Purification and Substrate Specificity of *endo*-Type Inulinase from *Penicillium purpurogenum*

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An inulinase was highly purified from the culture broth of *Penicillium purpurogenum* by chromatographies on DEAE-Sepharose CL-6B, Toyopearl HW-65, and Bio-Gel P-100. The enzyme was homogeneous by disc electrophoretic analysis. The molecular weight was 6.4×10^4 by SDS-disc electrophoresis and gel filtration on Bio-Gel P-150. The isoelectric point was pH 3.6 by isoelectric focusing. The enzyme hydrolyzed inulin rapidly, but did not affect sucrose. By paper chromatography analysis, the major products from inulin were tri-, tetra-, penta-, and hexa-saccharides. The substrate specificity of the enzyme on hydrolyses of fructo-oligosaccharides [$1^E(1-\beta\text{-D-fructofuranosyl})_n\text{sucrose}$ ($n=1$ to 6 and $\bar{n}$ (average of polymerization degree)=8)] were examined. The K_m values and relative maximum velocities for the hydrolyses of inulin and fructo-oligosaccharides (GF_n , $n=2$ to 7 and $\bar{n}=9$) were as follows: inulin, ($\overline{\text{DP}}=35$) 0.21 mM and 100; GF_7 , 0.24 mM and 86.5; GF_6 , 0.33 mM and 132; GF_5 , 0.85 mM and 71.2; GF_4 , 3.8 mM and 25.4; GF_3 , 2.8 mM and 28.8; GF_2 (1-kestose), 8.4 mM and 0.2. The molecular activities for the hydrolyses of fructo-oligosaccharides (GF_n , $n=2$ to 6) were increased depending on the degree of polymerization of fructosyl residues, and were nearly constant if the polymerization degree was over seven. These results strongly suggested that the *endo*-type inulinase from *Penicillium purpurogenum* had a subsite structure consisting of at least seven subsites.

In studies of fructan metabolism, we have reported the structures of fructo-oligosaccharides^{1~3)} and the properties of three kinds of fructosyltransferases^{4~7)} involved in the synthesis of the saccharides in asparagus root and onion bulbs, and have studied fructose-oligomer and -polymer hydrolyzing enzymes. Recently, we obtained *endo*- and *exo*-type inulinases from a strain of *Penicillium purpurogenum*.^{8,9)} Inulinase [2,1- $\beta\text{-D-fructan}$ fructanohydrolase EC 3.2.1.7] is known to catalyze the hydrolysis of 2,1- $\beta\text{-D-fructosidic}$ linkages in inulin, and has been found in higher plants^{10~12)} and microorganisms.^{13~16)} Inulinases from higher plants hydrolyze inulin and produce fructose, but do not hydrolyze sucrose. A number of inulinases from microorganisms also attacked inulin *exo*-wise, and affected sucrose.

Nakamura *et al.*^{17,18)} reported the properties of inulinases from *Aspergillus niger* and *Penicillium* sp. and that one of the extracellular inulinases from *Aspergillus niger*-12¹⁷⁾ did not hydrolyze sucrose or raffinose, and attacked inulin *endo*-wise producing inulo-oligosaccharides. The products from inulin hydrolyzed by inulinase from *Aspergillus niger*-12 were identified by paper chromatography analysis.

However, few studies on the kinetics of inulinases were reported and the reaction mode of the enzymes was not investigated in detail. To find the reaction mode of inulinases, we intend to study the kinetic properties of the enzymes. In this paper, we describe the purification and substrate specificity of the *endo*-type inulinase from *Penicillium purpurogenum*.

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MATERIALS AND METHODS

Microorganisms. *Penicillium purpurogenum* var. *rubriclerotium* (HOK-1, FERM P-8705)¹⁹⁾ obtained from soil (kindly supplied by Mitsui Toatsu Chemical Co., Ltd.) was aerobically cultivated with shaking at 28°C for 2 days in a medium of 2% inulin, 0.5% maltose, 0.5% $\text{NH}_4\text{H}_2\text{PO}_4$, 0.5% KH_2PO_4 , 0.02% Tween 80, and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The culture (125 ml) was inoculated into a jar fermenter containing 2.5 l of the same medium. The mold was cultivated aerobically at 28°C for 3 days. The mycelia were removed by centrifugation and the supernatant was used as the crude enzyme solution.

Chemicals. Inulin and sucrose were purchased from Wako Pure Chemical Ind., Ltd. Fructo-oligosaccharides, identified as $1^F(1\text{-}\beta\text{-D-fructofuranosyl})_n\text{sucrose}$ ($n=1$ to 6, and $\bar{n}=8$) were isolated from roots of asparagus.¹⁾

DEAE-Toyopearl, Toyopearl HW-65 TOSOH Co., Ltd., DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals), and Bio-Gel P-100 (Bio Rad Laboratories) were obtained from commercial sources. If not stated, other chemicals were purchased from commercial sources.

Analytical methods. The concentration of the enzyme was measured spectrophotometrically on the basis that $E_{1\text{cm}}^{1\%}$ at 280 nm was 14.4, which was obtained from the relation between the amount of protein measured by the procedure of Lowry *et al.*²⁰⁾ and absorbance at 280 nm of the purified enzyme preparation.

The enzyme reaction mixture, consisting of 0.5 ml of 0.5% substrate, 0.4 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution was incubated at 37°C for 5 min. Liberated reducing sugar was measured by the Somogyi-Nelson methods.^{21,22)} One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of reducing sugar as fructose or hydrolyzed 1 μmol of sucrose per min under the above conditions.

Polyacrylamide gel disc electrophoresis. Disc Electrophoresis²³⁾ in 7.5% polyacrylamide gel was done in Tris-glycine buffer (pH 8.3) at a current of 1 mA per tube (0.5 $\times$ 7 cm) at 4°C. Sodium dodecyl sulfate (SDS)-disc electrophoresis²⁴⁾ was done in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS with a current of 8 mA per tube (0.5 $\times$ 10 cm) for 5 hr at 4°C. Protein was stained with Coomassie Brilliant Blue G-250. Isoelectric focusing was done with a Phast System apparatus with Phast Gel IEF 3-9 media containing Pharmalyte carrier ampholyte (Pharmacia Fine Chemicals).

Paper chromatography. Paper chromatography (PC) was done by a multiple ascending technique (triple development) on Toyo Roshi No. 51 filter paper. The solvent system was 1-propanol-ethyl acetate-water (7:1:2). The sugars were detected with anisidine phosphate reagent.²⁵⁾

RESULTS

Purification of inulinase

All operations were done at 0 to 5°C throughout the purification steps.

Step 1. Chromatography on a DEAE-Sepharose CL-6B column. The culture broth (5 l) was filtered to remove mycelia. The filtrate was concentrated by ultrafiltration (Minitan, Millipore Ltd.), then the enzyme solution was dialyzed against 0.01 M sodium acetate buffer (pH 5.0). The dialysate (1800 ml) was put on a column (1.6 $\times$ 30 cm) of DEAE-Sepharose CL-6B equilibrated with 0.01 M sodium acetate buffer (pH 5.0). After removal of non-adsorbed protein, the elution was done with a linear gradient from 0 to 0.3 M NaCl in the same buffer (Fig. 1). The active fractions (Nos. 111 and 112) were combined.

Step 2. Chromatography on a Toyopearl HW-65 column. The enzyme solution (40 ml) obtained in Step 1 was dialyzed against 0.1 M sodium acetate buffer (pH 5.0) saturated to 40% with $(\text{NH}_4)_2\text{SO}_4$ over night. The enzyme solution was put on a column (1.6 $\times$ 30 cm) of

FIG. 1. Chromatography of *endo*-Type Inulinase from *Penicillium purpurogenum* on DEAE-Sepharose CL-6B Column.

The enzyme (1,800 ml) was put on a column (1.6 $\times$ 30 cm) of DEAE-Sepharose CL-6B equilibrated with 0.01 M sodium acetate buffer (pH 5.0). The elution was done with a linear gradient of NaCl in the same buffer at a flow rate of 40 ml/hr. —○—, absorbance at 280 nm; —●—, inulinase activity; —△—, invertase activity; —, concentration of NaCl.

FIG. 2. Chromatography of *endo*-Type Inulinase from *Penicillium purpurogenum* on Toyopearl HW-65 Column.

The enzyme (40 ml) was put on a column (1.6×30 cm) of Toyopearl HW-65 equilibrated with 0.1 M sodium acetate buffer (pH 5.0) saturated to 40% with $(\text{NH}_4)_2\text{SO}_4$. The elution was done with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ in the same buffer at a flow rate of 10 ml/hr.

—○—, absorbance at 280 nm; —●—, inulinase activity; —△—, invertase activity; —, concentration of $(\text{NH}_4)_2\text{SO}_4$.

FIG. 3. Gel Filtration of *endo*-Type Inulinase from *Penicillium purpurogenum* on Bio-Gel P-100 Column.

The enzyme (2 ml) was filtered on a column (3.0×65 cm) of Bio-Gel P-100 equilibrated with 0.05 M sodium acetate buffer containing 0.1 M NaCl and 2 mM 2-mercaptoethanol (pH 5.0), and eluted with the same buffer at a flow rate of 5 ml/hr. —○—, absorbance at 280 nm; —●—, inulinase activity; —△—, invertase activity.

Toyopearl HW-65 equilibrated with the same buffer. After removal of non-adsorbed protein, the elution was done with a linear gradient from 40 to 0% $(\text{NH}_4)_2\text{SO}_4$ in the same buffer (Fig. 2). The active fractions (Nos. 47~49)

were combined.

Step 3. Chromatography on a Bio-Gel P-100 column. The enzyme solution (30 ml) obtained in Step 2 was concentrated to 2 ml by a collodion bag and then filtered on a Bio-Gel P-100 column (3.0×63 cm) equilibrated with 0.05 M sodium acetate buffer containing 0.1 M NaCl and 2 mM 2-mercaptoethanol (pH 5.0) (Fig. 3). The eluates of fractions 30 and 31 were pooled and used as a purified enzyme preparation. The purification procedures of inulinase are summarized in Table I.

The purified inulinase was confirmed to be homogeneous by polyacrylamide gel disc electrophoresis. As shown in Fig. 3, the enzyme migrated as a single protein band.

Estimations of molecular weight and isoelectric point

The enzyme was electrophoresed on SDS-disc gel and was filtered on Bio-Gel P-150. The molecular weight was estimated to be 6.4×10^4 , which was nearly the same as the molecular weight of inulinases¹⁸⁾ from *Penicillium* sp.-1 (P-II, 6.35×10^4 ; P-III, 6.6×10^4). The molecular weights obtained by the two methods agreed well with each other. These re-

TABLE I. SUMMARY OF PURIFICATION PROCEDURE OF *endo*-TYPE INULINASE FROM *Penicillium purpurogenum*

Procedure	Total protein (mg)	Total activity for inulin (units)	Specific activity (units/mg)		I/S	Purification (-fold)	Yield (%)
			for inulin	for sucrose			
Filtrate	21,400 ^a	4,450	0.21	0.004	50	1	100
DEAE-Sepharose CL-6B	202 ^a	2,850	14.1	0.049	290	67	64.0
Toyopearl HW-65	51.3 ^a	2,130	41.5	0.022	1,890	198	47.9
Bio-Gel P-100	13.4 ^b	1,110	82.8	0.020	4,140	394	24.9

^a These values were calculated under the assumption that $E_{1\text{cm}}^{1\%}$ at 280 nm was 10.

^b The value was calculated on the basis of the fact that $E_{1\text{cm}}^{1\%}$ at 280 nm was 14.4.
I/S, the ratio of specific activity for inulin to that for sucrose.

FIG. 4. Effects of pH (A) and Temperature (B).

pH-Activity curve (—●—): the reaction mixture containing 0.5 ml of 0.5% inulin, 0.4 ml of Britton-Robinson buffer²⁶⁾ and 0.1 ml of enzyme (1.71 μ g) was incubated at 37°C for 5 min.

pH-Stability curve (—○—): after the mixture containing 0.02 ml of enzyme (1.71 μ g) and 0.08 ml of Britton-Robinson buffer had been kept at 4°C for 24 hr, 0.4 ml of 0.2 M sodium acetate buffer (pH 5.1) and 0.5 ml of 0.5% inulin were added, and the reaction mixture was incubated at 37°C for 5 min.

Temperature-stability curve (—○—): after the mixture containing 0.1 ml of enzyme (1.71 μ g) and 0.4 ml of 0.1 M sodium acetate buffer (pH 5.1) had been kept at various temperature for 10 min, 0.5 ml of 0.5% inulin was added, and the reaction mixture was incubated at 37°C for 5 min.

sults indicate that there is no subunit structure. The isoelectric point was estimated by isoelectric focusing on a polyacrylamide gel to be pH 3.6.

Effects of pH and temperature

The effects of pH on inulinase activity were examined. As shown in Fig. 4A, the pH optimum was observed to be 5.1. The enzyme was stable from pH 5.0 to 7.5. The enzyme was stable up to 55°C (Fig. 4B).

Action toward inulin, sucrose, and fructo-oligosaccharides

Figure 5A shows the courses of the hydrolysis of inulin and sucrose. Inulin was rapidly degraded in the initial phase in the reaction. At 12 hr of reaction, the degree of hydrolysis of inulin was 32.3%, and was scarcely increased afterward. On the other hand, sucrose was nearly unsusceptible to the action of enzyme. The products from inulin were analyzed by PC (Fig. 5B). The major products

FIG. 5. Time Courses of the Hydrolyses of Inulin and Sucrose (A) and Paper Chromatogram of the Products from Inulin (B) with *endo*-Type Inulinase from *Penicillium purpurogenum*.

The reaction mixture containing 6 ml of 0.5% inulin (—○—) or sucrose (—●—), 4.8 ml of 0.1 M sodium acetate buffer (pH 5.1) and 1.2 ml of enzyme (20.5 μ g) was incubated at 37°C. After the indicated times, 0.2 ml of the reaction mixture was pipetted out for measuring the liberated reducing sugar, and 1 ml of the reaction mixture for PC analysis.

PC analysis was done as described in MATERIALS AND METHODS. F₁, fructose; F₂, inulobiose; F₃, inulotriose; and through F₇. St, standard; F, fructose; S, sucrose.

FIG. 6. Time Courses of the Hydrolyses of Fructo-oligosaccharides with *endo*-Type Inulinase from *Penicillium purpurogenum*.

For 1, 2, 3, and 4, the reaction mixture containing 1 ml of 0.5% substrate, 0.8 ml of 0.1 M sodium acetate buffer (pH 5.1) and 0.2 ml of enzyme (0.28 μ g) was incubated at 37°C. At the indicated times, 0.1 ml of the reaction mixture was pipetted out for the determining the liberated reducing sugar.

For 5, 6, 7, and 8, the reaction mixture containing 3 ml of 0.5% substrate, 2.4 ml of 0.1 M sodium acetate buffer (pH 5.1) and 0.6 ml of enzyme (0.84 μ g) was incubated at 37°C. At the indicated times, 0.5 ml of the reaction mixture was pipetted out. GF_{*n*}, 1^F(1- β -D-fructofuranosyl)_{*n*}-1-sucrose (*n* = 2 to 7 and $\bar{n}$ = 9). 1, GF₇ (○); 2, inulin (▲); 3, GF₅ (●); 4, GF₆ (○); 5, GF₄ (○); 6, GF₅ (●); 7, GF₃ (nystose, ○); 8, GF₂ (1-Kestose, ●).

were identified at tri-, tetra-, penta-, and hexa-saccharides. These results indicate the enzyme attacks inulin *endo*-wise. In the final phase of the reaction, penta- and hexa-saccharides were hydrolyzed by slow degrees and not only tri- and tetra-saccharides but also a small amount of mono- and di-saccharides were produced. Figure 6 shows the courses of hydrolysis of fructo-oligosaccharides [1^F(1- β -D-fructofuranosyl)_{*n*}-1-sucrose, GF_{*n*}; *n* = 2 to 7 and $\bar{n}$ = 9]. The inulinase was capable of hydrolyzing GF₆, GF₇ and GF₉ as well as inulin. On the other hand, GF₄ and GF₅ were hydrolyzed more slowly, GF₃ (nystose) and GF₂ (1-kestose) were nearly unsuceptible to the action of the enzyme. The characteristics of the action of inulinase from *Penicillium purpurogenum* reasonably explained the results from PC analysis.

Rate parameters on hydrolyses of inulin and fructo-oligosaccharides

The hydrolytic activities at various concentrations of substrates, inulin and fructo-oligosaccharides (GF_{*n*}, *n* = 2 to 7 and $\bar{n}$ = 9) were examined. Figure 7 shows the relationship between the initial velocity (*v*) and the

FIG. 7. s/v versus s Plots for Hydrolyses of Inulin and Fructo-oligosaccharides by *endo*-Type Inulinase from *Penicillium purpurogenum*.

The reaction mixture containing 0.35 ml of substrate at various concentrations, 0.1 ml of 0.2 M sodium acetate buffer (pH 5.1) and 0.05 ml of enzyme (inulin, GF₉, GF₇ and GF₆, 0.51 μ g; GF₅ and GF₄, 1.03 μ g; GF₃, 13.5 μ g; GF₂, 25.9 μ g) was incubated at 37°C. s , mM; v , mg of reducing sugar as fructose/mg of protein/min. I, inulin. GF _{n} , 1^F(1- β -D-fructofuranosyl) _{n -1}sucrose ($n=2$ to 7 and $\bar{n}=9$).

TABLE II. RATE PARAMETERS OF *endo*-TYPE INULINASE FROM *Penicillium purpurogenum*

Substrate	K_m^a	V^b	k_0^c	k_0/K_m^d
Inulin	0.21	20.8 (100)	123	586
Fructo-oligosaccharide				
GF ₉	0.24	18.0 (86.5)	107	446
GF ₇	0.33	27.5 (132)	163	494
GF ₆	0.85	14.8 (71.2)	87.6	103
GF ₅	3.8	5.29 (25.4)	31.3	8.2
GF ₄	2.8	5.98 (28.8)	35.4	12.6
GF ₃ (nystose)	16	0.16 (0.8)	0.95	0.06
GF ₂ (1-kestose)	8.4	0.042 (0.2)	0.25	0.03

^a mM.

^b mg of reducing sugar as fructose/mg of protein/min. The values in parentheses indicate the relative V .

^c sec⁻¹. Molecular activity is expressed as V/e_0 , where V is the mole of the maximum velocity and e_0 is the mole of enzyme.

^d sec⁻¹·mM⁻¹.

GF _{n} , 1^F(1- β -D-fructofuranosyl) _{n -1}sucrose ($n=2$ to 7 and $\bar{n}=9$).

substrate concentration (s) for each substrate in s/v versus s plots. The Michaelis constants (K_m) and relative maximum velocities ($Rel V$) were as follows: inulin 0.21 mM and 100; GF₉ 0.24 mM and 86.5; GF₇ 0.33 mM and 132; GF₆ 0.85 mM and 71.2; GF₅ 3.8 mM and 25.4; GF₄ 2.8 mM and 28.8; GF₃ (nystose) 16 mM and 0.8; GF₂ (1-kestose) 8.4 mM and 0.2. In Table II are summarized the rate parameters, K_m , V , molecular activity [k_0 ($=V/e_0$; e_0 , enzyme concentration) calculated from the V value and the molar concentration of the enzyme], and k_0/K_m , for these substrates.

DISCUSSION

An *endo*-type inulinase from *Penicillium purpurogenum* was highly purified. The enzyme showed high activity toward inulin but nearly none toward sucrose. Nakamura and Nakatsu¹⁸) reported that inulinases (P-I, -II, and -III) from *Penicillium* sp.-1 could hydrolyze sucrose as well as inulin, and attacked inulin *exo*-wise. The enzyme obtained by us was clearly distinguished from those enzymes by reaction mode for inulin.

As for *endo*-type inulinases from molds, it

FIG. 8. Dependence of Rate Parameters (RP) on the Degree of Polymerization (n) of Fructo-oligosaccharides. k_0 , sec^{-1} ; K_m , M; I, inulin.

has been reported that *Aspergillus niger*-12¹⁷⁾ and *Aspergillus ficuum*²⁷⁾ formed such enzymes. The endo-type inulinase from *Penicillium purpurogenum* had a resemblance to one of the enzymes from *Aspergillus niger*-12 (P-III) in its reaction mode for inulin. Both enzymes hydrolyzed inulin rapidly, and much tri-, tetra-, penta-, and hexa-saccharide was produced from inulin. But the Michaelis constants for the hydrolysis of inulin were different from each other.

As shown in Table II, the substrate specificity of endo-type inulinase from *Penicillium purpurogenum* was studied in detail. The enzyme could hydrolyze fructo-oligosaccharides [$1^F(1-\beta\text{-D-fructofuranosyl})_n\text{sucrose}$ ($n = 5, 6$ and $\bar{n} = 8$) as well as inulin. The k_0 values were reduced according to the decrease of the degree of polymerization (DP), if the DP of fructo-oligosaccharides were under seven. The K_m values were also depend on the DP of fructo-oligosaccharides.

In amylases,²⁸⁻³⁰⁾ the $\log k_0$ values of an enzyme show a characteristic dependence on the DP of malto-oligosaccharides, and the facts are considered to indicate that the active site of the enzyme is made up of the intrinsic subsite structure.^{28,31)} Figure 8 shows the rate parameters on the DP of fructo-oligosaccharides. The $\log k_0$ values of endo-type inulinase from *Penicillium purpurogenum*

were also depend on the DP of fructo-oligosaccharides, if the DP of fructo-oligosaccharides were under seven, although the $\log k_0$ values were nearly constant when the DP was seven or more. These results strongly suggest that the active site of endo-type inulinase from *Penicillium purpurogenum* also has a subsite structure consisting of seven subsites, at least.

REFERENCES

- 1) N. Shiomi, J. Yamada and M. Izawa, *Agric. Biol. Chem.*, **40**, 567 (1976).
- 2) N. Shiomi, J. Yamada and M. Izawa, *Agric. Biol. Chem.*, **43**, 1375 (1979).
- 3) N. Shiomi, *Phytochemistry*, **20**, 2581 (1981).
- 4) N. Shiomi and M. Izawa, *Agric. Biol. Chem.*, **44**, 603 (1980).
- 5) N. Shiomi, *Carbohydr. Res.*, **96**, 281 (1981).
- 6) N. Shiomi, *Carbohydr. Res.*, **99**, 157 (1982).
- 7) N. Shiomi, H. Kido and S. Kiriyama, *Phytochemistry*, **24**, 695 (1985).
- 8) S. Onodera and N. Shiomi, Abstracts of Papers, the Annual Meeting of Hokkaido Branch of Agricultural Chemical Society of Japan, Sapporo, Dec., 1986, p. 3.
- 9) T. Yasuda, S. Onodera and N. Shiomi, Abstracts of Papers, the Annual Meeting of Hokkaido Branch of Agricultural Chemical Society of Japan, Sapporo, July, 1987, p. 5.
- 10) J. Edelman and T. G. Jefford, *Biochem. J.*, **93**, 148 (1964).
- 11) A. E. Flood, P. P. Rutherford and E. W. Weston, *Nature*, **214**, 1049 (1967).
- 12) P. P. Rutherford and A. C. Deacon, *Biochem. J.*, **126**, 569 (1972).
- 13) R. Dedonder, *Bull. Soc. Chim. Biol.*, **34**, 157 (1952).
- 14) T. Nakamura and S. Hoashi, *Nippon Nōgeikagaku Kaishi*, **43**, 599 (1969).
- 15) H. Negoro and E. Kito, *J. Ferment. Technol.*, **51**, 103 (1973).
- 16) D. G. Derycke and E. J. Vanamme, *J. Chem. Technol. Biotechnol.*, **34**, 45 (1984).
- 17) T. Nakamura, T. Kurokawa, S. Nakatsu and S. Ueda, *Nippon Nōgeikagaku Kaishi*, **52**, 159 (1978).
- 18) T. Nakamura and S. Nakatsu, *Nippon Nōgeikagaku Kaishi*, **51**, 681 (1977).
- 19) S. Morita, H. Tamatani, K. Takahashi, F. Yoshimi, T. Suzuki and K. Sato, Mitsui Toatsu Chemical Co., Ltd., Japan Kokai Tokkyo Koho, 87,228,293 (Oct. 7, 1987).
- 20) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 21) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

- 22) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).
 - 23) R. A. Reisfelt, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).
 - 24) K. Weber and M. Obsorn, *J. Biol. Chem.*, **244**, 4406 (1969).
 - 25) S. Mukherjee and H. C. Srivastava, *Nature*, **169**, 330 (1952).
 - 26) H. T. S. Britton and R. A. Robinson, *J. Chem. Soc.*, **1931**, 458.
 - 27) M. Ettalibi and J. C. Baratti, *Appl. Microbiol. Biotechnol.*, **26**, 13 (1987).
 - 28) K. Hiromi, *Biochem. Biophys. Res. Commun.*, **40**, 1 (1970).
 - 29) Y. Nitta, M. Mizushima, K. Hiromi and S. Ono, *J. Biochem.*, **69**, 567 (1971).
 - 30) S. Iwasa, H. Aoshima, K. Hiromi and H. Hatano, *J. Biochem.*, **75**, 969 (1974).
 - 31) K. Hiromi, Y. Nitta, C. Numata and S. Ono, *Biochim. Biophys. Acta*, **302**, 362 (1973).
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