Properties of 5'-Phosphodiesterase from *Aspergillus niger*[†]

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Among 920 strains of fungi tested only black koji-molds were found to be potent producers of 5'-phosphodiesterase. The enzyme from *Aspergillus niger* was partially purified from aqueous extract of the culture on wheat bran. The enzyme hydrolyzed 3'-hydroxyl-terminated 3'→5' and 2'→5' dinucleotides or 3'→5' and 2'→5' dinucleoside monophosphates without specificity toward bases or sugars. The enzyme also hydrolyzed *p*-nitrophenyl ester of nucleoside 5'-monophosphate, ADP, CDP-choline and UDPG to give nucleoside 5'-monophosphate. The enzyme was actually proved to split 2'→5' dinucleotides in nuclease P₁ digest of technical grade yeast RNA containing several per cent of 2'-5' phosphodiester linkages in addition to 3'-5' linkages.

Kuninaka *et al.*^{1,2)} demonstrated that technical grade yeast RNA contained several % of 2'-5' phosphodiester linkages resistant to nuclease P₁. Therefore, in order to obtain 5'-mononucleotides from RNA in good yield industrially, it is necessary to split 2'-5' phosphodiester linkages as well as 3'-5' phosphodiester linkages in RNA. The authors carried out screening for fungi capable of degrading 2'→5' dinucleotides from 920 strains of various fungi. As a result, high 5'-phosphodiesterase activity was found to be distributed exclusively in black koji-molds.

In the present paper, properties of 5'-phosphodiesterase from *Aspergillus niger* AHU 7117 and application of the enzyme for production of 5'-nucleotides from technical grade yeast RNA are described.

MATERIALS AND METHODS

Microorganisms. Four hundred and two of type culture of various fungi were kindly supplied from the Institute of Applied Microbiology, the University of Tokyo, the Institute of Fermentation, Osaka, Department of Agricultural Chemistry, Hokkaido University, Laboratory of Microbiology and Enzymology, Tokyo Nōkō University, Department of Fermentation Techno-

logy, Faculty of Engineering, Hiroshima University and Department of Agricultural Chemistry, Kyushu University. In addition to type cultures, 518 strains of fungi newly isolated from soil were subjected to screening test.

Screening. Each of fungi to be checked was cultured on 8 g of wheat bran supplemented with 5 ml of water in a 200-ml flask at 28°C for 3 to 5 day. The whole culture material was soaked in 80 ml of water for 1 hr at room temperature with occasional shaking and filtered. The filtrate was assayed for the enzyme activity attacking both adenosine 5'-*p*-nitrophenyl phosphate and (2'-5') A-A.

Substrates

i) *Dinucleoside monophosphates.* (2'-5') A-A, (2'-5') U-A, (2'-5') G-A, (2'-5') A-C, and (2'-5') C-A were prepared by the method of Miura and Ueda.³⁾

ii) *Dinucleotides bearing a 5'-phosphate group.* For preparing dinucleotides, aromatic phosphoramidates were employed as protecting group⁴⁾ as follows: (a) Preparation of (2'-5') pA-A and (3'-5') pA-A. Pyridinium N⁶-benzoyladenine 5'-phosphoramidate (1.32 mmoles) and tri-*n*-butylammonium 2', 3', N⁶-triacetyladenine 5'-phosphate (1.3 mmoles) were condensed by using TPS (3.3 mmoles) in absolute pyridine (7 ml) at room temperature for 2.5 hr. To the reaction mixture, water (10 ml) and tri-*n*-butylamine (1.6 ml) were added and the mixture was extracted with ether. The aqueous layer was dried by coevaporation with pyridine. The dried material was treated with isoamyl nitrite (4 ml) in pyridine-acetic acid (1:1) (20 ml) at room temperature for 4 hr. The solution was evaporated and the residue was treated with absolute

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methanolic ammonia at room temperature for 20 hr. The volatile materials were removed and the residue containing (2'-5') pA-A and (3'-5') pA-A was applied to a column (1.8×54 cm) of Dowex 1×2 (formate). Elution was carried out with a linear gradient of formic acid (0~1 M). Each of the eluted dinucleotide fractions was rechromatographed on a column (1.5×55 cm) of Dowex 1×2 (formate) with a linear gradient of ammonium formate (0.3~0.65 M). The eluates were desalted with activated charcoal column. The dinucleotides isolated from the desalted eluates were identified with (2'-5') pA-A and (3'-5') pA-A.^{1,2)} The yields of (2'-5') pA-A and (3'-5') pA-A were 1050 A_{260} and 1350 A_{260} , respectively. (b) Preparation of (2'-5') pA-C and (3'-5') pA-C. Pyridinium N⁶-benzoyladenine 5'-phosphoroanilidate (1.1 mmoles) and tri-*n*-butylammonium 2', 3', N⁴-triacetylcytidine 5'-phosphate (1 mmole) were condensed by using TPS (2.6 mmoles) in absolute pyridine (5 ml) at room temperature for 3 hr. To the reaction mixture, water (8 ml) and tri-*n*-butylamine (1.3 ml) were added and the mixture was allowed to stand at room temperature for 12 hr. The subsequent work-up was carried out as described above for preparing pA-A except that the column chromatography was carried out with a linear gradient of formic acid (0.02~0.5 M) and ammonium formate (0.2~0.55 M). The yields of (2'-5') pA-C and (3'-5') pA-C were 720 A_{260} and 780 A_{260} , respectively.

iii) Others. ADP, CDP-choline, UDPG and 3':5'-cyclic AMP were products of our laboratory. Technical grade yeast RNA preparation (free RNA content, about 85%) was purchased from Kohjin Co., Ltd. Nuclease P₁ digest of this preparation contained the 16 possible 2'-5' dinucleotides, each content of which was 0.1 to 0.6% the digest.^{1,2)} Other substrates were the same as employed previously.⁶⁾

Enzyme assay

Assay 1. This assay was based on measurement of the amount of inorganic phosphate released secondarily with *E. coli* alkaline phosphatase from AMP formed by hydrolysis of (2'-5') A-A. The standard condition for this measurement was as follows: The reaction mixture contained 0.1 ml of 10 mM (2'-5') A-A, 0.1 ml of M/35 veronal-acetate buffer (pH 5.0) and 0.1 ml of enzyme solution. After incubation at 37°C for 30 min, 0.1 ml of 0.2 M Tris-HCl buffer (pH 9.1) was added to the reaction mixture and the reaction was stopped by immersion for 3 min in a boiling water bath followed by rapid cooling. To the reaction mixture 0.02 ml of *E. coli* alkaline phosphatase solution (500 µg/ml) was added. After incubation at 37°C for 60 min, inorganic phosphate released was determined.

Assay 2. This assay was based on the formation of *p*-nitrophenol from adenosine 5'-*p*-nitrophenyl phosphate.

The standard condition for this measurement was as follows: The reaction mixture contained 0.1 ml of 10 mM adenosine 5'-*p*-nitrophenyl phosphate, 0.1 ml of M/35 veronal-acetate buffer (pH 5.0) and 0.1 ml of enzyme solution. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 0.7 ml of 0.1 N NaOH. The amount of *p*-nitrophenol was determined from absorbance at 400 nm, assuming a molar extinction coefficient of 17,600.

Assay 3. The activity toward RNA and DNA was assayed by the same method as employed for nuclease P₁ as described previously.⁶⁾

One unit of enzyme was defined as the amount of the enzyme which hydrolyzes 1 µmole of substrate in 1 min under the standard condition.

RESULTS

Screening for 5'-phosphodiesterase producing fungi

Result of screening test is summarized in Table I. Among fungi tested, high 5'-phosphodiesterase producers (above 300 units/ml by Assay 2) were found in the genus *Aspergillus* and new isolates. All these strains were proved to belong exclusively to black koji-molds. The activities of typical strains of black koji-molds are shown in Table II. *Aspergillus niger* AHU 7117, the most potent producer, was used in the following experiments.

Partial purification of 5'-phosphodiesterase from *Aspergillus niger*

Spores of *Aspergillus niger* AHU 7117 were inoculated on a mixture of 660 g of wheat bran and 400 ml of tap water in a tray and grown at 28°C for 3 days. The culture material was used as enzyme source. All operations were carried out at 4°C unless otherwise noted. The purification procedure was as follows:

Step 1. Extraction. The culture material (2 kg) was soaked with 6 liters of tap water with occasional stirring for 2 hr and squeezed through cloth. The crude filtrate was centrifuged to remove spores and mycelia.

Step 2. Heat treatment. The crude ex-

TABLE I. DISTRIBUTION OF 5'-PHOSPHODIESTERASE ACTIVITY IN VARIOUS FUNGI

Activity (units/ ml) ^{a)}	Number of strain				
	~ 100	101 ~ 300	301 ~ 1000	1001 ~ 3000	3001 ~
Culture					
<i>Aspergillus</i>	98	3	8 ^{b)}	69 ^{b)}	8 ^{b)}
<i>Rhizopus</i>	30	3	0	0	0
<i>Mucor</i>	36	1	0	0	0
<i>Monascus</i>	12	0	0	0	0
<i>Penicillium</i>	108	0	0	0	0
<i>Fusarium</i>	9	0	0	0	0
<i>Absidia</i>	4	4	0	0	0
<i>Neurospora</i>	2	0	0	0	0
<i>Trichoderma</i>	2	0	0	0	0
<i>Memmoniella</i>	2	0	0	0	0
<i>Corticium</i>	3	0	0	0	0
New isolates	476	25	2 ^{b)}	13 ^{b)}	2 ^{b)}

a) Enzyme units are expressed as in Assay 2 with adenosine 5'-*p*-nitrophenyl phosphate.

b) Black koji-molds.

TABLE II. 5'-PHOSPHODIESTERASE ACTIVITY OF VARIOUS BLACK KOJI-MOLDS

Strain	Activity (units/ml) toward	
	<i>p</i> -Nitrophenyl-pA	(2'-5') A-A
<i>Aspergillus niger</i> AHU 7106	3124	1930
<i>Asp. niger</i> AHU 7107	1809	484
<i>Asp. niger</i> AHU 7113	3104	974
<i>Asp. niger</i> AHU 7117	5078	2036
<i>Asp. niger</i> AHU 7220	3860	1253
<i>Asp. niger</i> AHU 7393	3582	1053
<i>Asp. niger</i> mut. <i>shemarii</i> (<i>schiemann</i>) Thom HUT 2055	1675	598
<i>Asp. phoenicis</i> Thom HUT 2078	955	370
<i>Asp. phoenicis</i> IFO 6648	2845	1184
<i>Asp. phoenicis</i> IFO 6649	3024	1270
<i>Asp. usamii</i> IAM 2185	1141	712
<i>Asp. usamii</i> Sakaguchi Iizuka and Yamazaki HUT 2107	2686	1156
<i>Asp. saitoi</i> R 3813	5133	1594
<i>Asp. awamori</i> var. <i>piceus</i> AHU 7014	2985	854
<i>Asp. awamori</i>	1890	547
<i>Asp. fuscus</i> IAM 2131	1608	484
<i>Asp. inuii</i> IAM 2255	1102	495
<i>Asp. batatae</i> IAM 2098	2547	1367
<i>Asp. carbonarius</i> IAM 2099	1692	1025
<i>Asp. usamii</i> Sakaguchi, Iizuka and Yamazaki HUT 2104	3303	1338

tract was adjusted to pH 6.3 and heated at 60°C for 30 min to inactivate contaminating phosphomonoesterase. After heat treatment, the enzyme solution was adjusted to pH 5.0.

Step 3. Charcoal treatment. To the heat treated enzyme solution (5100 ml) 76.5 g of

charcoal was added. After allowing it to stand with occasional stirring for 1 hr, charcoal was removed by filtration.

Step 4. Ethanol fractionation. Ten liters of ethanol (5°C) was added to the charcoal-treated enzyme solution (4900 ml). After

allowing it to stand for 2 hr, the precipitate formed was collected by centrifugation, dissolved in a small volume of water and dialyzed against running water.

Step 5. Ammonium sulfate fractionation. Finely powdered ammonium sulfate (220 g) was added with stirring to the ethanol fraction (580 ml) to give 0.5 saturation. After allowing it to stand for 5 hr, the precipitate formed was span down by centrifugation. An additional 36.5 g of ammonium sulfate was added to the supernatant to give 0.6 saturation. After allowing it to stand for 5 hr, the resulting precipitate was collected by centrifugation, dissolved in water and then dialyzed against running water overnight.

Step 6. DEAE-cellulose chromatography. The ammonium sulfate fraction (50 ml) was loaded on a column (4.5×55 cm) of DEAE-cellulose equilibrated with 0.05 M sodium acetate buffer, pH 6.0. A linear gradient elution was carried out with NaCl (0.1~0.5 M) in 0.05 M sodium acetate buffer, the total volume of the gradient being 2000 ml. A 15-ml fraction was collected at a flow rate of about 20 ml/hr. The elution profile is shown in Fig. 1. The enzyme peak fraction (No. 57 to 81) was concentrated to 15 ml with a colodion bag.

The results of the overall purification are summarized in Table III. The final preparation represented about 30-fold purification over the starting extract. This preparation was essentially free of phosphomonoesterase

FIG. 1. Chromatography of Ammonium Sulfate Fraction on DEAE-Cellulose.

△—△, activity toward (2'-5') A-A (Assay 1); ○—○, activity toward *p*-nitrophenyl-pA (Assay 2); ----, OD at 280 nm; · · · ·, NaCl.

activity.

Some properties of the partially purified enzyme

To compare 5'-phosphodiesterase from *Aspergillus niger* with the known phosphodiesterases from other sources, optimal conditions, stability and metal requirement of the enzyme were examined. pH optimum of the enzyme was at around 5 (Fig. 2). Temperature optimum of the enzyme was at around 70°C (Fig. 3). Stable pH range of the enzyme was 3.3 to 5.3 (Fig. 4). The enzyme was stable below 60°C (Fig. 5). Metal ions such as Li⁺, Sr²⁺, Co³⁺, Cd²⁺, Ca²⁺, Mn²⁺, Zn²⁺, Ni²⁺, Mg²⁺, Pb²⁺ and Ba²⁺ at the concentration of 1×10⁻³ M gave no significant effect on the activity. The *Aspergillus* enzyme seems to be distinguish-

TABLE III. SUMMARY OF PURIFICATION OF 5'-PHOSPHODIESTERASE FROM *Aspergillus niger*

Enzyme activity was measured by Assay 1.

Protein concentration was expressed in A_{280} units.

Purification step	Volume (ml)	Total A_{280}	Total activity (units)	Specific activity (units/ A_{280})
Crude extract	5100	163,863	82,408,554	503
Heat treatment (pH 6.3, 60°C, 30 min)	5100	163,863	37,750,302	230
Charcoal treatment (1.5%, pH 4.8)	4900	20,972	16,055,340	766
Ethanol	580	2170	1,989,000	917
Ammonium sulfate (0.5~0.6 sat)	50	295	932,670	3161
DEAE-Cellulose	15	37	552,120	14,923

FIG. 2. Effect of pH on Enzyme Activity.

The enzyme activity was measured by Assay 1 except that pH of veronal-acetate buffer was varied.

FIG. 3. Effect of Temperature on Enzyme Activity.

The enzyme activity was measured by Assay 1 except that the temperature of reaction was varied.

ed from the known phosphodiesterases in the pH optimum and the effect of metal ions.

Specificity and mode of action

As shown in Table IV, the enzyme preparation hydrolyzed both 2'→5' and 3'→5' dinucleotides including 3'-hydroxy-terminated dinucleotides and dinucleoside monophosphates without specificity toward bases and sugars, and also hydrolyzed *p*-nitrophenyl ester of nucleoside 5'-phosphate, ADP, UDPG, and CDP-choline to give nucleoside 5'-phosphate. No significant activity toward dinucleotides be-

FIG. 4. Effect of pH on Stability of the Enzyme.

The enzyme preparation (1000 units for (2'-5') A-A) was incubated in 1 ml of M/35 veronal-acetate buffer, at the indicated pH at 37°C for 2 hr. After incubation, the remaining activity was measured by Assay 1.

FIG. 5. Thermostability of the Enzyme.

The enzyme preparation (1000 units for (2'-5') A-A) was heated in 1 ml of M/35 veronal-acetate buffer, pH 5.0, for 30 min at the indicated temperature, and then the mixture was rapidly chilled. The remaining activity was measured by Assay 1.

aring 3'-phosphate, 2': 3'-cyclic AMP, 3': 5'-cyclic AMP, bis(*p*-nitrophenyl) phosphate and polynucleotides was recognized.

Application of the Aspergillus 5'-phosphodiesterase to degradation of 2'→5' dinucleotides in nuclease P₁ digest of technical grade RNA
Ten ml of the reaction mixture containing

TABLE IV. SUBSTRATE SPECIFICITY OF
5'-PHOSPHODIESTERASE FROM
Aspergillus niger

The hydrolysis rates toward oligonucleotides, CDP-choline, UDPG, 3': 5'-cAMP and 2': 3'-cAMP, *p*-nitrophenylesters, and nucleic acids were measured according to Assay 1, Assay 2 and Assay 3, respectively. The hydrolysis rate toward ADP was measured according to Assay 1 from which boiling procedure and alkaline phosphatase treatment were omitted.

Substrate	Relative activity
(2'-5') A-A	1.000
(2'-5') U-A	0.658
(2'-5') G-A	0.687
(2'-5') A-C	0.584
(2'-5') C-A	1.200
(3'-5') A-A	1.204
(3'-5') U-A	0.881
(3'-5') G-A	1.351
(3'-5') A-C	0.619
(3'-5') C-A	0.805
(3'-5') dA-dT	0.540
(3'-5') dT-dC	1.000
(2'-5') pA-C	0.394
(3'-5') pA-C	0.177
(3'-5') pA-A	0.451
(3'-5') A-Ap	0.001
(3'-5') A-Cp	0.001
ADP	0.497
CDP-choline	1.079
UDPG	1.342
<i>p</i> -Nitrophenyl-pU	1.000
<i>p</i> -Nitrophenyl-pA	2.583
Bis(<i>p</i> -nitrophenyl)phosphate	0.026
Tp- <i>p</i> -nitrophenyl	0.001
3': 5'-cAMP	0.005
2': 3'-cAMP	0.004
RNA	0.001
Heat-denatured DNA	0.001

400 mg of technical grade yeast RNA, 2×10^{-4} M ZnCl₂ and 40 μ g of nuclease P₁ was incubated at pH 5.0 at 70°C for 4 hr. Four ml of the nuclease P₁ digest was further incubated with or without 0.2 ml of the *Aspergillus* 5'-phosphodiesterase preparation (32,000 units/ml) at pH 5.0 at 70°C for 4 hr. After incubation, the digestion products were analyzed by DEAE-sephadex A-25 column chromatography in the presence of 7 M urea (Fig. 6). The further treatment of the nuclease P₁ digest with the *Aspergillus* enzyme resulted in 93% decrease of dinucleotides (peak 3), and 6% increase of

FIG. 6. DEAE-Sephadex A-25-7 M Urea Column Chromatography of Nuclease P₁ Digest of Technical Grade RNA (A) and the Digest Treated Further with *Aspergillus* 5'-Phosphodiesterase (B).

Each digest was applied to a 2×45 -cm column of DEAE-Sephadex A-25 equilibrated with the urea-Tris buffer system containing 7 M urea, 0.02 M Tris-HCl buffer (pH 7.6) and 0.14 M NaCl. The column was then connected to a 2000-ml linear gradient of 0.14 to 0.18 M NaCl in the urea-Tris buffer. After the linear gradient elution the elution was continued further with 1000 ml of the urea-Tris buffer containing 1 M NaCl and 0.5 M NaOH. Ten-ml fractions were collected at a flow rate of 40 ml/hr. The peaks 1, 2 and 3 were confirmed to be nucleoside, mononucleotide and dinucleotide fractions, respectively.

mononucleotides (peak 2) on the basis of A_{260} unit. The increase of mononucleotides is clearly due to degradation of 2'→5' dinucleotides resistant to nuclease P₁. In addition, it was confirmed that most of the dinucleotides isolated from the nuclease P₁ digest were 2'→5' dinucleotides and degraded into four 5'-mononucleotides.

From these results, it is found that technical grade yeast RNA containing 2'-5' phosphodiester linkages can be completely digested into 5'-mononucleotides by using both *Penicillium* nuclease P₁ and *Aspergillus* 5'-phosphodie-

sterase.

DISCUSSION

From the specificity studies it is suggested that the basic structural element for substrates of the *Aspergillus* enzyme is a "nucleoside 5'-phosphoryl" group as shown in Diagram 1.

DIAGRAM 1.

Bond cleavage occurs as shown to give a nucleoside 5'-phosphate and $R'OH$. The nature of R' was not critical, although it influenced the rate of hydrolysis. Variations of bases and sugars did not give great influence to the rates of hydrolysis. Like snake venom 5'-phosphodiesterase,⁷⁾ the presence of the 3'-monophosphoryl group seriously inhibited the rate of the hydrolysis. As discussed above, the *Aspergillus* 5'-phosphodiesterase seems similar to snake venom 5'-phosphodiesterase in the specificity and mode of action. However, difference exists in their behavior of the 5'-monophosphoryl group: the presence of the

phosphoryl group depresses the rate of hydrolysis with the *Aspergillus* enzyme action, while it enhances the rate of hydrolysis with the snake venom enzyme action. Besides snake venom phosphodiesterase, nonspecific 5'-phosphodiesterases have been found in hog kidney,⁸⁾ rat liver,⁹⁾ carrot,¹⁰⁾ *Lactobacillus acidophilus*¹¹⁾ and bovine milk¹²⁾ (Table V). However, the *Aspergillus* enzyme is clearly distinguished from these enzymes in pH optimum and requirement of metal ion: the *Aspergillus* enzyme has pH optimum at around 5 and does not require any metal ion for the activity, while others have pH optima at 8.0 to 10.0 and require Mg^{2+} or Mn^{2+} for the activity. In addition, the *Aspergillus* enzyme seems to be much less active toward polynucleotides than other enzymes.

The *Aspergillus* enzyme effectively hydrolyzed 2'-5' phosphodiester linkages which are contained in technical grade RNA and resistant to nuclease P_1 . In addition, this enzyme was quite thermostable and its optimal reaction temperature was at around 70°C. Therefore, this enzyme is expected to be used industrially to complete degradation of technical grade RNA with *Penicillium* nuclease P_1 .

The *Aspergillus* enzyme is the first 5'-phosphodiesterase that was found in fungi. It is of particular interest that high productivity of 5'-phosphodiesterase was distributed exclusively in black koji-molds among fungi tested.

TABLE V. COMPARISON OF *Aspergillus* 5'-PHOSPHODIESTERASE WITH OTHER 5'-PHOSPHODIESTERASE

Origin	pH Opt.	Metal requirement	Action toward				
			<i>p</i> -Nitro-phenyl-pT	Oligo		RNA	DNA
				ribo	deoxy		
Snake venom	8.6	Mg^{2+}	+	+	+	+	+
Bovine milk	9.5	Mg^{2+} , Ca^{2+}	+	+	+	+	+
Pig kidney	9.1	Mg^{2+}	+	+	+	+	+
Carrot	8.9	Mg^{2+} , Mn^{2+} , Zn^{2+}	+	+	+	+	+
Rat liver	10.0	Mg^{2+} , Ca^{2+}	+	+	+	—	+
<i>Lactobacillus acidophilus</i>	8.0	Mn^{2+}	+	+	+	—	+
<i>Aspergillus niger</i>	5.0	None	+	+	+	—	—

+, hydrolyzed; —, not hydrolyzed.

REFERENCES

- 1) A. Kuninaka, M. Fujimoto and H. Yoshino, *Agric. Biol. Chem.*, **39**, 597 (1975).
 - 2) A. Kuninaka, M. Fujimoto and H. Yoshino, *ibid.*, **39**, 603 (1975).
 - 3) K. Miura and T. Ueda, *Chem. Pharm. Bull.*, **19**, 2567 (1971).
 - 4) E. Ohtsuka, K. Murao, M. Ubasawa and M. Ikehara, *J. Am. Chem. Soc.*, **692**, 3441 (1970).
 - 5) M. Fujimoto, K. Fujiyama, A. Kuninaka and H. Yoshino, *Agric. Biol. Chem.*, **38**, 2141 (1974).
 - 6) M. Fujimoto, A. Kuninaka and H. Yoshino, *Agric. Biol. Chem.*, **38**, 777 (1974).
 - 7) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).
 - 8) W. E. Razzell, *ibid.*, **236**, 3031 (1961).
 - 9) M. Futai and D. Mizuno, *ibid.*, **242**, 5301 (1967).
 - 10) H. L. Malsman and L. Nussbaum, *Biochemistry*, **6**, 3689 (1967).
 - 11) Z. J. Sabatini and R. D. Hotchkiss, *ibid.*, **8**, 4831 (1969).
 - 12) S. Matsushita, F. Ibuki, T. Mori and T. Hata, *Agric. Biol. Chem.*, **29**, 436 (1965).
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