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Purification and *In Vitro* Translation of *Penicillium verruculosum* Cellulase mRNA

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Caboxymethyl cellulase (CMCase) I was purified from the induced culture filtrate of *Penicllium verruculosum* F-3 by ammonium sulfate precipitation, DEAE-Sephadex A-50 chromatography and Bio-gel P-150 filtration. The purified enzyme was assumed to be a glycoprotein consisting of 8.5% carbohydrate and having a molecular weight of 70.000 in SDS-polycrylamide gel electrophoresis (SDS-PAGE). The purified enzyme-specific anti-CMCase I IgG was obtained by rabbit immunization and protein A-sepharose CL-4B chromatography. The fungal poly(A^+) RNA was isolated from the total RNA of the mycelium grown under cellulase induction conditions by oligo(dT)-cellulosse chromatography. The translation products *in vitro* were prepared by translating the isolated poly(A^+) RNA in rabbit reticulocyte lysate and analyzed by SDS-PAGE and fluorography. Of the translation products, CMCase I was identified by the immunoprecipitation against anti-CMCase I IgG.

Biological conversion of cellulosic biomass into useful products, such as liquid fuel, chemical feedstock and food materials, can be one of the most promising methods to solve the resource shortage problems. However, enzymatic conversion of cellulose into low molecular materials has not been achieved yet commercially due to the high costs for producing cellulases by available microbial strains. Fungal cellulases are of major significance in industrial application in addition to their role in the ecological recycling of cellulosic materials. Several genetic approaches utilizing hyper-productive mutant isolation or protoplast recombination have been employed in the development of improved strains (17, 24, 26). Gene manipulation as a strategy of strain development to increase the cellulase yield and activity is useful and has been performed in procaryotic organisms (2, 7, 28). However, there are few reports of cellulase cloning in eucaryotic organisms except for *Trichoderma reesei*, of which the complete nucleotide sequence of the CBH I gene has been determined (23, 25). A cellulolytic fungus, *Penicilium verruculosum* F-3, has received attention recently due to its enzyme complex, which also have cellulase multiplicity as other fungal cellulase and degrades crystalline cellulose such as cotton and Avicel efficiently (5). The messener RNA-dependent cell-free translation system allows the combination of genetics, both classical and molecular, with biochemistry to answer many questions which remain on the mechanism and control of eucaryotic protein synthesis. In this study, we report the successful isolation of one cellulase component, mRNA coding the cellulase, and *in vitro* translation of the mRNA which yields a protein identical to the purified cellulase from *P. verruculosum*.

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Key words: *Penicillium verruculosum*, cellulase purification, poly (A^+) RNA, translation *in vitro*, immunoprecipitation

MATERIALS AND METHODS

Cellulase Purification

From the induced culture filtrate of *P. verruculosum*, extracellular protein containing the cellulase complex was precipitated with $(NH_4)_2SO_4$ to 80% of saturation and concentrated by ultrafiltration. Chromatography on DEAE-Sephadex A-50 resolved the CMCase into three fractions denoted I, II and III, of which Avicelase and β-glucosidase activity were mainly associated with fraction I (Fig. 1). An aqueous solution of fraction III was applied to a Bio-gel P-150 column equilibrated with 0.1

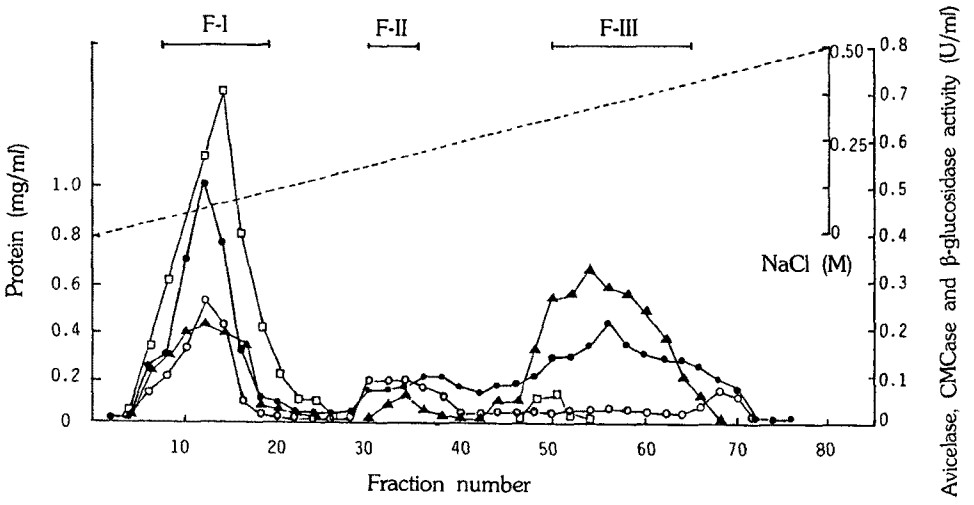

Fig. 1. Elution pattern of cellulolytic enzymes on DEAE-Sephadex A-50.

The column (2.0×80 cm) was eluted with 0.02 M citrate, pH 5.2, with an increasing NaCl-gradient. Fraction volume; 6 ml. Flow rate; 12.5 ml/h.

Symbols: ○, Avicel saccharifying activity; ▲, CM-cellulose saccharifying activity; □, β-glucosidase activity; ●, Protein; —, Pooled fraction.

M Na-acetate buffer (pH 5.2) and eluted with the same buffer. Fractions showing activity toward CM-cellulose were pooled and lyophilized. The homogeneity and carbohydrate content of purified enzyme was tested by SDS-polyacrylamide gel electrophoresis (13) and phenol-sulfuric acid method (8). The enzymatic hydrolysates of CM-cellulose by the purified CMCase I were identified by thin layer chromatography using a n-butanol-ispropanol-water (v/v, 3:12:4) system as the solvent and the amino acid composition of the protein was analyzed by amino acid analyzer (Hitachi, Japan).

Preparation of Specific Antibody

Antiserum against purified CMCase I was obtained from rabbits by a hypodermic injection of the purifed enzyme (1.5 mg) emulsified in incomplete Freund's adjuvant. The booster injection was repeated four times at intervals of 10 days and the animal bled 10 days after the last injection. Antisera were immediately precipitated with Na_2SO_4, collected and applied to the protein A-sepharose CL-4B column. The anti-CMCase I immunoglobulin G fraction was eluted with 0.1 M glycin-HCl (pH 3.0) and used as an anti-CMCase I antibody after dialysis. Specipicity of the anti-CMCase I IgG against CMCase I was tested by double immunodiffusion analysis (20).

Extraction of Total RNA

Total RNA of *P. verruculosum* was extracted by the guanidinum thiocyanate-HCl procedure modified by Chirgwin *et al.* (4). The fungal mycelium cultured in a medium containing cellobiose octaacetate as a carbon source described elsewhere (5) was harvested at times when the greatest increase in extracellular protein was obtained. One gram (wet weight) of the fungal mycellium was frozen immediately in liquid nitrogen and pulverized in a Waring blender. Powdered mycelium was dissolved in homogenization buffer (4.0 M of guanidine thiocyanate, 0.05% (w/v) sodium N-lauroyl sarcosine, 0.1% (v/v) β-mercaptoehanol, 0.1% (v/v) antifoam-A and 0.1 M Tris-HCl, pH 7.6) and centrifuged at 3,000×g for 20 min, and then the supernatant was precipitated by adding 0.05 volume of 2 M K-acetate (pH 5.5), 0.08 volume of acetic acid and 0.75 volume of ice cold absolute ethanol at −20℃ overnight. Following centrifugation at 10,000×g for 20 min the pellet was dissolved in guanidine-HCl buffer (7.5 M guanidine-HCl, 10 mM dithiothreiotol) and reprecipitated with addition of 0.05 volume of 2 M K-acetate and 0.5 volume of ice cold absolute ethanol at −20℃ for 4 h. The precipitated RNA was collected by centrifugation at 10,000×g for 20 min and completely resuspened in 3 ml of 20 mM EDTA solution (pH 7.0). The RNA solution was mixed with 2 volumes of chloroform-n-butanol (4:1, v/v) mixture and immediatly homogenized for 10 min. The mixture was centrifuged at 5,000×g for 10 min using a swing-out rotor and the aqueous phase was extracted repeatedly (usually 3 times) with the above organic solution until no protein remained at the interphase. The extracted RNA was precipitated by adding 0.1 volume of 2 M Na-acetate (pH 7.0) and 2.5 volume of ice cold absolute ethanol at −20℃ overnight and recentrifuged as above. After the

RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water, total RNA content was measured at 260 nm (an absorbance of 1 at 260 nm equals an RNA concentration of 40 μg/ml).

Oligo(dT)-cellulose Chromatography

Total RNA was subjected to chromatography on oligo (dT)-cellulose column by the modified method of Aviv and Leder (1). RNA solution was denatured at 68℃ for 2 min to promote the dissociation of rRNA and tRNA, and then applied to a preequilibrated oligo(dT)-cellulose column which was washed with elution buffer (0.01 M Tris-HCl, pH 7.6 and 0.01 M EDTA) and 0.1 N NaOH, and finally equilibrated with binding buffer (0.01 M Tris-HCl, pH 7.6, 0.5 M NaCl and 0.01 M EDTA). The column was washed with the binding buffer to elute out the nonabsorbed materials and poly(A^+)RNA retained by the column was fractionated with elution buffer at a velocity of 0.2 ml/min. The poly(A^+)RNA was precipitated with 2.5 volumes of ethanol, centrifuged and redissolved in DEPC-treated water.

Fractionation of mRNA

The poly(A^+) RNA was fractionated by gel electrophoresis on 1.5% agarose gel with 10 mM methylmercuric hydroxide as a denaturing agent. After electrophoresis, the gel was sliced into 2 mm fractions and the RNA in each slice was recovered essentially as described by Segal and Sagar (21). The slices were melted in NEB buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.5, 0.2% SDS) and the agarose particles were pelleted by centrifugation. After phenol/chloroform extraction of the supernatant, the RNA was precipitated with ethanol and redissolved in DEPC-treated water.

Translation *In Vitro*

In order to assess total mRNA as well as cellulase mRNA activity, rabbit reticulocyte lysate as an *in vitro* translation system was used. The standard assay mixture (total volume, 50 μl) contained 35 μl of nuclease treated lysate, 7 μl of distilled water, 1 μl of RNase inhibiter (RNAsin, 40 U/μl), 1 μl of 1 mM amino acid mixture (minus methionine), 2 μl of mRNA (1 mg/μl) and 10 μCi of [^{35}S]methionine. Incorporation of [^{35}S]-methionine into translated product was carried out by the modified method of Uchiyama (27). Aliquots of the reaction mixture were diluted with 10 volumes of 5% TCA, heated at 90℃ for 20 min, and finally cooled in ice-water for 5 min. The resultant precipitates were mixed thoroughly and collected onto Whatman GF/A filters. The filters were dried and counted in a toluene-based scintillation fluid [1 liter toluene containing 4 g of 2,5-diphenyloxazole and 0.1 g of dimethyl-1,4-bis[2-(4-methyl-5-phenyloxazole)]-benzene in a PACKARD scintillation counter.

Immunoprecipitation

Immunoprecipitation of cell-free translation products was performed as described by Clemens (6). At the end of the incubation for cell-free translation, he reaction mixtures were diluted with 8 volumes of Ip buffer (0.1 M NaCl, 1 mM EDTA, 1% Nonidet P-40 and 10 mM Tris-HCl, pH 7.5). After adding 2 μl of anti-CMCase I IgG, the mixture was allowed to stand at room temperature for 2 h to allow formation of antipen-antibody complexes. Protein A-Sepharose was added to adsorbe the complexes and the mixture was incubated with shaking at 20℃ for 40 min. The complexes were collected by centrifugation at 14,000×g for 1 min and the pellet was washed three times by resuspending it in Ip buffer without detergent and centrifugation. The pellet was dissolved and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

Identification of Translation Products by SDS-PAGE and Fluorography

For the identification of peptides synthesized in the cell-free translation system, total proteins were collected by TCA-precipitation and subjected to SDS-polyacrylamide gel electrophoresis. After reaction mixtures were precipitated with cold 10% TCA and redissolved in sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 50% glycerol and 20 mM Na-phosphate (pH 7.2). The samples again were heated to 100℃ for 3 min and analyzed by electrophoresis on 10% acrylamide/0.1% SDS gel by the method of Laemmli (13). Radioactivity in the gels was determined by cutting the gel into 3 mm slices, incubating in 0.5 ml of 30% H_2O_2 solution overnight at 68℃, adding 4.5 ml of scintillation cocktail (2 volumes of tolune scintillation fluid and 1 volume of Triton X-100) and then counting [^{35}S] activity of the gel with liquid scintillation counter (PACKARD Co.). Detection of [^{35}S]-methionine radioactive protein in gel was based on fluorography described by Hames (12). After SDS-polyacrylamide gel electrophoresis, the gel was soaked in 20 volumes of dimethylsulfoxide (DMSO) for 30 min, followed by a second 30 min immersion in fresh DMSO. The gel was transfered into 4 volumes fo 20% (w/w) PPO in DMSO for 3 h and then immersed in 20 volumes of water for 1 h. After gel drying, X-fay film was placed in contact with the gel and exposed at −70℃ for 3~14 days.

Enzyme Assays

Endo-1,4-β-glucanase (CMCase) was assayed by measuring the amount of reducing sugar produced from carboxymethyl cellulose (D.S. 0.6~0.7, DP. 4.50-500, Wako pure chemical Co.). The reaction mixture contained 0.25 ml of CM-cellulose (1%, w/v) in 0.05 M citrate buffer, pH 5.0, and 0.25 ml of enzyme solution. After incubating the mixture at 50℃ for 30 min with shaking at 150 rpm, the reaction was stopped by cooling in an ice bath and reducing sugars were determined.

Exo-1,4-β-glucanase(β-1,4 glucan cellobiohydrolase) was tested by the addition of 0.5 ml enzyme solution to 0.5 ml of 5% Avicel (w/v, Fluka Chemica, Avicel PH-101) in 0.05 M citrate buffer, pH 5.0. After the mixture was incubated at 50℃ for 1 h with shaking at 150 rpm, reducing sugars were determined.

β-glucosidase activity was measured with Salicin (Wako pure chemical Co.) as a substrate. The reaction mixture contained 0.4 ml of a Salicin (0.5%, w/v) in 0.05 M citrate buffer, pH 5.0, and 0.4 ml of enzyme solution. After the mixture was incubated at 50℃ for 30 min, reducing sugars were determined. Reducing sugars were determined by the method of Miller *et al.* (16). One unit of cellulose (CM-cellulose, Avicel ans Salicin) hydrolyzing activity was defined as the amount of enzyme that produced 1 μmole of reducing sugar per min under the given condition.

Protein was determined according to Lowry *et al.* (14). A standard curve prepared from determinations with bovine serum albumin.

RESULTS AND DISCUSSION

Purification of Cellulase and Enzyme Specific Antibody

One-endo type cellulase, tentatively called carboxymethyl cellulase (CMCase) I was purified by ammonium sulfate precipitation, DEAE-Sephadex A-50 ion exchange chromatography and Bio-gel P-150 filtration consecutively (Fig. 2). Table 1 shows the details of purification of the enzyme from culture broth of *P. verruculosum*. The purified enzyme (CMCase I) was assumed to be a glycoprotein having a molecular weight of 70,000 in SDS-PAGE (Fig. 3) and consisting of 8.5% carbohydrates as glucose. The CMCase I produced mainly glucose, cellobiose and a small amount of cellooligosaccharides from enzyme reactants against CM-cellulose (Fig. 4). CMCase I was shown to have pH and temperature optimums of pH 5.0 and 50℃, respectively, and was quite stable overlong periods at 50℃. From the amino acid analysis CMCase I showed a predominance of acidic and neutral amino acids and a low content of arginine, histidine and methionine (Table 2). The above results showed that the physicochemical properties of purified CMCase I were similiar to those of other fungal-originating CMCase or Cx components such as *T. vieide* (22) and *Aspergillus aculeatus* (18). The multiplicity of cellulase components has been reported of many cellulolytic fungi (9, 22). Okada (19) reported three endoglucanases of differing molecular weights and randomness of action toward hydrolysis of cellulose. Cellulase multiplicity of *P. verruculosum* was also revealed through cellulase activity staining on native gel after polyacrylamide gel electrophoresis, which showed seven seperates active against CM-cellulose (data not shown).

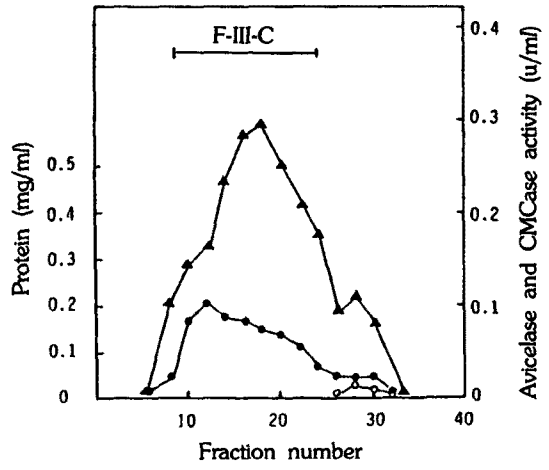

Fig. 2. Gel filtration chromatographys of F-III fraction on Bio-Gel P-150.
The effluent (F-III fraction) from the DEAE-Sephadex A-50 column was loaded on a column (2.5×110 cm) of Bio-Gel P-150 column equilibrated with 0.02 M citrate bffer, pH 5.2. Fraction volume; 3 ml, Flow rate; 20 ml/h, Symbols: ○, Avicel saccharifying activity; ▲, CM-cellulose saccharifying activity; ●, Protein; —, Pooled fraction.

Table 1. Purification of CMCase I from *P. verruculosum*

Fraction	Total protein (mg)	Total activity (units)	Specific activity (U/mg protein)	Yields of activity (%)	Purification (fold)
Culture filtrate	1,621	340	0.209	100	1
$(NH_4)_2SO_4$ precipitate	330.7	81.76	0.247	24.	1.2
DEAE-Sephadex A-50	20.7	32.9	1.59	9.6	7.6
Sephadex G-100	8.4	15.2	1.81	4.4	8.7

In order to obtain specific antibody against the purified enzyme, the enzyme was injected with Freund's complete adjuvant (FCA) into a rabbit. Anti-CMCase I IgG acquired from the antisera showed the specificity against CMCase I by double immunodiffusion analysis (Fig. 5).

Isolation of Poly(A$^+$) RNA

Total RNA was extracted from the induced cultures grown for 10 days using gusnidium salts, which was reported to be a more efficient denaturant than phenol and facilitated the isolation of intact and functional RNA from a wide range of biological sources (4). In order to minimize the possibilit of RNase contamination, all glassware were baked overnight at 150℃, plastic surface

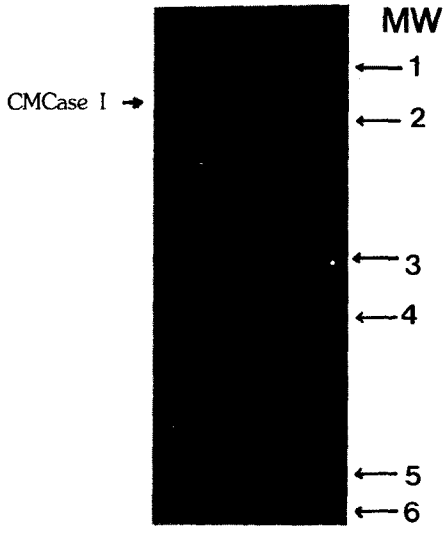

Fig. 3. SDS-polyacrylamide gel electrophoresis (10%) of CMCase I.
Molecular weight markers: 1, Phosphorylase B (92,500); 2, Bovine serum albumin (66,200); 3, Ovalbumin (45,000); 4, Carbonic anhydrase (31,000); 5, Soybean trypsin inhibitor (21,500); 6, Lysozyme (14,400).

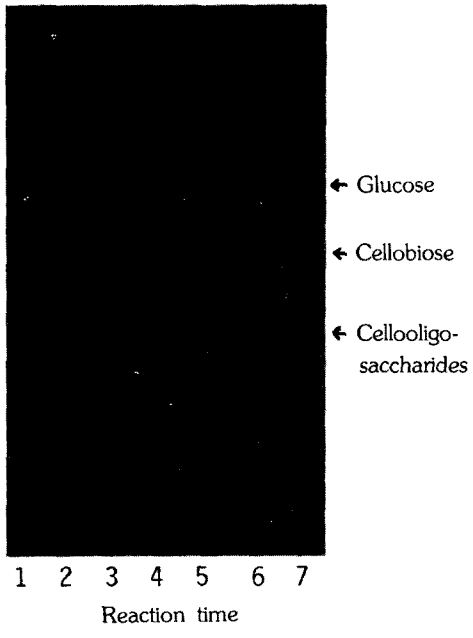

Fig. 4. Diagram of $AgNO_3$-stained thin layer chromatogram of the products fromed by CMCase I incubated with CM-cellulose.
Separation of products on Silica gel G-60 was carried out using n-butanol-isopropanol-water (v/v, 3:12:4) eluent. Reaction time: 1, 30 min; 2, 1 h; 3, 2 h; 4, 4 h; 5, 8 h; 6, 16 h; 7, 30 h.

Table 2. Amino acid composition of CMCase I

Amino acid	Molar ratio (%)
Cysteine	2.5
Methionine	1.2
Lysine	6.9
Histidine	2.1
Arginine	2.9
Aspartic acid	10.2
Threonine	6.4
Serine	10.4
Glutamic acid	13.2
Proline	9.7
Glycine	7.4
Alanine	6.2
Valine	4.7
Isoleucine	4.0
Leucine	5.1
Tyrosine	3.9
Phenylalanine	2.6

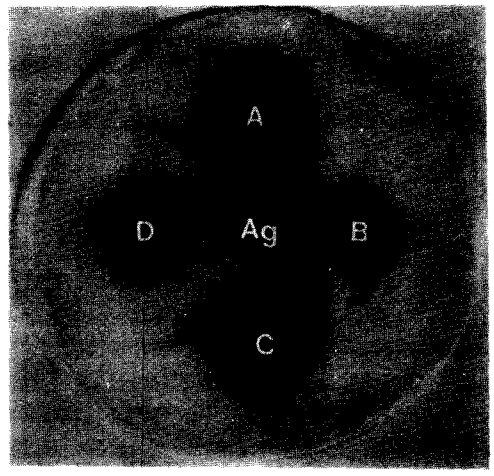

Fig. 5. Double immunodiffusion assay of CMCase I purified from *P. verruculoseum*.
Ag; Purified CMCase I, A, B, and C; Anti-CMCase IgG, D; Bovine serum albumin.

Table 3. Yields of total RNA and poly(A^+) RNA from *P. verruculosum* grown from cellulase induction culture

Total amount of mycelium[a]	1.0 g
Total amount of RNA	462.0 μg
Total amount of poly(A^+) RNA[b]	3.6 μg
Specific activity of poly(A^+) RNA[c]	102,570

[a]Wet weight.
[b]$A_{260}/A_{280}=2.0$.
[c]Specific activity was expressed as com/μg RNA in 2 μl aliquots of total reaction mixture (50 μl).

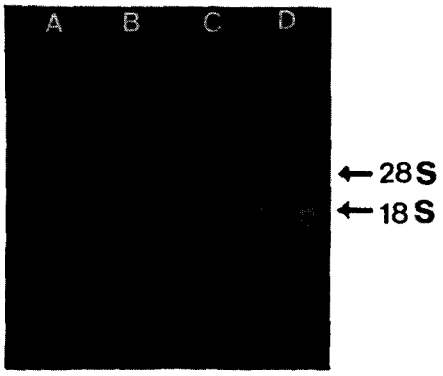

Fig. 6. Agarose densturing gel electrophoresis of total RNA from *P. verruculosum*.
RNA was loaded on 1.4% agarose gel containing 0.22 M formaldehyde and ran at 15 mA for 5 h. After electrophoresis, RNA was stained with EtBr.
RNA concentrations: A, 2 μg; B, 6 μg, C, 8 μg, D, 10 μg.

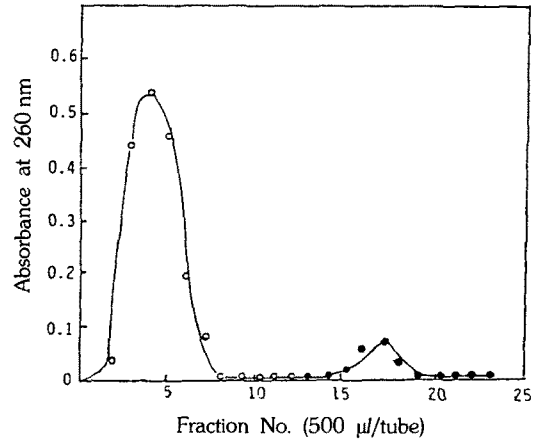

Fig. 7. Fractionation of total RNA extracted from *P. verruculosum* by oligo (dT)-cellulose affinity chromatography.
Symbols: ○; poly(A⁻) RNA, ●; poly(A⁺) RNA

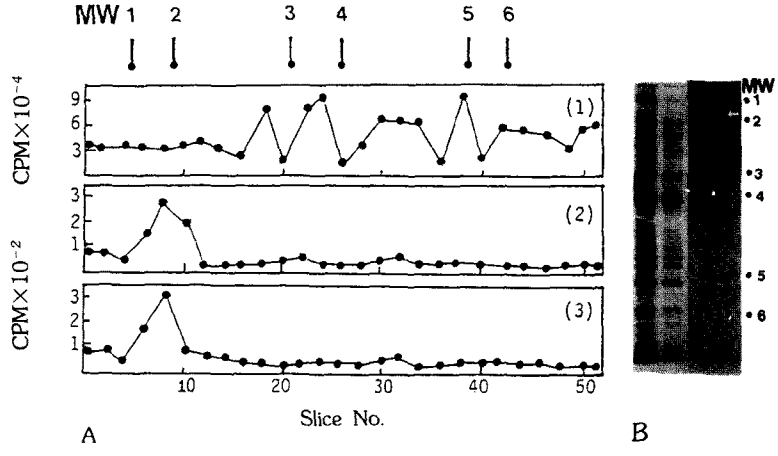

Fig. 8. SDS-polyacrylamide gel electrophoresis (A) and fluorography (B) of cell-free translation products directed by *P. verruculosum* poly(A⁺) RNA.
Plan (A): Cell-free translation products by TCA-precipitation (1) and immunoprecipitation (2) from total poly(A⁺) RNA, and TCA-precipitation from fraction 7 of size-fractionated poly(A⁺) RNA (3). Electrophoresis was from left to right. Plan (B): Fluorography of cell-free translation products of total (A⁺) RNA (1) and immunoprecipitation of fraction 7 of size-fractionated poly(A⁺) RNA (2). Molecular weight markers: 1, Phosphorylase B (92,500); 2, Bovine serum albumin (66,200); 3, Ovalbumin (45,000); 4, Carbonic anhydrase (31,000); 5, Soybean trypsin inhibitor (21,500); 6, Lysozyme (14,400).

and stock solutions except Tris-containing solutions were treated for 30 min with 0.2% DEPC and then throughly boiled to remove traces of the reagent. The yield of total nucleic acid from frozen mycelium approached 462 μg/g of the wet weight (Table 3). To confirm RNA degradation isolated RNA analyzed in 1.4% agarose gel containing 0.22 M formaldehyde and cellulase mRNA was presumed to be active since 18 S and 28 S rRNA were observed clearly (Fig. 6).

Because a number of eukaryotic mRNA have an extended poly(A)-rich region at their 3'-OH ends, fractionation of total RNA was carried out by oligo (dT)-cellulose affinity chromatography. As shown in Fig. 7, unadsorbed poly(A⁻) RNA was eluted at first as a larger peak and then the poly(A⁺) RNA which bound to coulmn was eluted as a smaller peak. Total poly(A⁺) RNA activity was assayed by measuring the incoporation of [^{35}S]-methionine into the TCA-precipitated material, while the

activity of cellulase specific mRNA was assayed by measuring the incoporation into the immunoprecipitants whith cellulase antisera. Messenger RNA can be extracted from whole cells, total polysome, or specially immunoadsorbed polysomes. Especially, precipitation of polysomes with antiserum specific for nascent protein and subsequent extraction of mRNA from the precipitates may be useful for the purification of a specific kind of mRNA from different sources of protein (27). Galis *et al.* (10) extracted polysomal mRNA from pools of frozen polysomal pellets of yeast. But mRNA isolation by oligo (dT)-cellulose chromatography from whole cell or mycelium was the most widely used in fungi such as *Neurospora* (15) and *Asperillus* (3). While in principle methods based on immunological purification of specific polysomes could be used, these methods are made less attractive by the difficulty of isolating high-order polysomes from fungi (11).

Identification of Products Synthesized *In Vitro*

Total peptides synthesized in the cell-free translation system under the direction of added poly(A^+) RNA containing CMCase I mRNA were recovered by TCA-precipitation or by immunoprecipitation with specific anti-CMCase I IgG, and subjected to SDS-polyacrylamide gel electrophoresis. At frist, purified mRNA from oligo (dT)-cellulose chromatography was incubated to test the ability of cell-free translation in micrococcal nuclease treated rabbit reticulocyte lysate. Standard incubations of mRNA-dependent reticulocyte lysate were set up with [^{35}S]-methioine and various conclentrations of poly(A^+) RNA. After 60 min incubation, samples were withdrawn for assay of [^{35}S] incorporation and plotted against poly (A^+) RNA concentration. Dose-response of each poly (A^+) RNA showed that 0.7 μg poly(A^+) RNA was the optimum concentration (data not shown). As shown in Fig. 8, the TCA-precipitated products gave a heterogeneous mixture of peptides. However, the immunoprecipitated products exhibit only a radioactivity band corresponding to a molecular weight of approximately 70,000, which was the same as the molecular weight of purified CMCase I estimated using several standard proteins (Fig. 8, A-1 and A-2). Thus, the immunoprecipitated protein was proven immunologically and electrophoretically to be identical with purificed CMCase I. The translation products of size fractionated poly(A^+) RNA on methylmercuric hydroxide gel showed that the most of peptides of fraction 7 corresponded to immunoprecipitated material and authentic purified CMCase I (Fig. 8, A-3). These results were also confirmed in fluorgraphy (Fig. 8, B). Consequently, the CMCase I mRNA of *P. verruculosum* was isolated, which will be a suitable template for the generation of a specific cDNA for the CMCase I mRNA sequence.

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