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Pharmaceutical Studies on Aminopeptidase from *Aspergillus japonica*. I[1]

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To utilize aminopeptidase from *Aspergillus japonica* as a digestive enzyme preparation, the properties of the crude enzyme were examined from pharmaceutical aspects. The enzyme was most active at pH 8.0 and 50°, and was stable in a pH range of 5.5 to 8.5 and below 50°. The enzyme was activated by Co^{2+}, but was strongly inhibited by some metal ions, *o*-phenanthroline, and N-bromosuccinimide. Hydrolytic cross-reaction between the enzyme and various proteases was carried out using casein as a substrate. The hydrolysis ratio (60 %) was 2-fold greater than that by only the individual proteases. Therefore, the enzyme is considered to be useful for digesting a protein, together with other proteases. From the examination of the stability of the enzyme powder and that of the enzyme to various pharmaceutical factors, the enzyme was found to be highly stable to diluents, binders, and wetting agents at a high concentration, and was also stable to low first pressure in tabletting.

From these results, it is suggested that this aminopeptidase can be satisfactorily utilized as a digestive enzyme preparation.

As a protease, pepsin and pancreatin from animals, papain from plants, and proteases from *Aspergillus*, *Bacillus* and *Rhizopus* are mixed in commercial digestive enzyme preparations.[3–5] Generally, these endopeptidases hydrolyze inner peptide bonds of high molecular weight including proteins, liberating low molecular weight peptides, but do not act on terminal peptide bonds of the peptides.[6,7] Therefore, exopeptidase can be usefully mixed with endopeptidases to liberate free amino acids from peptides of low molecular weight which are liberated from proteins by the action of endopeptidases.

During a search for exopeptidase from a number of molds, we found that *Aspergilus japonica* produced a potent aminopeptidase. To know whether or not the aminopeptidase can be utilized as digestive enzyme preparation, we examined the properties of this crude enzyme, and effect of pharmaceutical factors such as diluents, wetting agents, binders, organic solvents, and pressure on the enzyme.

Materials and methods

Enzyme and Reagents——*Asp. japonica* (S-44) was grown in a medium, containing 5% of defatted soybean, 3% of potato starch, 1% of glucose, and 0.5% of KH_2PO_4 in a fermenter. This mixture was incubated at 30° for 80 hr. Three volumes of ethanol was added to the concentrated culture filtrate, and the resulting precipitate was collected and dried to be used as the crude enzyme preparation.

Trypsin and α-chymotrypsin were products of Esai Co., Tokyo. Bromelain was a product obtained from South Star Chemical Co., Taiwan. Semi-alkaline protease was purchased from Amano Pharm. Co., Nagoya, and pepsin from Pentex, ILL., U.S.A. Hammersten quality milk casein was a product of E. Merk AG., Darmstadt, Germany and L-leucyl β-naphthylamide was that of Wako Pure Chemical Industries, Osaka, Diisopropyl

1) This work forms part CXVIII of a series entitled "Studies on Enzymes" by M. Sugiura.
2) Location: *1432-1, Horinouchi Hachioji-shi, Tokyo 192-03, Japan.*
3) S. Naito, "Rinsyo Yakuzaigaku," Hirokawa Shoten, Tokyo, 1970, p. 325.
4) "New Drugs in Japan," Yakuji Nippo-sha, Tokyo, Vol. 21, 1970, p. 133, Vol. 22, 1971, p. 134, Vol. 23, 1972, p. 147, Vol. 24, 1973, p. 131.
5) B. Kobo, *Yakkyoku*, **20**, 1097 (1969).
6) B. Hagiwara, "Koso Kenkyuho," Vol. 2, ed. by S. Akabori, Asakura Shoten, Tokyo, 1957, p. 237.
7) K. Takahashi, *Tanpakushitsu, Kakusan, Koso*, **7**, 763 (1962).

fluorophosphate (DFP) was obtained from Sigma Chemical Comp. Mo., U.S.A. The other reagents used were of reagent grade.

Determination of Aminopeptidase Activity——The activity was measured by the method of Goldbarg.[8] To 1 ml of 0.02% L-leucyl β-naphthylamide in 0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM $CoCl_2$, 1 ml of the enzyme solution was added. After incubation at 37° for 30 min, the reaction was stopped and the enzyme protein was precipitated by the addition of 1 ml of 40% CCl_3COOH. This mixture was filtered after standing 20 min at 37°. To 1 ml of this filtrate, 1 ml of 0.1% $NaNO_2$, 1 ml of 0.5% ammonium sulfaminate, and 2 ml of EtOH solution of 0.05% N-(1-naphthyl)ethylenediamine were added and the reaction mixture was allowed to stand for 10 min. Absorbance of this solution was measured at 560 nm with Hitachi 101 spectrophotometer.

Results

Effect of pH and Temperature on Activity and Stability of the Aminopeptidase

As shown in Fig. 1, the enzyme was most active at pH 8.0 and the optimum temperature of the enzyme was estimated to be 50°. The enzyme was stable in a pH range of 5.5 to 8.5 for 24 hr at 4° and was also stable up to 50° for 15 min at pH 8.0, but it was completely inactivated at 60°.

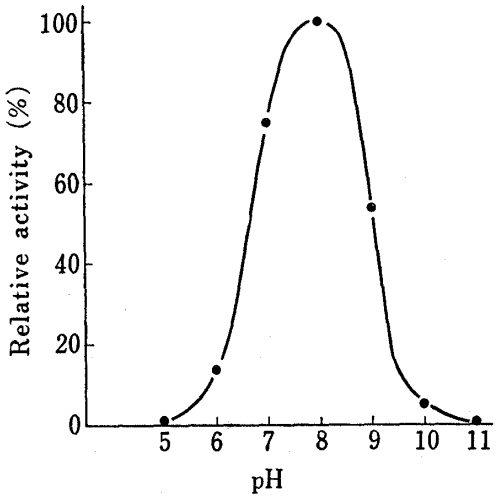

Fig. 1. Effect of pH on Activity of Aminopeptidase from *Aspergillus japonica*

The reaction was carried out at various pH in Britton-Robinson buffer.

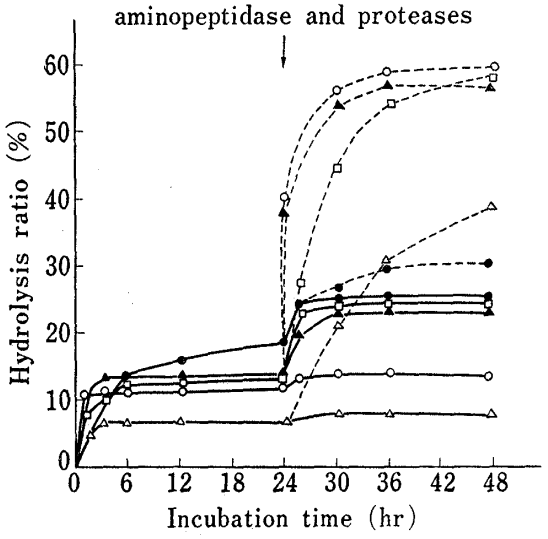

Fig. 2. Effect of Aminopeptidase on Enzymatic Hydrolysis of Casein

To 100 ml of 1% casein solution containing 0.02 M Tris-HCl buffer (pH 8.0) (excepting 0.02 M lactate buffer (pH 1.8) for pepsin), 50 mg of various proteases was added and the mixture was incubated at 37° for 24 hr. The aminopeptidase (0.05 g/g casein) was added and the hydrolysis was continued for another 24 hr at pH 8.0. Hydrolysis rate was followed by formol titration method.[9] The value of 100% was taken when casein was hydrolyzed by 6 N HCl at 110° for 48 hr.

—○—: trypsin
---○---: trypsin+aminopeptidase
—●—: α-chymotrypsin
---●---: α-chymotrypsin+aminopeptidase
—△—: pepsin
---△---: pepsin+aminopeptidase
—▲—: bromelain
---▲---: bromelain+aminopeptidase
—□—: semi-alkaline protease
---□---: semi-alkaline protease+aminopeptidase

Effect of Some Metal Ions and Reagents on the Enzyme Activity

To examine the effect of some metal ions and reagents on the aminopeptidase, the activity was measured in the presence of 1 mM metal salt or reagent listed in Table I and percentage of the activity was calculated compared with the activity in the absence of the metal salt or reagent. The enzyme was activated 2-fold by Co^{2+} but was not affected by Mg^{2+}. However, the enzyme was fairly inactivated by other metal ions. Among various reagents, the enzyme was strongly inactivated by *o*-phenanthroline

8) J.A. Goldbarg and A.M. Rutenburg, *Cancer*, **11**, 283 (1958).
9) M. Nomoto, Y. Narahashi and M. Murakami, *J. Biochem.* (Tokyo), **20**, 820 (1960).

Table I. Effect of Various Metal Ions and Reagents on Activity of Aminopeptidase from *Aspergillus japonica*

Metal salt	Remaining activity (%)	Reagent	Remaining activity (%)
None	100	EDTA	36
$ZnCl_2$	2.0	*o*-phenanthroline	0
$NiCl_2$	8.0	cysteine	52
$CoCl_2$	200	2-mercaptoethanol	22
$SnCl_2$	21	thioglycolic acid	49
$MgCl_2$	95	potassium cyanide	96
$CuCl_2$	1.0	N-bromosuccinimide	0
$FeCl_3$	58	diisopropyl fluorophosphate	109
$CdCl_2$	4.6	*p*-chloromercuribenzoate	100
$PbCl_2$	0	monoiodoacetic acid	105
$HgCl_2$	0		
AgCl	27		
$MnCl_2$	32		

Activity was measured by the method of Goldbarg[8] in the presence of 1 mM of metal salt or reagent using 40 μg of the enzyme.

and N-bromosuccinimide, while the enzyme was not affected by DFP, *p*-chloromercuribenzoate (PCMB), or monoiodoacetic acid.

Cross-reaction between the Aminopeptidase and Various Proteases

Hydrolysis ratio of casein by the aminopeptidase and various proteases was determined by measuring carboxylic groups liberated from the substrate by the formol titration method.[9] To determine the cross-reactivity between the enzyme and various proteases, casein was first hydrolyzed with 0.05 g/g casein of various proteases at 37° and pH 8.0 (except for pepsin at pH 1.8) for 24 hr and then an equal amount of the aminopeptidase was added and hydrolysis was continued for another 24 hr at pH 8.0. This result is shown in Fig. 2. The hydrolysis ratios of casein by trypsin, bromelain, pepsin, and semi-alkaline protease, followed by the aminopeptidase, were about 2-fold greater than those only by the respective proteases.

Stability of Crude Enzyme Powder

Effect of humidity and temperature on stability of the crude enzyme powder was determined at three conditions; relative humidity (RH) of 92% at 30°, RH 75% at 20°, and RH 52% at 5°, for 5 weeks, as proposed by Kubo.[10] As shown in Fig. 3, the enzyme was quite stable at RH 52% at 5° and RH 75% at 20°. However, at RH 92% and 30°, 10% and 80% of the original activity was lost after 1 week and 5 weeks, respectively.

Effect of Artificial Gastric and Intestinal Juice, and Human Intestinal Juice on the Enzyme

Effect of Artificial gastric juice (JP VIII, solution I), artificial intestinal juice (JP VIII, solution II), and human intestinal juice on stability of the aminopeptidase was examined at 37° and the result is presented in Fig. 4. The enzyme lost its activity immediately after being mixed with the artificial gastric juice, but the enzyme was stable in the artificial intestinal juice for 6 hr. In human intestinal juice, the enzyme was highly stable for 2 hr and only 20% of the original activity was lost after 6 hr.

Effect of Various Proteases on the Enzyme

Since trypsin, α-chymotrypsin, bromelain, and semi-alkaline protease were expected to be mixed with the aminopeptidase in a multienzyme preparation, the effect of various proteases

10) F. Kubo, T. Ueno, M. Horioka, B. Kobo and T. Ishihara, *Yakuzaigaku*, **19**, 276 (1959).

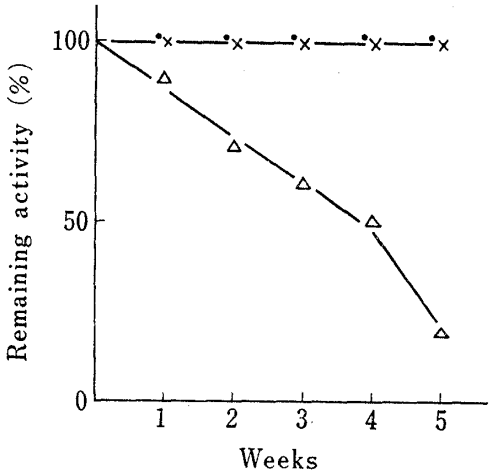

Fig. 3. Effect of Humidity and Temperature on Stability of Aminopeptidase from *Aspergillus iaponica*

—●—: RH 52%, 5°
—×—: RH 75%, 20°
—△—: RH 92%, 30°

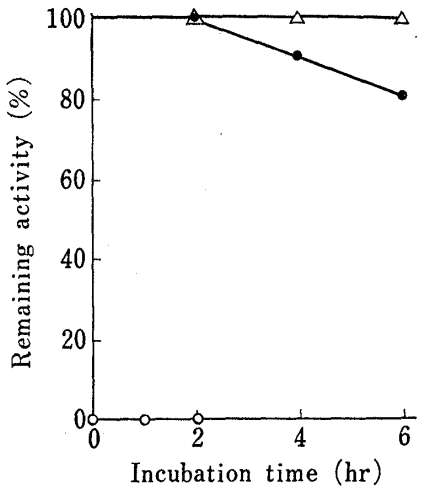

Fig. 4. Stability of Aminopeptidase in Artificial Intestinal Juice (JP VIII), Artificial Gastric Juice (JP VIII), and Human Intestinal Juice

Five milliliters of artificial gastric or intestinal juice and human intestinal juice was incubated with 25 mg of the enzyme. After 200-fold dilution with cold 0.05 M Tris-HCl buffer (pH 8.0), the remaining activity was measured in a usual manner.

—●—: human intestinal juice
—○—: artificial gastric juice
—△—: artificial intestinal juice

on stability of the enzyme was studied. To 5 ml of the aminopeptidase solution (1 mg/ml) containing 0.1M Tris-HCl buffer (pH 8.0), 5 mg of a protease was added and the mixture was incubated at 37° for 1 hr. The mixture was diluted 40-fold with cold water and the remaining activity was measured. As shown in Table II, the enzyme was almost unaffected in the presence of trypsin, semi-alkaline protease, bromelain, and α-chymotrypsin.

TABLE II. Effect of Proteases on Activity of Aminopeptidase

Protease	Remaining activity (%)
Control	100
Semi-alkaline protease	100
Bromelain	94
Trypsin	103
α-Chymotrypsin	96

Effect of Diluents on the Enzyme

An equal amount of the enzyme powder and various diluents was mixed and incubated at 20°, 40°, and 60° for 1 hr, then the mixture was dissolved and diluted with cold 0.02M Tris-HCl buffer (pH 8.0), and the remaining activity was determined. As shown in Table III, the enzyme was little affected by any of the diluents.

Statistically significant difference was found among diluents and among temperatures at 1% probability.

Effect of Wetting Agents on the Enzyme

To 200 mg of the aminopeptidase, 1 ml of various wetting agents was added to 30%, 60%, and 90% concentration. The mixture was incubated at 40° for 1 hr and then the agents were evaporated to dryness under a reduced pressure. This result is shown in Table IV.

Table III. Effect of Diluents on Activity of Aminopeptidase

Diluent	Temperature (°C)		
	20	40	60
Lactose	100	99	99
Sucrose	92	91	89
Glucose	100	86	86
Starch	91	88	85
Dextrine	96	96	89
Cellulose	100	96	96
Calcium carbonate	99	96	92
Kaolin	98	96	92
Synthetic aluminium silicate	92	86	86

Values are remaining activity (%)

Analysis of Variance

Factor	s.s	d.f	m.s	*Fo*
Diluent	447.3	8	55.9	10.5
Temperature	148.2	2	74.1	13.9
Error	85.0	16	5.3	
Total	680.5	26		

$F(8.16; 0.01)=3.89$ $F(2.16; 0.01)=6.23$

Table IV. Effect of Wetting Agents on Activity of Aminopeptidase

Wetting agent	Concentration (%)		
	30	60	90
Ethanol	72	88	100
Isopropanol	65	83	100
Acetone	78	95	100

Values are remaining activity (%)

Analysis of Variance

Factor	s.s	d.f	m.s	*Fo*
Wetting agent	104.3	2	52.12	3.94
Concentration	1220.3	2	610.15	46.05
Error	53.0	4	13.25	
Total	1377.6	8		

$F(2.4; 0.01)=18.00$ $F(2.4; 0.05)=694$

The enzyme was not affected by all the wetting agents at 90% concentration, while it was strongly inactivated by the agents at 30% concentration. Statistically significant difference was found among concentration of the agents at 1% probability, but not among wetting agents.

Effect of Binders and Disintegrators on the Enzyme

An equal volume of 1% solution of various binders and disintegrators was mixed with 1% enzyme solution containing 0.1M Tris-HCl buffer (pH 8.0) and incubated at 40° for 15, 30, or 60 min. The mixture was diluted 200-fold with cold water and the remaining activity

was determined. As shown in Table V, the enzyme was strongly inactivated by sodium lauryl sulfate and Aerosol OT but not so much by the others. From the result of statistical analysis, significant difference was found among binders and disintegrators at 1% probability, and also among incubation times at 5% probability.

TABLE V. Effect of Binders and Disintegrators on Activity of Aminopeptidase

Binder and disintegrator	Time (min)		
	15	30	60
Acacia	93	94	94
Methyl cellulose	98	95	90
Sodium carboxymethyl cellulose	100	99	98
Polyvinyl alcohol	103	102	101
Polyvinylpyrrolidone	100	98	96
Gelatin	105	105	105
Sodium lauryl sulfate	34	27	17
Aerosol OT	81	77	67

Values are remaining activity (%)

Analysis of Variance

Factor	s.s	d.f	m.s	*Fo*
Time	135.3	2	67.6	5.9
Binder	14328.3	7	2046.9	179.1
Error	160.1	14	11.4	
Total	14623.7	23		

$F(7.14; 0.01)=4.28$ $F(2.14; 0.01)=6.51$ $F(2.14; 0.05)=3.74$

TABLE VI. Effect of Organic Solvents on Activity of Aminopeptidase

Solvent	Temperature (°C)		
	20	40	60
Methanol	96	60	0
Ethanol	94	94	82
Isopropanol	94	88	80
Ethyl acetate	94	84	78
Benzene	100	95	73
Chloroform	90	83	83
Ethylene chloride	87	76	62
Petroleum ether	95	83	75
Ligroine	94	94	86

Values are remaining activity (%)

Analysis of Variance

Factor	s.s	d.f	m.s	*Fo*
Solvent	3628.7	8	453.5	2.3
Temperature	2860.7	2	1430.4	7.2
Error	3173.3	16	198.3	
Total	9662.7	26		

$F(8.16; 0.01)=3.89$ $F(8.16; 0.05)=2.59$ $F(2.16; 0.01)=6.23$

Effect of Organic Solvents on the Enzyme

Effect of organic solvents used for coating of a tablets on the enzyme was examined in the same way as the effect of wetting agents and the result is presented in Table VI. The enzyme was strongly inactivated by methanol at 40° and 60°, and also highly inactivated by ethylene chloride. Statistically significant difference was not found among the solvents, but was found among temperatures at 1% probability.

Effect of Pressure on the Enzyme

The enzyme powder (100 mg) was directly compressed with a single stroke tabletting machine (UPF-6, Nichiei Seiko Co., Tokyo) with an 8 mm dies, at a pressure from 0.5 to 5 tons. The first compressed tablet was crushed and was secondarily compressed. Pressure was measured electronically using Radio Rapilcorder KMU-300 V.[11] First and second compressed tablets were crushed to powder and the remaining activity was determined. This result is shown in Table VII. The effect of first pressure on the enzyme was greater than that of the second pressure. From the statistical analysis, significant difference was found among first pressures and among second pressures at 1% probability.

Table VII. Effect of Pressure on Activity of Aminopeptidase

2nd pressure (tons)	1st pressure (tons)				
	0.5	1	2	3	5
0	95	94	93	84	72
0.5	93	91	91	83	72
1	88	87	86	82	71
2	84	84	84	79	71
3	83	82	76	76	68
5	72	71	64	60	59

Values are remaining activity (%)

Analysis of Variance

Factor	s.s	d.f	m.s	*Fo*
1st pressure	1648.2	5	529.6	49.6
2nd pressure	1167.0	4	291.8	43.9
Error	133.0	20	6.7	
Total	2948.2	29		

$F(5.20; 0.01)=4.10$ $F(4.20; 0.01)=4.43$

Discussion

To utilize aminopeptidase from *Asp. japonica* as a digestive enzyme preparation,enzymic properties and stability were examined from pharmaceutical aspects. Properties of the enzyme from *Asp. japonica* differed slightly from those of the other *Aspergillus* enzymes. Leucine aminopeptidase from *Asp. oryzae* was activated by Ca^{2+}, Co^{2+}, and Mn^{2+}, but was inactivated by EDTA and *o*-phenanthroline, while the enzyme was unaffected by PCMB and monoiodoacetic acid.[12] However, the aminopeptidase from *Asp. japonica* was activated by Co^{2+} and unaffected by Mg^{2+} but was strongly inactivated by Zn^{2+}, Ni^{2+}, Mn^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+}, *o*-phenanthroline, and N-bromosuccinimide. Leucine aminopeptidase from *Asp. sojae*

11) Y. Kanaya, Y. Imai and K. Asahina, *Yakuzaigaku*, **32**, 31 (1972).
12) T. Nakadai, S. Nasuno and N. Iguchi, *Agr. Biol. Chem.* (Tokyo), **37**, 767 (1973).

was slightly activated by DFP and α,α′-dipyridyl.[13] The aminopeptidase from *Asp. japonica* is considered to be a metal enzyme, because it was activated by Co^{2+} and inactivated by EDTA and *o*-phenanthroline.

The enzyme was most active at pH 8.0 and stable in a pH range of 5.5 to 8.5. Therefore, it is suggested that this enzyme is sufficiently active towards substrates in the duodenum and upper part of the small intestine. From the result of cross-reaction between the aminopeptidase and various proteases, 60% of hydrolysis ratio towards casein by the cross-reaction was obtained, showing potentiation of the hydrolysis ratio. By mixing the aminopeptidase with digestive enzyme preparations, ingested protein can be easily hydrolyzed to dipeptides or free amino acids and absorption of the products from intestinal tract can be facilitated.

The enzyme powder was fairly stable for 4 weeks at RH 92% and 30°, showing 50% of the original activity, and did not show any loss of activity at RH 75% and 20° and RH 52% at 4°. The enzyme solution was also stable to artificial and human intestinal juice, but was inactivated by artificial gastric juice. The enzyme was almost unaffected by diluents, binders, wetting agents at high concentration, and organic solvents except for methanol. Therefore, stable aminopeptidase preparation can be obtained by taking following precautions: (a) sodium lauryl sulfate and Aerosol OT are not suitable as a disintegrator, (b) tablets must be compressed using a diluent such as micro-crystalline cellulose and low first pressure, and (c) enteric coating must be carried out using coating agents other than methanol.

In summary, aminopeptidase from *Asp. japonica* was stable to artificial and human intestinal juice and various pharmaceutical factors. When the enzyme was mixed with other proteases, casein was thought to be hydrolyzed at least to dipeptides by a cross-reaction. It may be concluded that the aminopeptidase is sufficiently useful for digestive enzyme preparations.

13) Y. Ozawa, K. Suzuki, T. Mizumura and K. Mogi, *Agr. Biol. Chem.* (Tokyo), 37, 1285 (1973).