 2. L-Asparaginase formation by *Hypomyces* species after 3 and 5 days of culture

Strain	Activity (i.u./ml)	
	3-day	5-day
<i>Hypomyces aurantius</i> IFO6847	0.02	0.02
<i>H. aurantius</i> IFO6890	0.05	0.05
<i>H. aurantius</i> IFO7773	0	0.02
<i>H. haematococcus</i> IFO5980	0.88	0.88
<i>H. rosellus</i> IFO6911	0.02	0
<i>H. solani</i> f. <i>mori</i> IFO7707	1.89	1.75
<i>H. solani</i> f. <i>mori</i> IFO7708	0.52	1.38
<i>H. solani</i> f. <i>xanthoxyli</i> IFO7709	0.32	0.41
<i>H. solani</i> f. <i>xanthoxyli</i> IFO7710	0.69	0.97
<i>H. trichothecoides</i> IFO6892	0	0.02

Laboratories, Detroit, Michigan, U.S.A.), 5; malt extract (Difco Laboratories), 2. Single, approximately 5 to 10 mm², pieces of mycelial mat were inoculated into 50 ml of DP medium in 300 ml shaking flasks. DP medium (pH 6.0) contained (g/l distilled water): dextrin, 30; Pharmamedia (Traders Mill Co., Fort Worth, Texas, U.S.A.), 40; KH₂PO₄, 5; K₂HPO₄, 1.5; MgSO₄·7H₂O, 0.5; NaCl, 5. Flasks were shaken at 120 rev./min on a reciprocating shaker with 8 cm stroke for 3 and 5 days at 28 °C.

Enzyme preparations. Culture broths were filtered through Toyo no. 2 filter papers (Toyo Roshi Kaisha, Osaka, Japan) and the filtrates were then centrifuged at 8000g for 10 min. The supernatant fluids were used as enzyme preparations.

L-Asparaginase assay. A reaction mixture containing 0.8 µmol of L-asparagine, 10 µmol of tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.2) and an enzyme preparation in a total volume of 0.1 ml was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 3.7 ml of 0.008 M-trichloroacetic acid. A blank test, in which L-asparagine was added after the addition of trichloroacetic acid, was run for each experiment. Twenty minutes after the addition of 0.2 ml of Nessler's reagent (Wako Pure Chemicals, Osaka, Japan), with incubation at 15 to 20 °C the extinction at 450 nm was measured with a Shimadzu Bausch-Lomb Spectronic 20 colorimeter in a ½ in cell. The amount of ammonia liberated from L-asparagine was determined from the difference in the extinctions of experimental and blank reaction mixtures. One international unit (i.u.) of L-asparaginase is the amount which liberates 1 µmol of ammonia in 1 min in the above described reaction conditions.

RESULTS AND DISCUSSION

Formation of L-asparaginase by Fusarium species. Out of 50 strains of *Fusarium* species, 47 strains formed more than 0.1 i.u. of extracellular L-asparaginase/ml (Table 1). There were many strains which produced high (more than 0.5 i.u./ml) levels of L-asparaginase: *Fusarium oxysporum*, 9 out of 24 strains; *F. solani*, 16 out of 18 strains; *F. causicum*, the one strain; *F. culmorum*, 1 out of 2 strains; *F. roseum*, 2 out of 4 strains. Several species, such as *F. oxysporum* IFO5264, 9331, *F. oxysporum* f. *lini* IFO5880, *F. oxysporum* f. *narcissi* IFO5265, *F. solani* IFO5977, 7154, 7163, 8510, *F. causicum* IFO5979 and *F. roseum* IFO5421, formed extremely high levels of L-asparaginase activity (more than 2 i.u./ml) in their culture filtrates.

It thus appears that the extracellular formation of L-asparaginase is one of the common properties within the genus *Fusarium*.

Table 3. *L-Asparaginase formation by Nectria species after 3 and 5 days of culture*

Strain	Activity (i.u./ml)	
	3-day	5-day
<i>Nectria cinnabarina</i> IFO6821	0·03	0·09
<i>N. ditissima</i> IFO5995	0·14	1·20
<i>N. elegans</i> IFO7187	0·91	1·06
<i>N. haematococca</i> IFO6891	0·94	1·20
<i>N. inventa</i> IFO6306	0	0·05
<i>N. pityrodes</i> IFO6000	0·74	0·92

Table 4. *L-Asparaginase formation by Gibberella species after 3 and 5 days of culture*

Strain	Activity (i.u./ml)	
	3-day	5-day
<i>Gibberella fujikuroi</i> IFO5268	0·02	0·03
<i>G. fujikuroi</i> IFO6349	0	0·06
<i>G. fujikuroi</i> IFO6356	0	0·32
<i>G. fujikuroi</i> IFO6603	0	0
<i>G. fujikuroi</i> IFO6604	0·09	0·15
<i>G. fujikuroi</i> IFO6605	0	0·08
<i>G. fujikuroi</i> IFO6606	0	0
<i>G. fujikuroi</i> IFO6607	0·02	0·09
<i>G. lateritium</i> IFO7188	0	0
<i>G. lateritium</i> f. <i>cerealis</i> IFO7704	0·03	0·41
<i>G. lateritium</i> f. <i>mori</i> IFO7705	0	0·15
<i>G. zeae</i> IFO4474	0·02	0·14
<i>G. zeae</i> IFO5269	0	0·23
<i>G. zeae</i> IFO6608	0·03	0·46
<i>G. zeae</i> IFO7160	0	0·60
<i>G. zeae</i> IFO7520	0	0·37
<i>G. zeae</i> IFO7772	0	0
<i>G. zeae</i> IFO8640	0·03	0·05

Formation of L-asparaginase by Fusarium-related species. There are several ascomycete genera, *Hypomyces*, *Nectria* and *Gibberella*, which have a *Fusarium* state in the imperfect cycle. Species of these genera have been examined to see whether they produce extracellular L-asparaginase or not.

In Table 2 the formation of L-asparaginase by *Hypomyces* species is shown. *Hypomyces haematococcus* IFO5980, *H. solani* f. *mori* IFO7707, 7708 and *H. solani* f. *xanthoxyli* IFO7709, 7710 are known to have a *Fusarium* asexual state. These species formed a high level of extracellular L-asparaginase. On the other hand, *H. aurantius* IFO6847, 6890, 7773, *H. rosellus* IFO6911 and *H. trichothecoides* IFO6892—all species with imperfect states not of the *Fusarium* type—rarely formed L-asparaginase.

Table 3 indicates the extracellular formation of L-asparaginase by *Nectria* species. Those species having a *Fusarium* or related imperfect state, such as *Nectria ditissima* IFO5995, *N. elegans* IFO7187, *N. haematococca* IFO6891 and *N. pityrodes* IFO6000, formed extracellular L-asparaginase, while those having no *Fusarium* state, such as *N. cinnabarina* IFO6821 and *N. inventa* IFO 6306, did not. Thus the ability of species of *Hypomyces* and *Nectria* to form extracellular L-asparaginase is closely related to whether they have a *Fusarium* state or not.

Gibberella species are also known to have a *Fusarium* state, but their ability to form L-asparaginase was small, as shown in Table 4.

These results show that the occurrence of a certain enzyme may reflect the taxonomy of micro-organisms. We understand that mere knowledge of enzyme activities solves no taxonomic problems, and that the use of enzyme activities in taxonomic keys sometimes presents problems. However, the accumulation of knowledge of enzyme activities and properties may give some hints in classifying interrelationships among micro-organisms and in solving problems of evolution of micro-organisms.

The L-asparaginase of *Fusarium* species suppresses the growth of 6C3HED lymphosarcoma in mice. Its enzymatic properties and antitumour activities will be reported elsewhere.

We thank Drs S. Tatsuoka, R. Takeda, and M. Isono of Central Research Division, Takeda Chemical Industries Ltd, for their continued interest and encouragement throughout the course of our work. We also thank Mr H. Ono, Miss H. Sotoma and Miss Y. Nakamura for their skilful technical assistance.

REFERENCES

- ADAMSON, R. H. & FABRO, S. (1968). Antitumor activity and other biological properties of L-asparaginase. *Cancer Chemotherapy Reports* **25**, 617–626.
- ARIMA, K., SAKAMOTO, T., ARAKI, C. & TAMURA, G. (1972). Production of extracellular L-asparaginases by microorganisms. *Agricultural and Biological Chemistry* **36**, 356–361.
- BACH, D. (1928). Les conditions d'action de l'asparaginase de l'*Aspergillus niger*. *Compte rendu hebdomadaire des séances de l'Académie des sciences* **187**, 955–956.
- DE-ANGELI, L. C., POCCIARI, F., RUSSI, S., TONOLO, A., ZURITA, V. E., CIARANFI, E. & PERIN, A. (1970). Effect of L-asparaginase from *Aspergillus terreus* on ascites sarcoma in the rat. *Nature, London* **225**, 549–550.
- DOX, A. W. (1909). The intracellular enzymes of lower fungi, especially those of *Penicillium camemberti*. *Journal of Biological Chemistry* **6**, 461–467.
- IMADA, A., IGARASI, S., NAKAHAMA, K. & ISONO, M. (1973). Asparaginase and glutaminase activities of micro-organisms. *Journal of General Microbiology* (in the press).
- SCHEETZ, R. W., WHELAN, H. A. & WRISTON, J. C., JUN. (1971). Purification and properties of an L-asparaginase from *Fusarium tricinctum*. *Archives of Biochemistry and Biophysics* **142**, 184–189.
- SCHMALFUSS, K. & MOTHES, K. (1930). Über die fermentative Desamidierung durch *Aspergillus niger*. *Biochemische Zeitschrift* **221**, 134–153.
- WRISTON, J. C., JUN. (1971). L-Asparaginase. In *The Enzymes*, 3rd edn, vol. IV, pp. 101–121. Edited by P. D. Boyer. New York and London: Academic Press.