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Microbial Aspartase and Its Activity on Deamination of L-Aspartyl-L-Phenylalanine Methyl Ester

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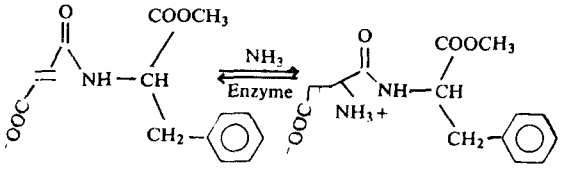
Abstract □ Examination of many microorganisms and soil isolates for the activity of aspartase proved that *R. rubra, G. suboxydans, A. versicolor, P. purpurogenum, E. coli, Ps. aeruginosa, A. gigantus, A. unguis, A. parasiticus* and a soil isolate(S-90) had high activity of aspartase. Comparision of the activity of the aspartase by cell free extracts of these microorganisms with the activity of the enzyme catalyzing the deamination of aspartame by the same cell free extracts showed similar kinetic characteristics. The aspartase existing in the cell free extracts seemed to catalyze the deamination of aspartame, too.

Keywords □ screening of aspartase, L-aspartic acid, aspartame, activity of L-aspartylphenylalanine methyl ester ammonia lyase.

Aspartase (EC 4.3.1.1) catalyzes the divalent metal-dependent deamination of L-aspartate to yield fumarate and ammonia[1]. Aspartase has been regarded as a catabolic enzyme in both bacteria and plants, whereas little information is available for its existence in animals. The deamination reaction is reversible unlike most of other catabolic reactions and the formation of L-aspartate from fumaric acid and ammonia is favored with $\Delta G° = -3.2$ kcal/mol[2]. Aspartase catalyzes the *trans*-addition of ammonia across the double bond of fumarate[3]. Englard[4] have shown that the addition of NH_4^+ to fumarate and its elimination from L-aspartate are stereo-specific. The enzyme shows substrate specificity on aspartate and fumarate, but NH_2OH and hydrazine can be substituted for ammonia[5].

Although aspartase shows strict specificity on its substrate, we wanted to develop a new enzyme or aspartase which can deaminate the amino group of the aspartyl moiety in aspartame or which can add ammonia to the fumaroyl moiety of N-fumaroylphenylalanine methyl ester. We thought that it should be possible to develop a new enzymatic technique for the production of aspartame (L-aspartylphenylalanine methyl ester) by adding ammonia to the double bond of N-fumaroylphenylalanine methyl ester by employing the aspartase or the aspartame ammonia lyase.



Currently aspartame is produced organic chemically by coupling of L-phenylalanine methyl ester with L-aspartic acid but this process needs the protection and the deprotection of the other functional groups of aspartic acid that are not participating in the coupling reaction. For the development of a new enzymatic technique for the production of aspartame by employing an ammonia lyase, we needed to assay the activity of the deamination of aspartame in various microbial cell-free extracts. Thus, we examined the activity of aspartase in cell free extracts of various microorganisms first, and then we examined the activity of the deamination of aspartame with the cell free extracts of the same organisms. From this assay, we wanted to select microorganisms having a high deamination activity on aspartame and we were going to pursue the possibility for the biotechnological production of aspartame by *trans*-addition of ammonia across the double bond of N-fumaroyl-L-phenylalanine methyl ester. In this report we want to report the result obtained from examination of many microbial cell

free extracts for the activity of aspartase and for the activity of the deamination of aspartame.

MATERIALS AND METHODS

General

The UV spectrophotometry employed was LKB Biochrom Ultrospec 4050 equipped with Controller 4070. Autoclave was done at 121 °C/15LB for 15min by using a model made by Astell Hearson Co. (England). Cells were cultured on a rotary shaker (180 rpm) at 28 °C. Centrifugation was done by using Beckman J-21 Centrifuge and MSE Europa 65 Ultracentrifuge. Sonicator employed was MSE-MK2 Sonicator. pH was measured by using Digital pH Meter 202 Nova Scientific Co.

Microorganisms and maintenance

Rhodotorula rubra(IFO 0001, IFO 0889 and IFO 0911), *Rh. glutinis*(IFO 0389), *Rh. glutins* var *dairensis* (IFO 0415), *Rh. minuta* var *texensis* (IFO 0932), *Rh. aurantiaca* (IFO 0559) *B. subtilis* (IFO 3134), *Proteus vulgaris* (IFO 3167), *E. coli* (IFO 3301 and IFO 12734), *Gluconobacter suboxydans* (IFO 3172), *G. melanogenus* (IFO 3292), *G. rubiginosus* (IFO 3243) and *Pseudomonas aeruginosa* (IFO 13738) were purchased from Institute for Fermentation in Osaka, Japan. *Aspergillus gigantus* (M 1), *A. nidulans* (M 3), *A. versicolor* (Cz 5), *A. flavipes* (Cz 7 and Cz 8), *A. alliaceus 1(Cz 10), A. foetidus* (Cz 11), *A. famarii* (Cz 15), *A. sclerotiorum* (Cz 18), *A. fumigatus mui hevola* (Cz 20), *A. oryzae* (Cz 21), *A. panamensis* (M 31), *A. rugulosus* (M 32), *A. parasiticus* (Cz 33), *A. avenaceus* (Cz 34), *A. ustus* (Cz 54), *A. clavatus* (Cz 56), *A. nidulans* (Cz 57), *A. oryzae* (Cz 58), *Penicillium striatum* (M 23), *P. purpurogenum* (Cz 26), *P. notatum* (Cz 29), *P. rogueforti* (Cz 37), *P. digitiatum* (M 44), *Paecilomyces varioti* (Cz 27, Cz 43 and M 46), *Byssoclamys fulva* (Cz 28), *Entomophthora apiculata* (M 30), *Conidiobolus* sp. (M 48), *Thamnidium elyans* (M 49), *Botrytis spectsbilis* (M 52) and *Mucor rammannianus* (M 53) were the fungi stocked in our laboratory. The soil isolates (135 species) were the microorganisms isolated from soil collected from various places in Korea.

The strains of *Rhodotorua* and *Rhodosporidium* geni were stocked on malt extract agar slants. The strains of Cz series were those stocked on Czapeck-Dox agar slants and strains of M series were stocked on malt extract agar slants. Strains designated by S were the microorganisms isolated from soil and stocked on V-8 agar slants or nutrient agar slants. Other bacteria were stocked on nutrient agar slants.

The malt extract medium contained 2% malt extract. The Czapeck-Dox medium contained 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$-$7H_2O$, 0.05% KCl, 0.01% $FeSO_4$ and 3% sucrose in distilled water (pH 7.0). The V-8 medium was composed of 20% (v/v) V-8 juice and 0.3% $CaCO_3$ in distilled water. Agar slants and agar plates were prepared by adding 2 to 3% agar to these liquid media.

Isolation of microorganisms from soil

Microorganisms in soil were pure-cultured by the following method. A small amount of soil collected from various places in Korea was suspended in 10 m*l* of sterilized distilled water containing 1 μg of chloramphenicol per m*l* to prevent the growth of fast growing bacteria and mixed very well. After the suspension was diluted 10^4-fold with distilled water, 0.5 m*l* of the liquid was spreaded on a V-8 agar plate or an oat meal agar plate(2% oat meal, 0.1% yeast extract and 2% in distilled water).

Plates were incubated at 28 °C until some colonies formed. Colonies having different growth patterns were transfered on new V-8 agar plates or oat meal agar plates, pure-cultured and stocked on V-8 agar slants or nutrient agar slants.

Screening of the activity of aspartase in soil isolates

The 135 microorganisms (bacteria 34 spp.; fungi 40 spp.; *Streptomyces* 61 spp.) isolated from soil and preserved on nutrient agar slants, oat-meal agar slants or V-8 agar slants were employed for the screening of the activity of aspartase. Aspartase was screened at first by observation whether fumarate was accumulated in aspartase screening medium or not. Aspartase screening medium was composed of 2% L-aspartate, 0.2% KH_2PO_4, 0.1% K_2HPO_4, 0.01% $MgSO_4$-$7H_2O$, 0.05% yeast extract (pH 6.0). The soil isolates were inoculated in the aspartase screening medium (5.0 m*l*) and incubated at 28 °C for 3 days. Fumarate produced in the cultured medium was examined by thin-layer chromatography (TLC) by spotting 5.0 μ*l* of the medium on a TLC plate (silica gel). The plate was developed with Pr^i-OH-NH_4OH-H_2O(20:1:1, v/v %) and the spots of fumaric acid were compared with that of the authentic sample by UV.

Growth of the microorganisms and the preparation of cell free extracts

The microorganisms used for the preparation of cell free extracts were grown under aerobic condition at 28 °C in the Asp medium[6] containing ammonium fumarate 1.6%, KH_2PO_4 0.2%, $MgSO_4$-

$7H_2O$, 0.05%, yeast extract 2%, corn steep liquor 2%, (pH was adjusted to 7.0) or in a modified Asp medium composed of 2% corn steep liquor, 2% yeast extract (pH was adjusted to 7.0 with 1.0 N KOH)[7]. For the seed culture of microorganisms, the Asp medium in which NH_4OH (67 mM) was added instead of ammonium fumarate (1.6% or 132 mM) was employed. Microorganisms were seed-cultured at 28 °C for 1 day and transferred to the Asp medium. After the cells were grown fully in Asp medium (1 day), they were harvested by centrifugation (8,000 rpm, 15 min) and washed twice with Tris-HCl buffer (50 mM, pH 7.6). The wet cell-paste was resuspended in Tris-HCl buffer (50 mM, pH 7.6, 0.5 m*l* per 100 mg of the wet cell paste) for the preparation of cell free extracts. The cells suspended in the solution were disrupted with MSE-MK2 Sonicator for 5 min at 30 amplitude in an ice-water bath. Cell debris was removed by centrifugation at 15,000 rpm for 30 min to give a supernatant which was stored in an ice-water bath and used as a cell free extract.

A stopped enzyme assay method

After the assay mixture was incubated at 30 °C for 30 min, the activity of aspartase was determined by measurement of the increase of the absorption at 240 nm due to the formation of fumarate[8]. The assay mixture contained 0.1 M L-aspartate (0.5 m*l*), 2.0 mM $MgCl_2$-$6H_2O$ (0.5 m*l*), 50 mM Tris-HCl buffer (pH 7.6, 0.9 m*l*), a cell free extract (0.1 m*l*) in a total volume of 2.0 m*l*. The blank contained no L-aspartate. The enzyme reaction was stopped by boiling the reaction mixture in a boiling water bath for 30 min. The precipitiate produced was removed by centrifugation. The absorption of the supernatant was measured at 240 nm.

An uncoupling continuous enzyme assay method

The activity of aspartase was determined by measuring the increase in absorption at 293 nm due to fumarate formation at 10, 20, 30, 40, 50, 60 min with LKB Model No 4070 at 30 °C[9]. The assay mixture contained 10 mM L-aspartate (1 m*l*) 50 mM Tris-HCl buffer (pH 7.6, 0.7 m*l*), 2 mM $MgCl_2$-$6H_2O$ (1.0 m*l*) and a cell free extract (0.3 m*l*). The amount of protein was determined by the method of Lowry[10]. One unit of enzyme was defined as the amount of protein producing 1 μ mol of fumarate per min under the standard assay condition[11].

Assay of the activity of the deamination of aspartame

The activity of the deamination of aspartame by the cell free extract was determined spectrophotometrically by measuring the increase of absorption at 293 nm. The assay mixture contained 10 mM aspartame (1.0 m*l*), 50 mM Tris-HCl buffer (pH 7.6, 0.7 m*l*), 2 mM $MgCl_2$-$6H_2O$(1.0 m*l*) and a cell free extract (0.3 m*l*). The cell free extracts from *Ps. aeruginosa, E. coli, G. suboxydans, R. rubra* (IFO 0911, IFO 0001), *By. fulva, P. striatum, Botrytis spectabilis,* S-90 and S-111 were employed.

Growth of G. suboxydans and E. coli

The medium containing 0.2% K_2HPO_4, 0.05% $MgSO_4$-$7H_2O$, 2% corn steep liquor, and 2% yeast extract (pH 7.0 adjusted with 1.0 N KOH) was inoculated with *G. suboxydans* or *E. coli* and incubated at 28 °C on a rotary shaker. After 24 hrs' culture, the medium was used as a seed culture transfered to 190 m*l* of Asp medium to give 5% of the total volume. Samples were taken out from the cultured medium at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 42, 48 hrs' incubation and their turbidity at 660 nm and their activity of aspartase was measured by the uncoupling continuous enzyme assay method.

Examination of the activity of aspartase during growth of G. suboxydans and E. coli

The cells in each sample were harvested by centrifugation (8,000 rpm for 15 min), washed twice with Tris-HCl (pH 7.6, 50 mM), suspended in the same buffer, sonicated at 30 μ for 5 min in an ice-water bath. The cell debris was removed by centrifugation at 15,000 rpm for 30 min. The supernatant stored in an ice-water bath was immediately used for the aspartase assay. The aspartase assay was performed by measurement of the increase in absorption at 293 nm. The assay mixture contained 10 mM L-aspartate (1.0 m*l*), 50 mM Tris-HCl buffer (pH 7.0, 0.7 m*l*), 2.0 mM $MgCl_2 \cdot 6H_2O$ (1.0 m*l*) and the supernatant (0.3 m*l*). The amount of aspartase in the cell free extract was obtained from the initial velocity of the formation of fumaric acid. The initial velocity was obtained from the slope of the graph obtained from the results of the enzyme assay, or μmol of fumarate produced per min.

RESULTS AND DISCUSSION

Selection of microorganisms producing aspartase

The activity of aspartase in soil isolates was determined by the identification of fumarate produced in Asp medium by TLC. From this study, 48 soil isolates were found to produce fumaric acid in the medium and we regarded these organisms to

produce aspartase. The microoranisms and the soil isolates (48 spp). regarded to produce aspartase were reexamined for the activity of aspartase by the stopped enzyme assay method. Examination for the activity of aspartase with cell free extracts of these soil isolates, yeasts, bacteria and fungi revealed that the microorganisms, *R. rubra* (IFO 0911, IFO 0001) *G. suboxydans, E. coli* (IFO 12734, IFO 3301), *Ps. aeruginosa, A gigantus, A. versicolor, A. unguis, P. purpurogenum, A. parasiticus,* S-16, S-32, S-40, S-90, S-104, S-108, S-111, S-112, S-120, S-123, had higher activity of aspartase than the rest of organisms. Aspartase seemed to be distributed universally in yeast, bacteria and fungi. The organisms showing good activity of aspartase were reexamined with cell free extracts by the uncoupling continuous enzyme assay method. The data obtained from this study for the microorganisms showing good activity of aspartase were presented in Figure 1 and their specific activities of aspartase are presented in Table I. As shown in Figure 1, *E. coli Ps. aeruginosa* and *R. rubra* are especially prominent in the activity of aspartase. From this sudy, it can be said that aspartase is much more abundant in bacteria and in some yeasts.

Growth of E. coli and G. suboxydans and the change of activity of aspartase at each growth times

The turbidity, the pH and the wet weight of cells in each medium obtained at various incubation time

Table I. The specific activity of aspartase

Organisms	Wet weight (g)	Volume* (m*l*)	Protein** (my)	Activity*** (units)	Specific activity (units/mg)
R. rubra (IFO 0911)	0.2647	1.32	2.97	0.968	0.326
R. rubra (IFO 0001)	0.2647	1.32	2.97	0.402	0.0437
G. suboxydans (IFO 3172)	0.3345	1.67	3.72	0.662	0.178
A. versicolor (Cz 5)	0.7014	3.51	0.42	0.519	0.124
P. purpurogenum (Cz 26)	0.8818	4.41	1.27	0.184	0.145
E. coli (IFO 12734)	0.3184	1.59	8.78	2.80	0.319
S-123	0.3360	1.68	6.72	0.544	0.081
S-104	0.3100	1.55	3.85	—	—
S-108	0.3702	1.85	5.14	0.537	0.104
S-90	0.7742	3.87	8.87	0.503	0.0567
S-111	0.6423	3.21	10.6	0.264	0.0249
S-112	0.1619	0.81	2.54	—	—
S-121	0.3307	1.65	2.21	—	—
S-40	0.2144	1.07	8.87	0.337	0.038
S-120	0.4055	2.03	6.84	0.652	0.0953
E. coli	0.5206	2.60	12.8	1.369	0.107
E. coli (IFO 3301)	0.5218	2.61	10.8	0.997	0.0886
Ps. aeruginosa (IFO 13738)	0.3626	1.81	5.56	2.29	0.411
S-32	0.2876	1.44	6.25	0.538	0.086
S-16	0.2771	1.39	4.15	0.211	0.0509
A. gigantus (M1)	0.3849	1.92	0.458	0.128	0.279
A. unguis (Cz 20)	0.5365	1.92	0.458	0.128	0.279
A. parasiticus (Cz 33)	0.4466	2.23	0.453	0.0743	0.164

* The total volume of the cell free extract; **The amount of the total protein in the total volume of the cell free extract; ***The total activity of aspartase in the cell free extract

for *E. coli,* and *G. suboxydans* were examined and their growth curves were presented in Figures 2 and 3. *E. coli* and *G. suboxydans* got the maximum growth at 27 hrs' incubation. The pH was increased slowly as cells were growing. The correlation between growth of cells and the activity of aspartase was presented in Figures 2 and 3. The result indicated that at the time of maximum growth of the microorganisms showed the highest activity of aspartase. The amount of aspartase was increased as cells grew, and decreased as the number of cells was decreased. This relationship seems to suggest that the amount of aspartase was related with the number of cells. But whether aspartase was induced in these microorganisms was not clearly suggested.

Examination of the cell free extract for the activity of the deamination of aspartame

To examine the activity of the deamination of the amino group of the aspartyl moiety of aspartame (L-aspartyl phenylalanine methyl ester), we incubated the assay mixture containing aspartame instead of L-aspartic acid, and the increase in absorption at 293 nm of the assay mixture was monitored continuously with a UV spectrophotometer.

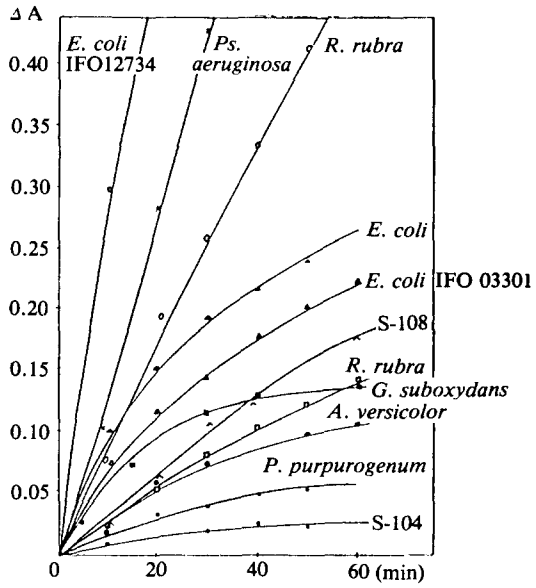


Fig. 1. The increase of the absorption at 293 nm of the assay mixture added with L-aspartic acid and the cell free extract of the microorganisms indicated.

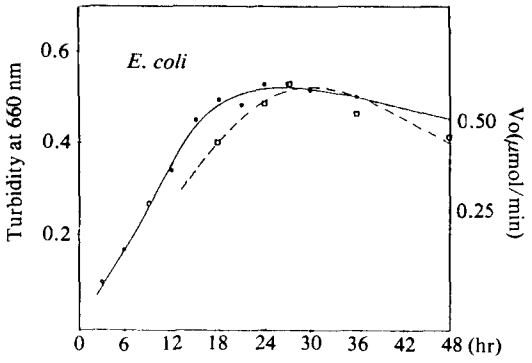


Fig. 2. The growth (–) and the activity of aspartase(- -) of *E. coli* (IFO 12734).

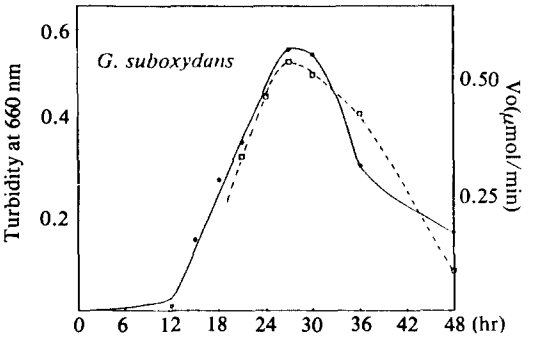


Fig. 3. The growth (–) and the activity of aspartase (- -) of *G. suboxydans* (IFO 3172).

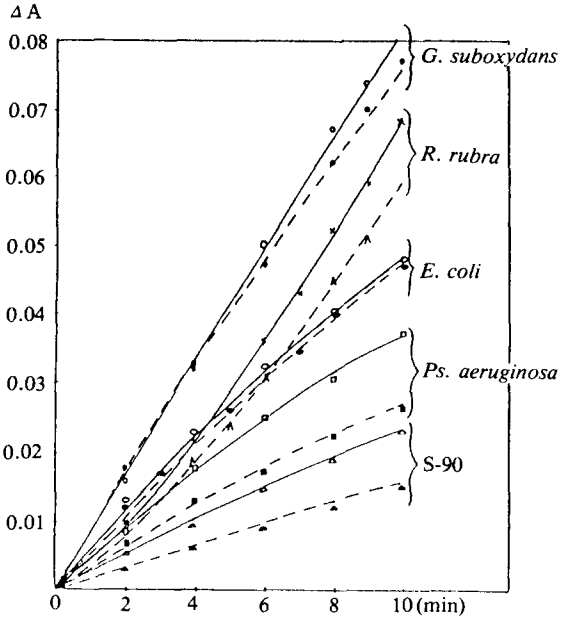


Fig. 4. Comparision of the increase of the absorption at 293nm of the assay mixture added with L-aspartic acid with that added with aspartame.

The data are presented in Figure 4.

The increase of absorption at 293 nm of the assay mixture added with asartame instead of L-aspartic acid showed the same pattern of increase of UV absorption as that of L-aspartate. But the increase of the UV absorption of the assay mixture added with aspartate, which is due to the formation of fumarate from L-aspartate, was just a little faster than that of the assay mixture added with aspartame instead of L-aspartic acid which is mainly due to the formation of the product N-fumaroylphenylalanine methyl ester, produced from aspartame. These data suggested the possibility that aspartame can be deaminated very well by aspartase also.

Since production of L-aspartic acid from fumarate by employing aspartase is well established and it should be possible to produce aspartame from N-fumaroyl-L-phenylalanine methyl ester by employing aspartase. Examination of the metabolized products formed from aspartame in the assay mixtures by incubation with cell free extracts suggested the participation of other hydrolyzing enzymes. Currently, we are further trying to identify and characterize the aspartase and the aspartame-ammonia lyase and investigating the enzymatic transformation of N-fumaroyl-L-phenylalanine methyl ester to aspartame.

ACKNOWLEDGEMENT

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