KOR. JOUR. MICROBIOL. Vol. 21, No. 3, 135~142, 1983

Purification and properties of polyphosphate phosphohydrolase from *Chlorella ellipsoidea.*

LIM, Young Bock and Yung Nok LEE

(Department of Biology, Korea Universty)

*Chlorellaellipsoidea*로 부터 polyphosphate phosphohydrolase의 분리, 정제 및 성질

임 영 복·이 영 록

(고려대학교 생물학과)

ABSTRACT

The presence of polyphosphate phosphohydrolase (PPPH) and tripolyphosphate phosphohydrolase (TPPH) in *Chlorella ellipsoidea* were confirmed from the cell-free extract of the algal cells and three forms of PPPH were isolated, purified, and measured Km-Vmax value and inhibitory effect by metal ions, respectively.

PPPH was most active at pH 7.2, whereas TPPH at pH 7.6. Both enzymes exhibited their maximum activity at 37°C. For the manifestation of catalytic activity, divalent metal ions are needed, and the best activator for both enzymes was Co^{++} ions (10^{-3}M). These enzymes were inhibited by Hg^{++} ions (10^{-3}M) considerably.

PPPH from *Chlorella ellipsoidea* was purified by ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephadex A-25, and gel filtration on Sephadex G-100, and some properties of the three different fraction with PPPH activity ($PPPH_1$, $PPPH_2$, and $PPPH_3$) were found, i.e., PPPH has multiple form.

The Km values of $PPPH_1$, $PPPH_2$, and $PPPH_3$ obstained were 6.25 x10^{-4}M, 5.26 x10^{-4}M, and 3.33 x10^{-4}M, and Vmax were 3.33 mM/min, 3.33 mM/min, and 2.67 mM/min, respectively. It was shown that the types of inhibition of Hg^{++} on the activities of three forms of PPPH were competitive inhibition.

INTRODUCTION

The pathways of the biosynthesis, utilization, and degradation of high molecular weight inorganic polyphosphates in heterotrophic organisms, such as bacteria, actinomycetes, and fungi, have been studied rather thoroughly (Kulaev, 1979).

In particular, polyphosphate phosphohydrolase (PPPH) and tripolyphosphate phosphohydrolase (TPPH) associated with degradation of polyphosphates and tripolyphosphates have been isolated and studied to some degree in a

few microorganisms, such as *Corynebacterium xerosis* (Muhammed et al., 1959), *Aerobacter aerogenes* (Harold, 1964; Harold & Harold, 1965), *Saccharomyces cerevisiae* (Felter et al., 1970), *Neurospora crassa* (Kulaev & Konoshenko, 1971; Egorov & Kulaev, 1976), *E. coli* (Nesmeyanova et al., 1973; 1974), *Endomyces mangusii* (Afanas'eva et al., 1976), *Thermus flavus* (Egorova et al., 1981).

However, there is even less information about enzymatic reactions of polyphosphate utilization in phototrophs. Jungnickel (1973) found polyphosphatase activity in cells of higher plants. Rubtsov and Kulaev (1977) suggested that the degradation of polyphosphates in *Acetabularia mediterranea* apparently occured with the participation of PPPH, which was found in a cell-free extract. Although there are many reports on the polyphosphate metabolism in *Chlorella* cells (Lee, 1964), the presence of PPPH and TPPH in the algal cells have not yet been confirmed. Therefore, we will confirm the presence of PPPH and TPPH in *Chlorella* cells and characterize some properties of these enzymes in the present study.

MATERIAL AND METHODS

1. Experimental organism

Chlorella ellipsoedea was used in this study. The cells were grown in a M4N medium for 5~6 days as described previously(Lee & Lim, 1982).

2. Preparation of cell free extracts

To obtained the cell free extract of *Chlorella*, the harvested cells (2~3 m*l* in packed-cell volume) were washed twice with 2mM K_2SO_4 Solution and suspended in 50mM tris-maleate buffer, pH7.2 at 0~2°C. The suspended cells were sonicated at 20 KHz for 30 min, and the homogenate obtained was centrifuged at 27,000 Xg for 30 min. The cell free extract obtained was used to determine enzyme activity.

3. Enzyme assay

The activities of polyphosphates phosphohydrolase (PPPH) and tripolyphosphates phosphohydrolase (TPPH) were determined from the amount of orthophosphate formed from the appropriate substrate during incubation in a cell-free extract of *Chlorella*.

The incubation mixture for detection of the enzymatic activities contained the following composition: for PPPH 50 mM pH 7.2 tris-maleate buffer, 1 mM $MgCl_2$, polyphosphate (n=15) (1mg/m*l*), and cell-free extract 200~400 μg protein; for TPPH 50 mM pH 7.6 tris-HCl buffer, 1 mM $MgCl_2$, sodium tripolyphosphate (1mg/m*l*), and cell-free extract 200~400μg protein. The reaction mixture, with a total volume of 0.5 m*l*, was incubated for 20 min at 37°C and then the reaction was stopped by the addition of 0.5 m*l* of 7% $HClO_4$.

Inorganic orthophosphate was determined according to the method of Berenblum and Chain (1935). The protein content of the cell-free extract was determined by Lowry's method (1951). The amount of the enzyme that liberated 1μM of orthophosphate per min was taken as the unit of enzymatic activity (U).

4. Purification of the enzymes

Tris-maleate buffer was used to isolate PPPH, since the enzyme retained its activity better in this buffer than in the other buffer mixtures. In addition, $MgCl_2$ and 2-mercaptoethanol were introduced into the buffer as additional stabilizing agents. All stages of the experiment were conducted at 2~4°C.

Ammonium sulfate Fractionation; Ammonium sulfate was added to the cell-free extract in small portions with mixing to 50% of saturation. After the last portion of the salt dissolved the mixture was left for 20 min in an ice bath and then centrifuged. The sediment was discarded and ammonium sulfate was added again to the supernatant to 80% of saturation.

The precipitate was dissolved in 50 mM tris-maleate buffer (pH 7.2) containing 1mM $MgCl_2$ and 1mM 2-mercaptoethanol (working buffer), and it was dialyzed against the same buffer for 48hr.

Treatment with DEAE-Sephadex; A-25 The fraction which collected during salting out, was applied to a column(1 x 20 cm) of DEAE-Sephadex A-25 epuilibrated with the working buffer. A continuous linear NaCl gradient from 0 to 0.2M in the working buffer was used to elute the proteins. The total volume of the eluent was 400 m*l* and the flow rate was 18 ml/h. Each fraction was collected 4m*l*.

Gel filtration; Fractions which contained PPPH activity, collected in the stage of treatment with DEAE-Sephadex A-25, applied to a column (1x20 cm), filled with Sephadex G-100. The column has been preliminarily equilibrated with working buffer. The flow rate was the same as treatment with DEAE-Sephadex A-25.

The amount of protein in the elutes from the columes during purication and fractionation of PPPH was measured by the absorption of the solutions at 280 nm.

RESULTS AND DISCUSSIONS

1. Effect of pH and temperature on the activities of PPPH and TPPH

We detected activities of PPPH and TPPH in a cell free extract of *Chlorella*. An investigation of the effect of the hydrogen ion concentraction on the activities of the enzymes was conducted at 37°C in the pH range from 2.0 to 9.0. In the region of pH 2.0 glycine-HCl buffer was used, in pH 3~5 acetate buffer, in pH 6.0~7.2 tris-maleate buffer, and in pH 7.2~9.0 tris-HCl buffer. As can be seen from Fig. 1, PPPH showed its maximum activity at pH 7.2 and TPPH at pH 7.6 in the presence of 1 mM $MgCl_2$. Therefore, 50mM tris-maleate buffer (pH 7.2) for PPPH and 50 mM tris-HCl buffer (pH 7.6) for TPPH were used for the subsequent experiments.

The effect of temperature on the rate of hydrolysis of the enzymes was verified in the

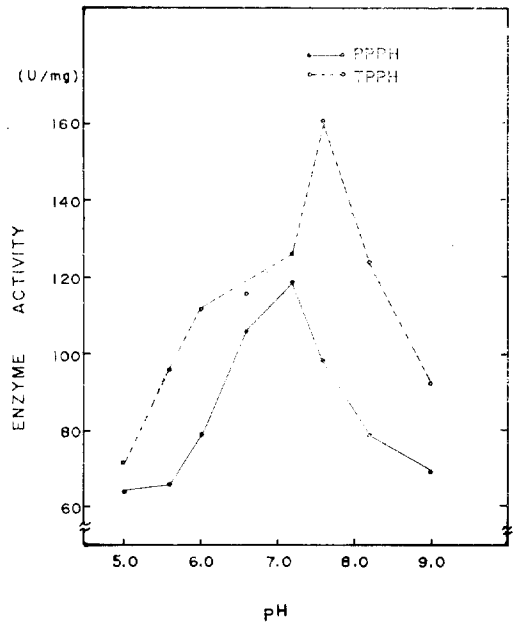

Fig. 1. Effect of pH on the activivies of PPPH and TPPH from *Chlorella*.

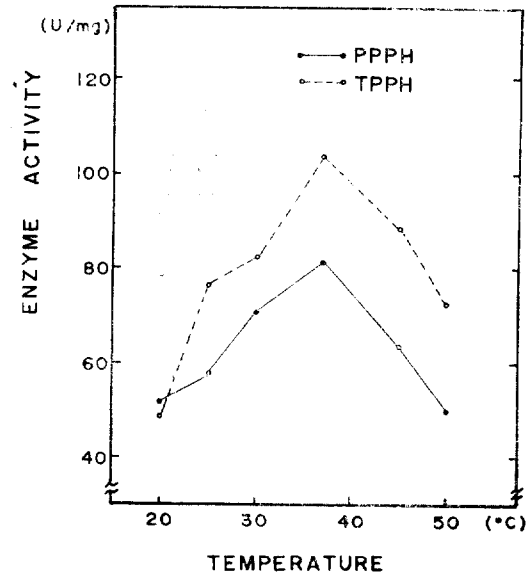

Fig. 2. Effect of temperature on the activities of PPPH and TPPH from *Chlorella*.

interval from 20° to 50°. From Fig. 2 it can be seen that the activitis of PPPH and TPPH were most active at 37°C.

The properties of these enzymes are quite similar to the properties of the analogous enzymes from other organisms. For example, the optimum pH and temperature on the activity of PPPH and TPPH from *C. ellipsoidea* were quite similar to those from *Neurospora* (Kulaev & Konoshenko, 1971), *Propionibacterium* (Kulaev *et al.*, 1973), *Escherichia* (Nesmeyanova, 1974), and *Aerobacter* (Harold & Harold, 1965).

2. Effect of metal ions on PPPH and TPPH activity

The effects of various divalent metal ions on the enzyme activity of a cell-free extract were examined, in concentration of 10^{-3}M (Table 1). It was found that the cation Co^{++}, Fe^{++}, and Mg^{++} stimulated activities of both PPPH (30~40%) and TPPH (20~30%), while the Hg^{++} ion inhibited the activities of these enzymes (25~50%) considerably.

It was found that Co^{++} ion is the most effective activator for PPPH and TPPH from *Chlorella*, although it was reported that PPPH from *Aerobacter* (Harold & Harold, 1965) was

Table 1. Effects of metal ions on the activities of PPPH and TPPH of *Chlorella*.

Metal ions Conc. (1×10^{-3}M)	Enzyme Activities for PPPH		Enzyme Activities for TPPH	
	unit	%	unit	%
None	144.5	100	151.8	100
$CoCl_2$	202.9	140.4	195.8	129.0
$FeSO_4$	195.9	135.6	193.4	127.4
$MgCl_2$	191.0	132.2	184.4	121.5
$ZnCl_2$	183.7	127.1	178.7	117.7
$MnCl_2$	174.3	120.6	171.4	112.9
$CuCl_2$	136.4	94.4	114.5	75.4
$CaCl_2$	129.5	89.6	117.8	77.6
$HgCl_2$	105.9	73.3	78.5	51.7

Table 2. Purification of PPPH from *Chlorella*.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Degree of purification	Yield (%)
Cell-free extract	928	170,800	184	1	100
Dialysis	36.70	13,200	359	2	7.72
DEAE-Sephadex A-25					
$PPPH_1$	6.05	3,276	542	2.95	2.03
$PPPH_2$	3.72	4,094	1,101	6.02	2.40
$PPPH_3$	2.93	4,315	1,475	8.02	2.53
Sephadex G-100					
$PPPH_1$	1.60	3,024	1,890	10.37	1.77
$PPPH_2$	1.34	2,828	2,132	11.60	1.67
$PPPH_3$	1.16	2,090	1,803	9.70	1.22

activated by Mg^{++}, and PPPH from *Endomyces* (Kulaev *et al.*, 1972) by Mn^{++}. Ions of other divalent metals were also useful for the detection of PPPH and TPPH activity in *Chlorella*, just as in other biological objects.

3. Purification of PPPH

The purification steps of PPPH were summarized in Table 2. In the ammonium sulfate fractionation the degree of purification was increased to 2-fold, however the yield was very low showing about 8% of total PPPH activity of the cell-free extract. The fraction collected during salting out within the limits of 50~80% of saturation by $(NH_4)_2 SO_4$ was dissolved in a minimal amount of working buffer and used for further experiment.

The typical elution profile on treatment with DEAE-Sephadex A-25 is shown in Fig. 3, from which it is seen that PPPH was separated into three peaks (Ⅰ, Ⅱ, and Ⅲ). Peak Ⅰ ($PPPH_1$) emerged at a very low concentration of NaCl (0.04M), and peak Ⅱ ($PPPH_2$) and peak Ⅲ ($PPPH_3$) emerged at 0.07M and 0.11M, respectively. The specific activities of $PPPH_1$, $PPPH_2$, and $PPPH_3$ obtained in this stage were about 3-, 6-, and 8- times higher than the specific activity of the cell-free extract. The fractions of each peak with high PPPH activity were pooled and were further purified. A typical chromatogram on Sephadex G-100 is shown in Fig. 4.

Further purication was difficult because of the very great losses of the total activity of the enzyme during its isolation. Only average 1.5% of initial activity of the homogenate remained in the preparation obtained.

Felter *et al.*, (1970) and Afanas'eva *et al.* (1976) in isolating the enzyme from *Saccharomyces cerevisiae* and *Endomyces magnusii*, respectively, also encountered similar high lability of PPPH. The yields of total activity were only 1% for *S. cerevisiae* and 3.2% for *E. magnusii*. With regard to the PPPH of other organisms, for example in mold fungi, the enzyme is considerably more stable (Umnov *et al.*, 1974). Well purified preparation of PPPH with a high yield of total activity, which comprised 20~30% of the initial activity, have also been isolated from a number of bacteria (Muhammed *et al.*, 1959; Harold & Harold, 1965).

4. Km and Vmax value of PPPH

Samples of $PPPH_1$, $PPPH_2$, and $PPPH_3$,

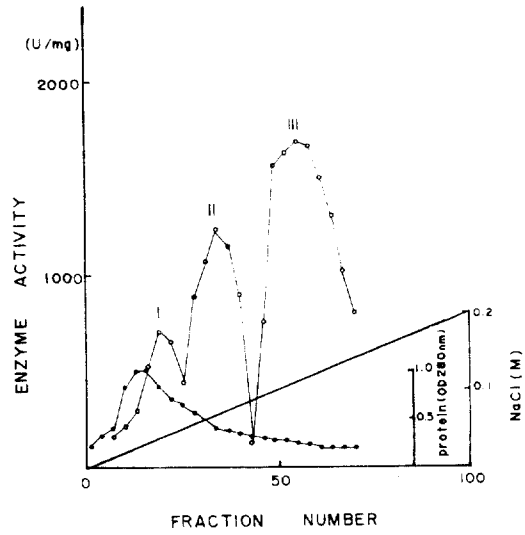

Fig. 3. Ion-exchange chromatography of PPPH on DEAE-Sephadex A-25. (open circle; enzyme activity, closed circle; protein content)

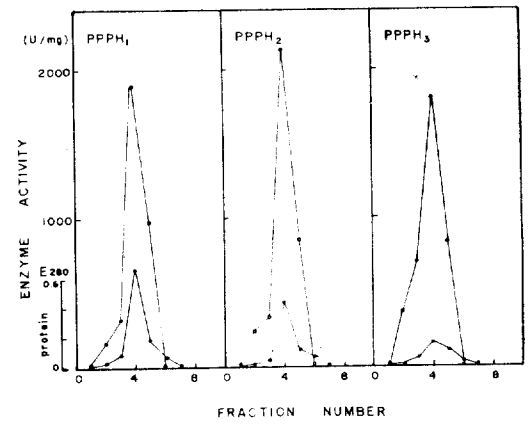

Fig. 4. Gel filtration of PPPH on Sephadex G-100. (open circle; enzyme activity, closed circle; protein content)

obtained by gel filtration, were used for determination of Km and Vmax value. Mg^{++} ions were used as the activator. The dependence of the rate of hydrolytic cleavage on the substrate by Lineweaver-Burk plots is shown in Fig. 5. From Lineweaver-Burk plots, the Km values of $PPPH_1$, $PPPH_2$, and $PPPH_3$ were 6.25×10^{-4}M, 5.26×10^{-4}M, and 3.33×10^{-4} M, respectively, whereas the Vmax values were 3.33 mM/min, 3.33mM/min, and 2.67 mM/min, respectively. The Vmax value of $PPPH_1$ is equal to that of $PPPH_2$.

The fractionation of the isolated enzyme preparation conducted in the present work showed that it contained at least three peaks, which differ in Km and Vmax value. It was found that the Km value of $PPPH_3$ is the lowest among the three forms. Accordingly $PPPH_3$ is the highest affinity to the substrates. The presence of multiple forms of PPPH in *Chlorella* is in accord with the result of Afanas'eva *et al.* (1976) in *Endomyces*.

5. Type of enzyme inhibition by mercury ion

As mentioned above in Table 1, significant inhibitory effect of Hg^{++} on the PPPH activity in cell-free extract was observed. As shown in Fig. 6, 7, and 8, the types of the inhibition of Hg^{++} on the activities of $PPPH_1$, $PPPH_2$, and $PPPH_3$ were all competitive inhibition.

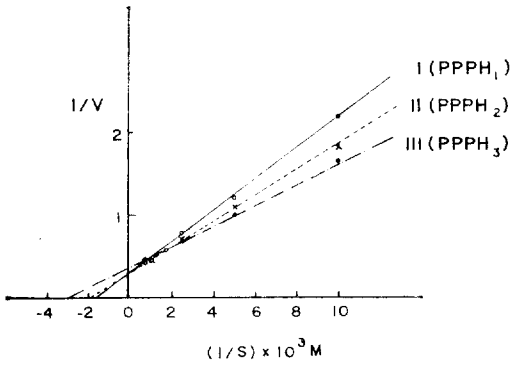

Fig. 5. Lineweaver-Burk plots for the actions of $PPPH_1$, $PPPH_2$, and $PPPH_3$.

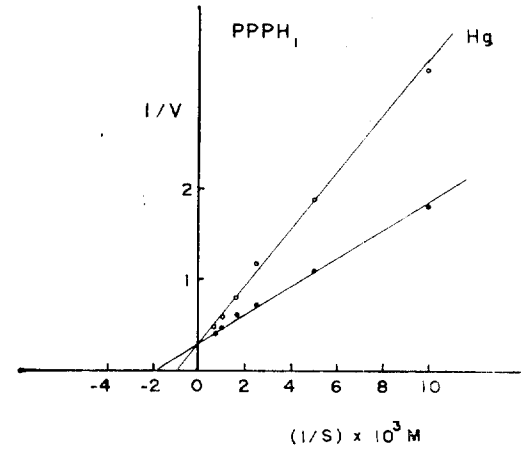

Fig. 6. Plot for the inhibition constant of Hg^{++} ion on the activity of $PPPH_1$.

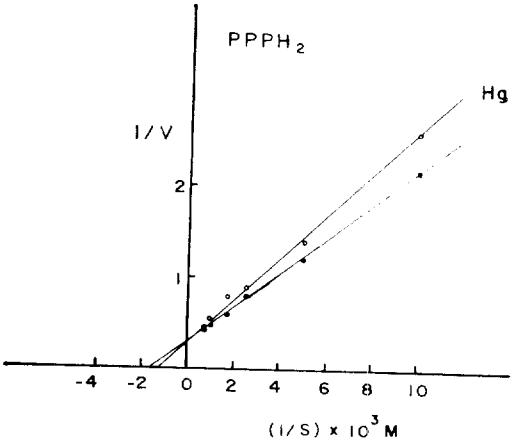

Fig. 7. Plot for the inhibition constant of Hg^{++} ion on the activity of $PPPH_2$.

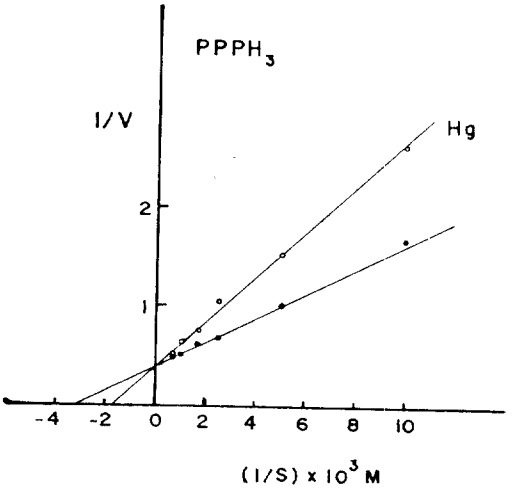

Fig. 8. Plot for the inhibition constant of Hg^{++} ion on the activity of $PPPH_3$.

摘 要

*Chlorella ellipsoidea*의 세포추출액으로 부터 polyphosphates phosphohydrolase (PPPH)와 tripolyphosphate phosphohydrolase (TPPH)의 존재를 확인하고 세가지 다른 형태의 PPPH를 분리, 정제하여 그들의 Km 및 Vmax 값과 여러가지 금속 이온에 대한 억제 작용등을 조사하였다.

이들 두 종류의 효소활성에 대한 최적 pH는 PPPH의 경우 pH 7.2였고, TPPH의 경우 pH 7.6이였다. 또한 두 효소는 모두 37°C에서 가장 높은 활성을 나타내었다. 이들 PPPH와 TPPH의 활성은 Co^{++} (10^{-3}M)에 의해 가장 크게 증가되며, Hg^{++} (10^{-3}M)는 두 효소의 활성을 억제시켰다.

분리·정제된 PPPH는 세가지 분획구(Ⅰ, Ⅱ, Ⅲ)에서 높은 활성도를 나타내었는데, 세포 추출액에 비해 9.7～11.6배 순화되었다. $PPPH_1$, $PPPH_2$ 및 $PPPH_3$의 Km 값은 각각 6.25×10^{-4}M, 5.26×10^{-4}M 및 3.33×10^{-4}M이었고, $PPPH_1$ 및 $PPPH_2$의 Vmax 값은 다같이 3.33mM/min였고, $PPPH_3$는 2.67mM/min이었다. 따라서 $PPPH_3$가 기질에 대한 친화력이 가장 높았다.

금속의 영향중 억제 영향이 가장 심했던 Hg^{++}의 억제형태는 세가지 분획구에서 모두 경쟁적 억제를 나타내었다.

REFERENCES

Afanas'eva, T.P., S.O. Uryson, I.S. Kulaev, 1976. The detection of multiple forms of polyphosphate phosphohydrolase in *Endomyces magnusii*. *Biokhimiya*. **41**, 6, 1078～1086.

Berenblum, I. and E. Chain, 1938. An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295～298.

Egorov, S.N. and I.S. Kulaev, 1976. The isolation and properties of tripolyphosphatase from the fungus *Neurospora crassa*. *Biokhimiya*, **41**, 1958～1967.

Egorova, L.A., M.A. Bobyk, L.G. Loginova, and I.S. Kulaev, 1981. The metabolism of polyphosphates in the thermophilic bacterium *Thermus flavus*. *Mikrobiologiya*, **50**, 4, 603～606.

Felter, S., G. Dirheimer, and J.P. Ebel, 1970. Recherches sur les polyphosphatases de levure de boulangerie. *Bull. Soc. Chim. Biol.*, **52**, 433.

Harold, F.M., 1964. Enzymic and genetic control of polyphosphate accumulation in *Aerobacter aerogenes*. *J. Gen. Microbiol.*, **35**, 81～90.

Harold, F.M. and R.L. Harold, 1965. Degradation of inorganic polyphosphates in mutants of *Aerobacter aerogenes*. *J. Bacteriol.*, **89**, 5, 1262～1270.

Jungnickel, F., 1973. Reaction mechanism and control properties of phosphotransferases, Int. Symp. Reinhardsbrunn, Akademie-Verlag, Berlin, 87～91.

Kulaev, I.S., 1979. The Biochemistry of Inorganic Polyphosphates. John Wiley & Sons. (Translation by R. F. Brookes) 122～173.

——, and G.I., Konoshenko, 1971. The detection and properties of polyphosphatases of *Neurospora crassa* which hydrolyse inorganic polyphosphates to orthophosphate. *Biokhimiya*, **36**, 6, 1175～1182.

——, T.P. Afanas'eva, and A.N. Belozersky, 1972. Polyphosphate phosphohydrolases in *Endomyces magnusii*. *Fiziol. Rast.*, **19**, 984

——, L.I. Vorob'eva, L.V. Konovalova, M.A. Bobyk, G.I. Konoshenko, and S.O. Uryson, 1973. Polyphophate metabolizing enzymes in the development of *Propionibacterium shermanii*, under normal conditions and in the presence of polymyxin M. *Biokhimiya*, **38**, 712～717.

Lee, Y.N., 1964. Studies on the phosphate metabolism in *Chlorella*, with special reference to polyphosphate. *Kor. J. Microbiol.*, **2**, 1～11.

Lee, C.S. and Y.B. Lim, 1982. Effects of heavy metals on contents of various phosphate compounds and biosynthesis of organic compounds in *Chlorella* cells. ibid., **20**, 27～40.

Lowry, O.H., N.I. Rosenbrough, A.L. Farr, and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265～275.

Muhammed, A., A. Rodgers and D.E. Houghes, 1959. Purification and properties of a polymetaphosphatase from *Corynebacterium xerosis*. *J. Gen. Microbiol.*, **20**, 482.

Nesmeyanova, M.A., A.D. Dmitriev, and I.S. Kulav, 1973. High-molecular polyphosphales and polyphosphate-metabolizing enzymes during the growth of a culture of *Escherichia coli*. *Mikrobiologiya*, **42**, 213~219.

——, 1974. The regulation of phosphorus metabolism and the level of polyphosphates in *Escherichia coli* K-12 by exogenous orthophosphate. ibid., **43**, 2, 227~234.

Rubtsov, P.M. and I.S. Kulaev, 1977. Some pathways of biosynthesis and breakdown of polyphosphates in the green alga *Acetabularia mediterranea*. *Biokhimiya*, **42**, 6, 1083~1089.

Umnov, A.M., S.N. Egorov, S.E. Mansurova, and I.S. Kulaev, 1974. Some properties of polyphosphatases from *Neurospora crassa*. ibid., **39**, 2, 373~377.