KOR. JOUR. MICROBIOL. Vol. 18, No. 3, 133~147, 1980

Purification and Properties of Carboxymethylcellulases from *Aspergillus nidulans* FGSC 159

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Aspergillus nidulans FGSC 159의 Carboxymethylcellulases의 分離 純化 및 그 性質에 關한 硏究

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ABSTRACT

Washed mycelia of *Aspergillus nidulans* FGSC 159 were incubated in CMC minimal liquid medium and the culture filtrate which contained induced extracellular cellulase was fractionated by a three-step procedure including chromatography on Bio-Gel P-150, chromatography on DEAE-Sephadex A-50 and chromatography on Sephadex G-100. Three CMCase components: F-I-Ia, F-I-Ib and F-II-Ia were prepared.

No enzyme activity toward avicel could be detected in these components. Similarly, there was no β-glucosidase activity. pH-optima of the three components were all 5.0 in acetate buffer. Temperature-optima for the activities of F-I-Ia, F-Ⅰ-Ib and F-Ⅱ-Ia were 45°C, 40°C and 50°C, respectively.

F-II-Ia was shown to be more thermostable than the other two components. F-II-Ia was proved to have quite a different substrate specificity and action property from those of F-I-Ia and F-I-Ib by product analysis on liquid chromatography.

INTRODUCTION

Recently many investigations have been performed about the multiplicity of cellulase components and their action modes in various organisms such as *T. viride*(Okada *et al.* 1968, Toda *et al.* 1971, Okada 1975, Nakayama *et al.* 1976, Berghem *et al.* 1976, Wood *et al.* 1972), *Pseudomonas fluorescens* var. *cellulosa* (Yamane *et al.* 1971, Yoshikawa *et al.* 1974), *Irpex lacteus*(Nisizawa *et al.* 1959, Kanda *et al.* 1976), *Pellicularia filamentosa*(Tanaka *et al.* 1977, 1978), *Dolabella* sp.(Okada *et al.* 1966), *Fusarium solani*(Wood 1969, 1971), *Cellvibrio gilvus*(Storvick *et al.* 1963), *Sporotrichum pulverulentum*(Eriksson *et al.* 1975), and *Myrothecium verrucaria*(Halliwell 1961).

Among these microorganisms, *T. viride* is the most frequently investigated one because of its high yield of cellulase. However, sexual or parasexual cycle of this organism has not been

discovered yet. Therefore, there are little informations about the genes which control the production of various cellulase components and about their genetic regulation mechamism. In this respect, *A. nidulans* has some advantages in that it shows sexuality and parasexuality in its life cycle. Moreover, since many strains of *A. nidulans* which contain various markers have been developed and genetically studied by a great number of investigators, it seems desirable to use this fungus in genetic approach to cellulase system.

In this investigation, three CMCase components were isolated from the culture filtrate of *A. nidulans* FGSC 159 and several characteristics of these components were examined.

MATERIALS AND METHODS

1. Organism and growth conditions

Conidial suspension of *A. nidulans* FGSC 159 was inoculated in *Aspergillus* complete medium (dextrose, 10g; yeast extract, 1.5g; casamino acid, 1.5g; minimal salt stock solution, 20m*l*; vitamin stock solution, 10m*l*; 1% adenine solution, 2m*l*; to 1,000m*l* distilled water) adjusted to pH 6.0(3.0×10^5 conidia/m*l* medium). Cultures were grown in 1,000m*l* culture flask and incubated for 18 hours at 37°C with aeration and agitation.

Mycelia at a late logarithmic phase of the cultures were harvested by filtration through sterile gauge, and washed with sterile 0.2 M citrate buffer, pH 6.0. Washed mycelia were resuspended in 500m*l* of CMC minimal medium (CMC, 10g; minimal salt stock solution, 20m*l*; 1% adenine solution, 2m*l*; nicotinic acid, 1mg; p-aminobenzonic acid, 5mg; pyridoxin, 5mg; riboflavin, 1mg; lysine, 70mg; phenylalanini, 80mg; Na_2SO_3, 150mg; to 1,000 m*l* distilled water) in 1,000m*l* culture flask and incubated for 12 hours under the same conditions as in the previous cultivation.

2. Enzyme purification

1) Crude enzyme

After incubation for 12 hours, the mycelia were separated off by filtration through a filter paper(No. 2 Toyo Roshi Co., Ltd). 3,500 m*l* of crude enzyme was obtained from the whole culture.

2) Fractionation with ammonium sulfate

The crude enzyme preparation was treated with ammonium sulfate(90%). The supernatant, which had no CMCase activity was removed by centrifugation at 12,000×g for 20 min at 4°C. The resulting precipitate was dissolved in a small amount of 0.02M acetate buffer, pH 5.0. The solution was dialyzed against 0.02M acetate buffer, pH 5.0 for 48 hours at 4°C.

3) Molecular sieve chromatography on Bio-Gel P-150

40m*l* of dialyzed enzyme solution was fractionated by molecular sieve chromatography on a column(4.5×65cm) packed with Bio-Gel P-150, 100-200 mesh (Bio-Rad Laboratories). The column was equilibrated with 0.02M acetate buffer, pH 5.0 and eluted with the same buffer, at a flow rate of 40m*l*/hr.

4) Ion-exchange chromatography on DEAE-Sephadex A-50

Further purifications of the CMCase fractions, F-I and F-II, were achieved by DEAE-Sephadex A-50(Pharmacia Fine Chemicals) ion-exchange chromatography. The column (2.2×35cm) was equilibrated with 0.02M acetate buffer, pH 5.0 and eluted with a linear concentration gradient of 0.02-2.5 M acetate buffer, pH 5.0 at a flow rate of 28 m*l*/hr.

5) Molecular sieve chromatography on Sephadex G-100

The CMCase fractions, F-I-I and F-II-I, obtained in the previous step were concentrated by lyophilization. Each concentrated

CMCase solution was applied on a Sephadex G-100 column (2.2×75cm) equilibrated with 0.02 M acetate buffer, pH 5.0 and the column was eluted with the same buffer at a flow rate of 25m*l*/hr.

The CMCase fractions, F-I-Ia, F-I-Ib and F-II-Ia, obtained in this step were concentrated by lyophilization and dialyzed.

3. Substrates

Sodium carboxymethylcellulose, CMC(d.s. 0.63), was supplied by Daiichi Kogyo Seiyaku Co., Ltd.

p-Nitrophenyl-β-D-glucoside, PNPG, was purchased from Nakarai Chemicals, Ltd.

Cellooligosaccharides, from cellobiose to cellohexaose, were isolated from fuming HCl hydrolysate of cellulose (Sigma Chemicals Co.) by the procedures of Miller *et al.*(1960). Each cellooligosaccharide solution was lyophilized and dissolved in a small amount of 0.02 M acetate buffer, pH 5.0.

4. Analytical methods

1) Enzyme assays

가) CMCase

The reaction mixture consisted of 0.8 m*l* of 0.5% CMC solution in 0.05 M acetate buffer, pH 5.0 and 0.2 m*l* of enzyme solution. After incubation of the mixture at 37°C for 30 min, reducing sugars produced by the enzyme were analyzed according to the method of Somogyi-Nelson(1952).

One unit of enzyme activity was defined as the amount of enzyme releasing 1.0μg or glucose (or glucose equivalents) in 30 min under the standard assay conditions. It was calculated from the standard curve that 1 OD unit change is equivalent to 750 Units/m*l* of CMCase concentration.

나) β-Glucosidase

The reaction mixture contained 0.8m*l* of 1mM PNPG solution in 0.05M acetate buffer, pH 5.0 and 0.2m*l* of enzyme solution. After incubation at 37°C for 30min, 2.0m*l* of 1M Na_2CO_3 solution was added to the mixture.

The mixture was diluted with 10.0m*l* of distilled water and the amount of p-nitrophenol liberated was estimated from the absorbance at 420nm.

One unit β-glucosidase activity was defined as the amount of enzyme liberating 1μ mole of p-nitrophenol in 30min. Standard curve indicated that 1 OD unit change is equivalent to 930 Units/m*l* of β-glucosidase concentration.

2) Analysis of reaction products by liquid chromatography

Substrates used for this experiment were cellobiose (G_2), cellotriose (G_3), cellotetraose (G_4), cellopentaose (G_5) and cellohexaose (G_6), The reaction mixture contained 0.2m*l* of substrate solution in 0.02M acetate buffer, pH 5.0 and 0.2m*l* of enzyme solution. The mixture was incubated at 37°C. 50μ*l* aliquots of the mixture were removed and product analyses were performed on a high pressure liquid chromatograph (Model 6000A, Waters Associates Co.). The products were separated on a Waters μ-Bondapak carbohydrate analysis column (3.9mm IB by 30cm), using acetonitrile-water (65 : 35) as an eluting solvent at a flow rate of 1m*l*/min and detected by Waters differential refractometer (R401).

3) Determination of protein

Protein concentrations were determined from absorption measurements at 280nm. When necessary, the method of Lowry *et al.* (1951) was also used with bovine serum albumin as standard.

RESULTS

1. Enzyme purification

Fig. 1 shows the result of chromatography on Bio-Gel P-150 of the enzyme material after ammonium sulfate precipitation. CMCase com-

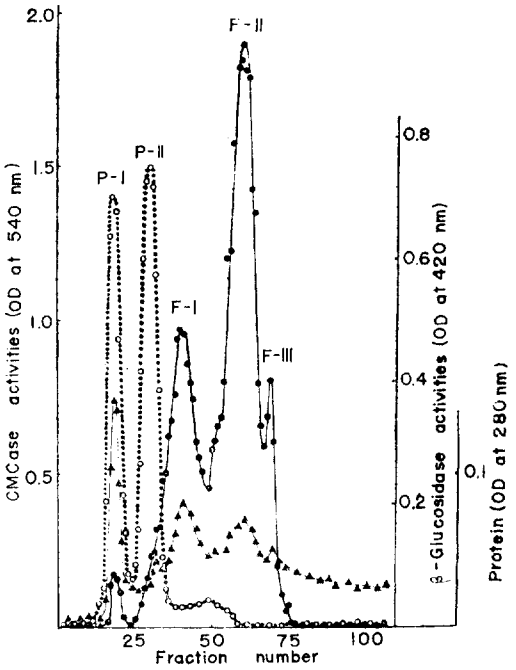

Fig. 1. Bio-Gel P-150 chromatography of crude enzyme preparation. ●——● : CMCase activity, ○·····○ : β-glucosidase activity, ▲——▲ : protein, 1 fraction=10m*l*, F-I: 35-48, F-II : 50-67, F-III : 68-73

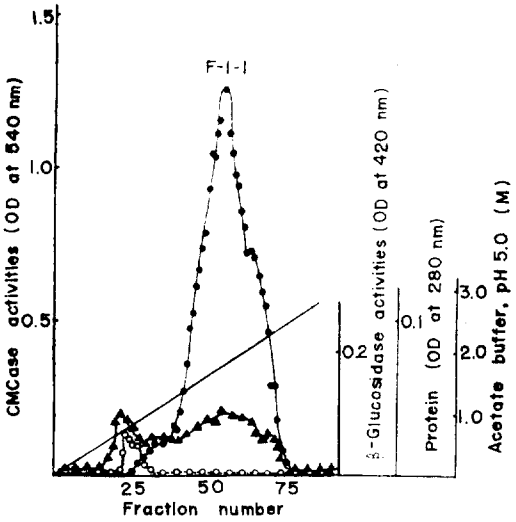

Fig. 2. DEAE Sephadex A-50 ion-exchange chromatography of F-I. All abbreviations are the same as in Fig. 3. 1 fraction=10m*l*, F-I-I : 43-70

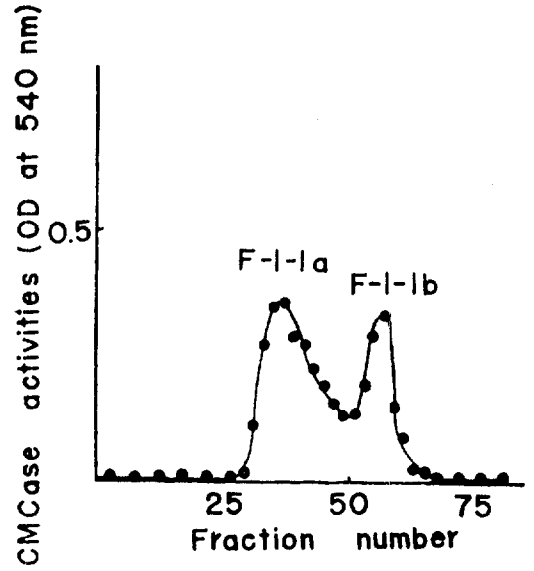

Fig. 3. Sephadex G-100 chromatography of F-I-I. 1 fraction=5m*l*, F-I-Ia : 33-45, F-I-Ib : 53-59.

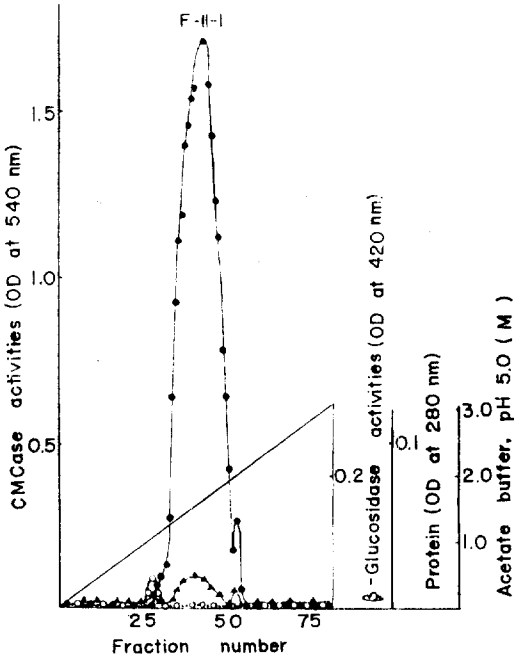

Fig. 4. DEAE-Sephadex A-50 ion-exchange chromatography of F-II. All abbreviations are the same as in Fig. 3. 1 fraction=8m*l*, F-II-I : 33-51

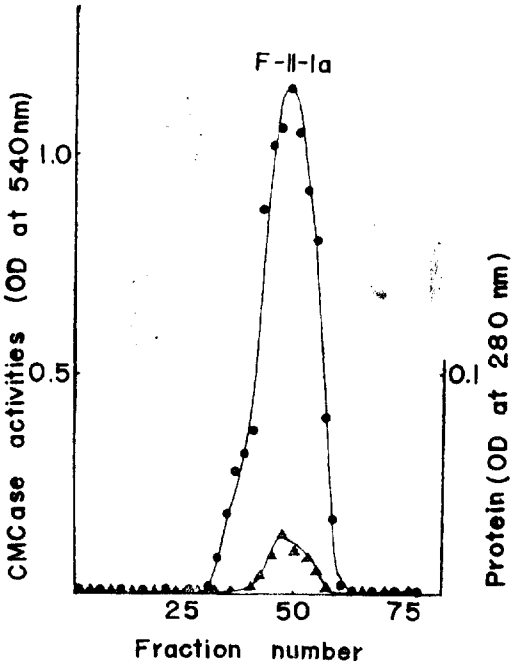

Fig. 5. Sephadex G-100 chromatography of F-II-I. All abbreviations are the same as in Fig. 3. 1 fractaion=5 ml, F-II-Ia : 35-59

ponents were effectively separated from β-glucosidase components in this step. Among the 3 CMCase peaks, F-I and F-II were used for the next steps.

Further purification of F-I was achieved by ion-exchange chromatography on a DEAE-Sephadex A-50 column. As shown in Fig. 2, contaminated β-glucosidase was completely removed and a CMCase peak, F-I-I, was obtained. Total fractions in F-I-I were combined and concentrated by lyophilization. 15m*l* of concentrated preparation was rechromatographed on a Sephadex G-100 column and the result is shown in Fig. 3. It is shown that F-I-I was divided into two CMCase peaks, F-I-Ia and F-I-Ib. The two CMCase peaks were collected and lyophilized separately.

F-II was also rechromatographed on a DEAE-Sephadex A-50 column and F-II-I which had no β-glucosidase activity was obtained as shown in Fig. 4. The result of molecular sieve chromatography of concentrated F-II-I on Sephadex G-100 column is presented in Fig. 5. CMCase activity was contained in a symmetrical peak, F-II-Ia.

Table 1. Purification procedure and yields of CMCase

Enzyme preparation	Total protein mg	Total CMCase kU	Specific activity kU/mg	Yields %
Crude enzyme	80.5	1,520	18.9	100
Bio-Gel P-150				
F-I	13.2	385	29.7	25
F-II	16.5	815	49.4	54
DEAE-Sephadex A-50				
F-I-I	9.4	795	84.6	52
F-II-I	4.7	640	136.2	42
Sephadex G-100				
F-I-Ia	0.7	74	105.9	5
F-I-Ib	0.4	35	87.5	2
F-II-Ia	2.3	325	141.3	21

Table 1 shows the yields of the purification procedures. In case of DEAE-Sephadex A-50 chromatography of F-I, the resulting F-I-I had twofold increased total enzyme units. Probably, this result was due to the fact that this component might be more active in the increased buffer concentration than in the lower one.

2. Effect of pH on CMCase activity

The pH dependence of each CMCase component was examined by measuring the reducing sugar formed at various pH values of 0.05 M acetate buffer. Reaction mixtures were incubated at 37°C for 30min. As shown in Fig. 6, the three components showed their highest activities at pH 5.0 and relatively high sensitivities to pH change.

3. Effect of temperature on CMCase activity

The reaction mixtures were incubated at various temperatures. Fig. 7 shows the effect of temperature on the three CMCase com-

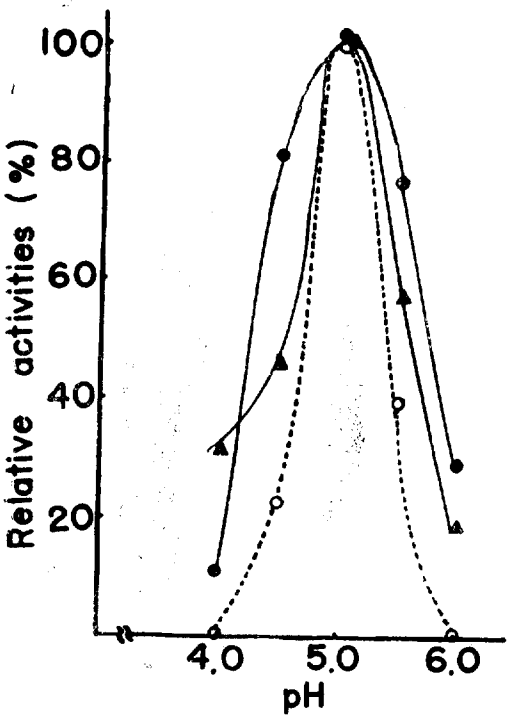

Fig. 6. Effect of pH on the activities of CMCase components. ●——● : F-I-Ia, ○·····○ : F-I-Ib, ▲——▲ : F-II-Ia

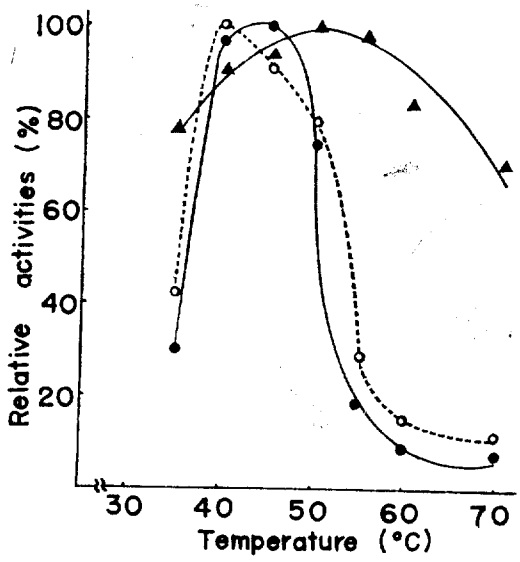

Fig. 7. Effect of temperature on the activities of CMCase components. ●——● : F-I-Ia, ○·····○ : F-I-Ib, ▲——▲ : F-II-Ia

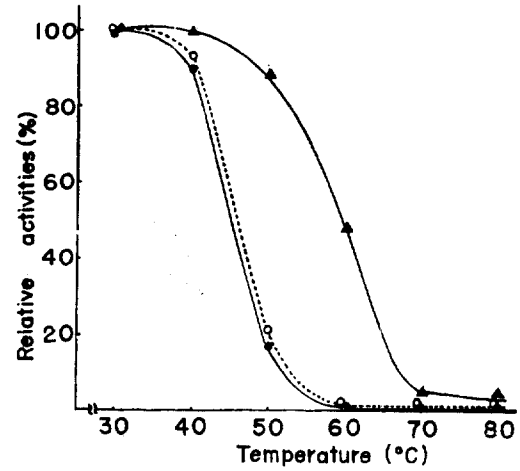

Fig. 8. Thermostabilities of CMCase components. ●——● : F-I-Ia, ○·····○ : F-I-Ib, ▲——▲ : F-II-Ia

ponents. The temperature-optima were 45°C for F-I-Ia, 40°C for F-I-Ib and 50°C for F-II-Ia, respectively. F-I-Ia and F-I-Ib showed dramatically decreased activities at temperatures below 35°C and above 55°C, while F-II-Ia activity was shown to be relatively insensitive to the change of incubation temperature.

4. Thermal stability

Each CMCase component was incubated at various temperatures for 30min in acetate buffer at pH 5.0 in the absence of substrate. After preincubation, the residual enzyme activities were measured under the standard assay conditions. As presented in Fig. 8, F-I-Ia and F-I-Ib lost 80% of their activities after treatment at 50°C, and showed no reducing activities after preincubation at 60°C. F-II-Ia was shown to be more thermostable than the other two components, because half of the activity still remained after 30-min incubation at 60°C.

5. Hydrolysis products from cellooligosaccharides

Hydrolysis products from several kinds of cellooligosaccharides were identified by liquid

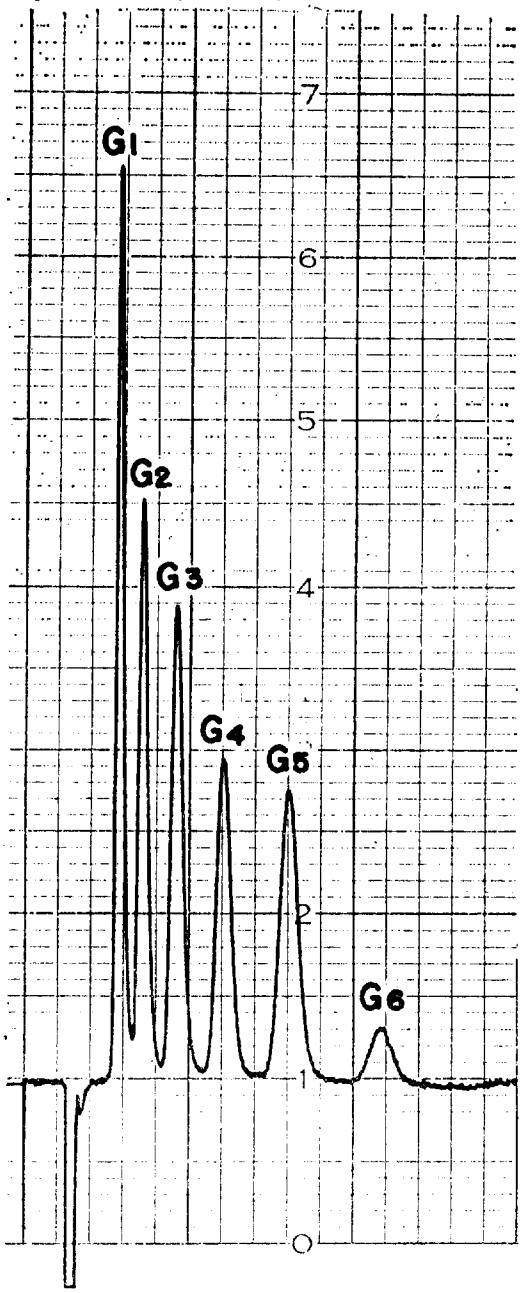

Fig. 9. Liquid chromatography of glucose and cellooligosaccharides. Flow rate: 1m*l*/min. Chart speed: 0.25cm/min

chromatography after incubation with each CMCase component. As shown in Fig. 9, each cellooligosaccharide had its own peak recorded at a specific position when analyzed by liquid chromatography.

Fig. 10 indicates that none of the CMCase components could hydrolyze G_2(cellobiose) at all.

G_3(cellotriose) was slowly split into G_1 and G_2 by F-I-Ia and F-I-Ib, but was not attacked by F-II-Ia at all, as shown in Fig. 11. Although the analysis was not achieved qualitatively, it seems that the production of G_2 was greater than that of G_1.

Fig. 12 illustrates that F-I-Ia and F-I-Ib readily attacked G_4 and produced G_1, G_2 and G_3 after prolonged incubation. But, G_1 was not detectable during early incubation period. F-II-Ia attacked G_4 very slowly and produced G_2 and G_3. G_1 was not produced even after prolonged incubation.

The products from G_5 and G_6 are shown in Figs. 13 and 14, respectively. F-I-Ia produced G_1, G_2, G_3 and a small amount of G_4 from G_5 and G_6 after prolonged incubation. F-I-Ib attacked G_5 and G_6 and accumulated G_2, G_3, G_4 and a trace amount of G_1 after 12-hour incubation. But, G_1 was not produced from G_6 by this component. F-II-Ia easely hydrolyzed G_5 and G_6 to produce G_2, G_3 and G_4 as end products. However, G_1 was not detected at all.

The results illustratrated in Figs. 10, 11, 12, 13 and 14 are summerized in Table 2. F-II-Ia was shown to have quite a different substrate specificity and an action property from those of the other two components. However, F-I-Ia and F-I-Ib showed similar substrate specificities and action properties. The only difference between the two enzyme solutions seems to be that they have different reaction velocities.

DISCUSSION

In the present work, it was found that *A. nidulans* can inductively produce CMCase when its mycelia were incubated in CMC minimal

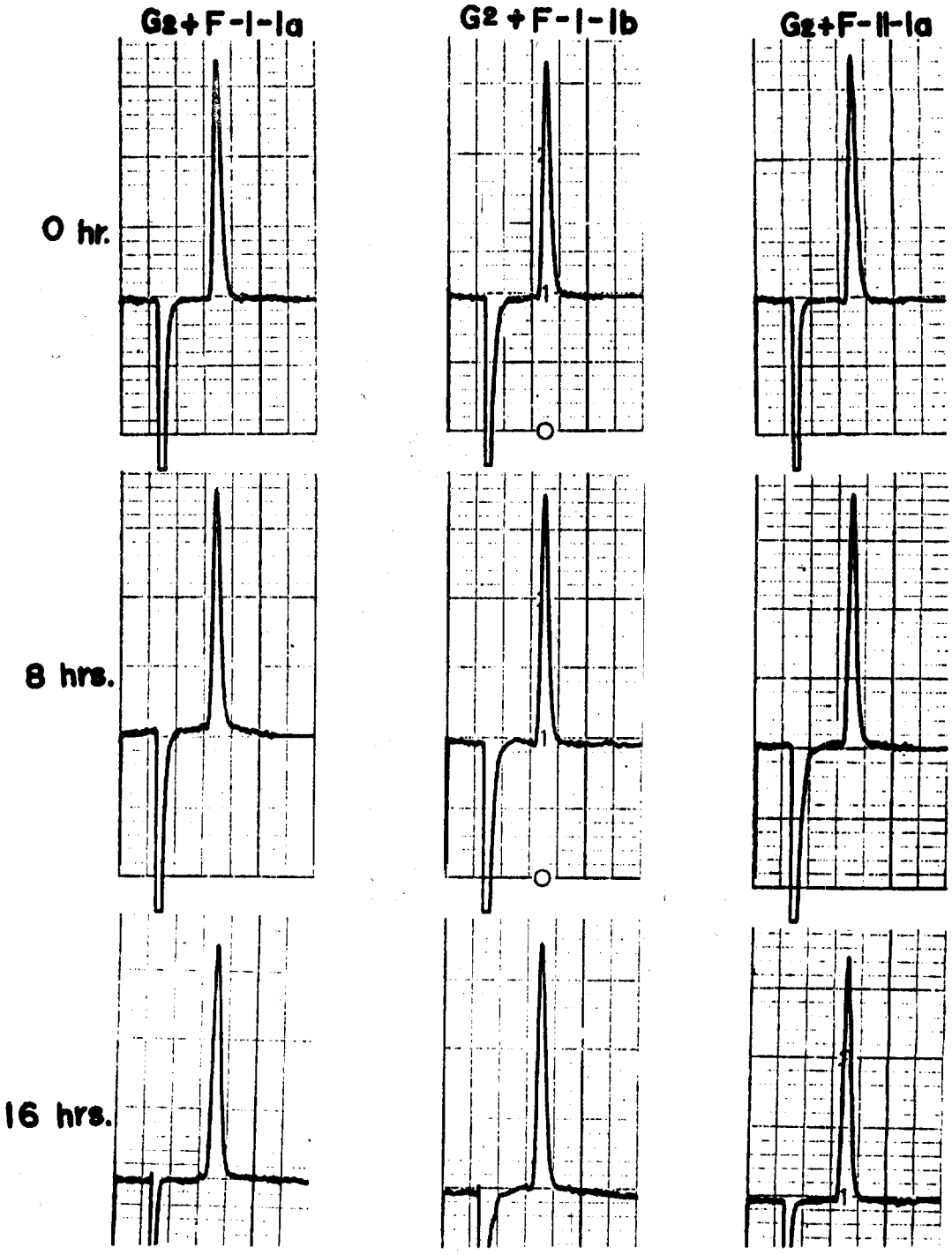

Fig. 10. Hydrolysis products from G_2 after treatment with CMCase components

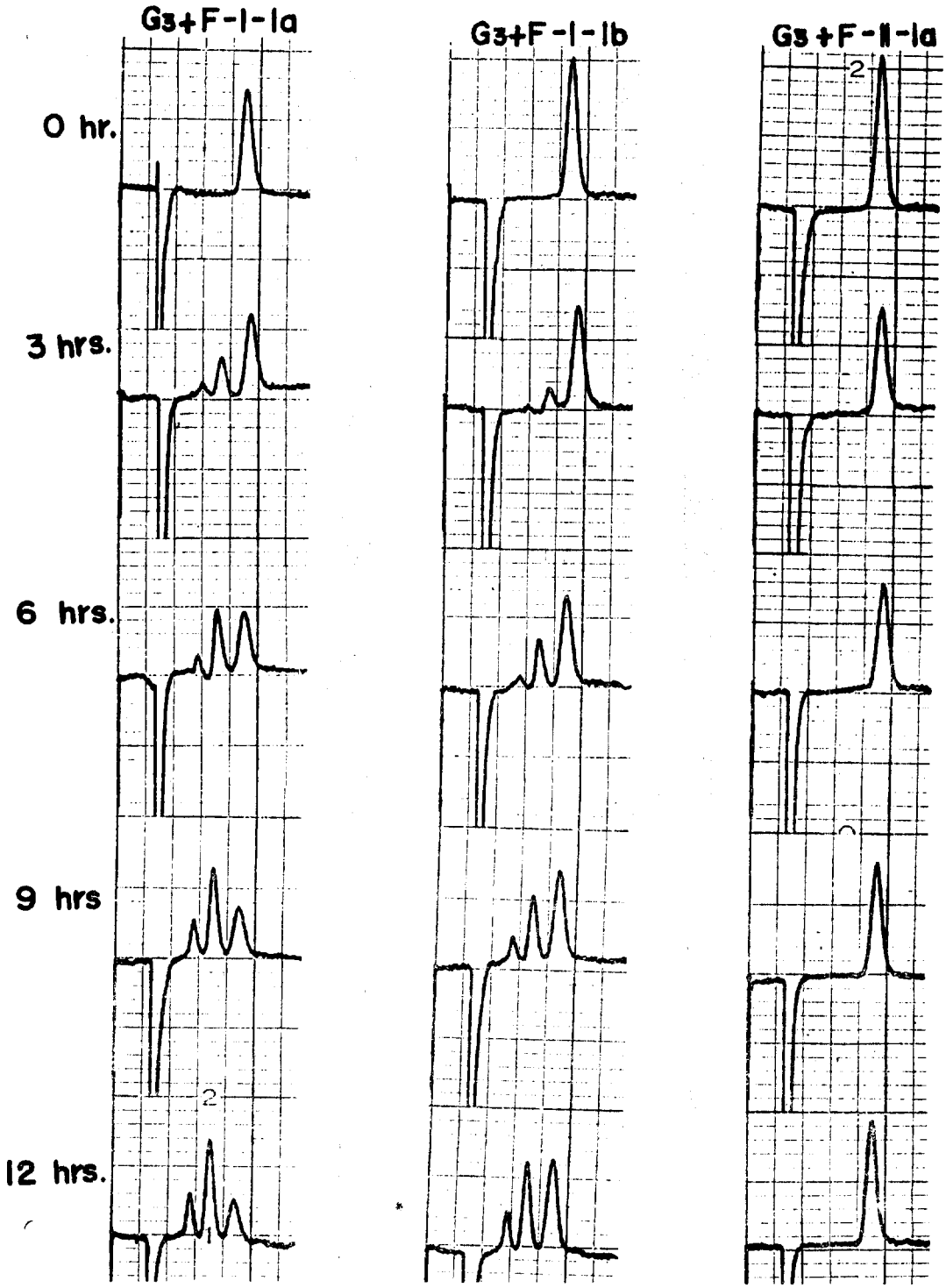

Fig. 11. Hydrolysis products from G_3 after treatment with CMCase components

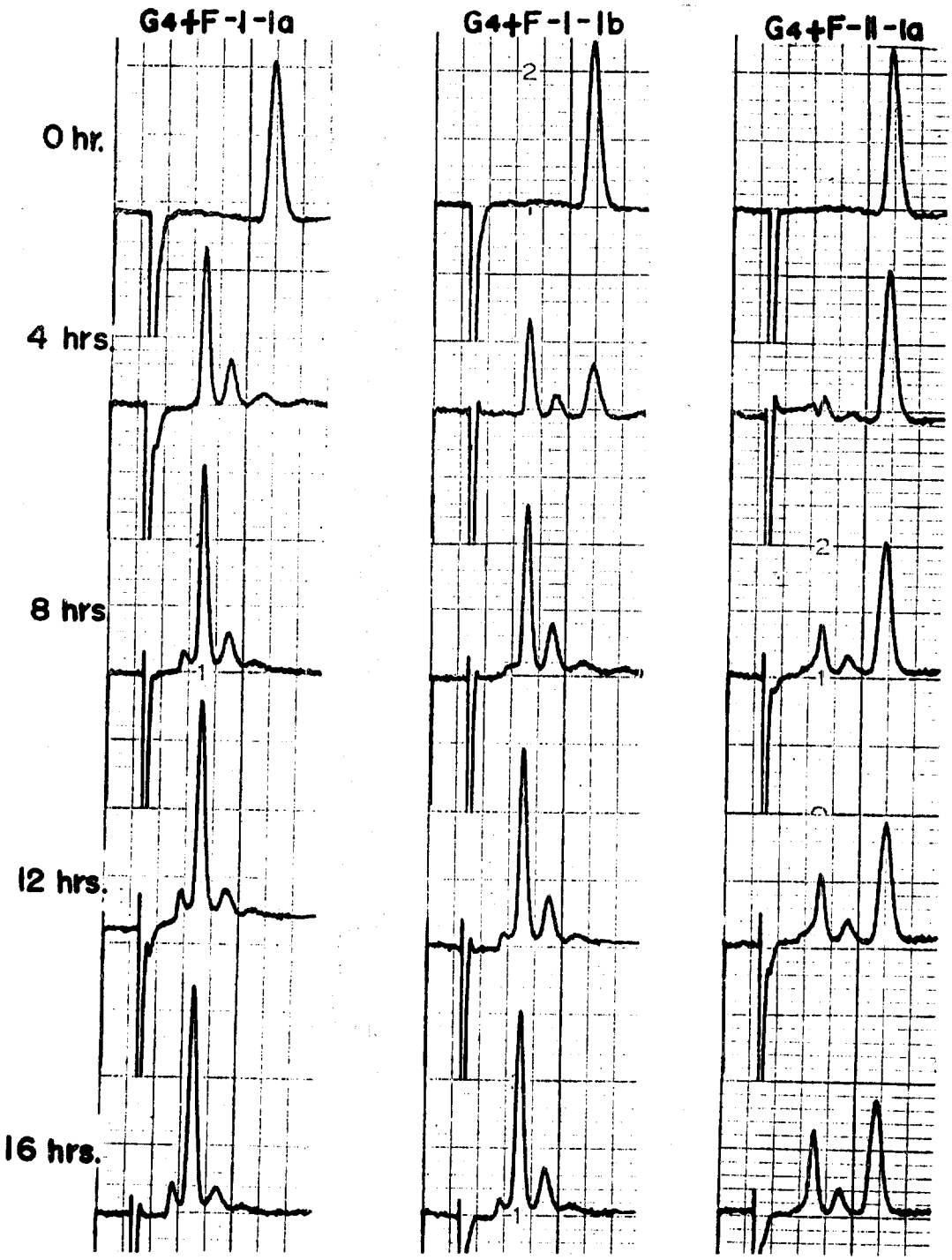

Fig. 12. Hydrolysis products from G_4 after treatment with CMCase components

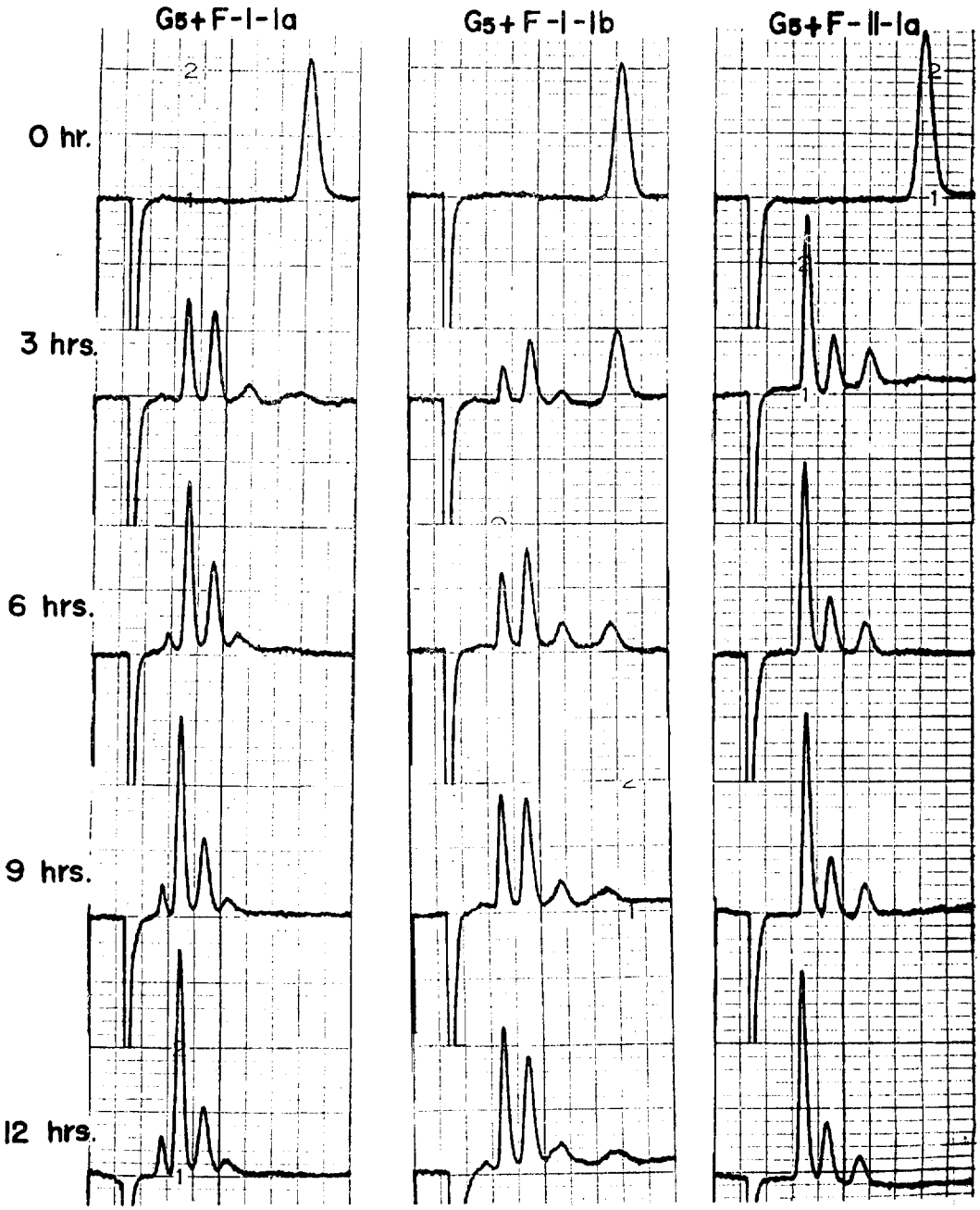

Fig. 13. Hydrolysis products from G_5 after treatment with CMCase components

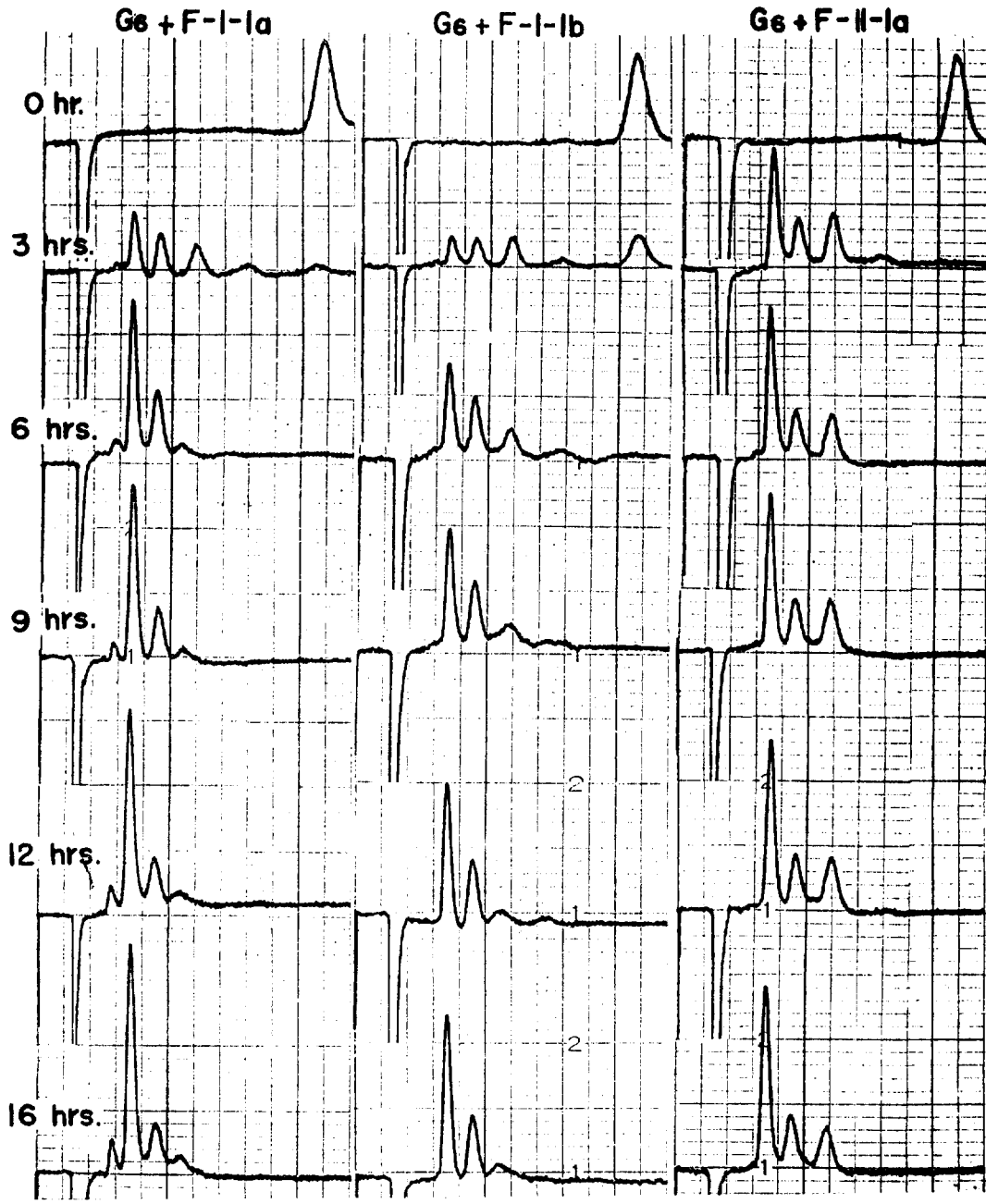

Fig. 14. Hydrolysis products from G_6 after treatment with CMCase components

Table 2. Summerized results about the substrate specificities and action properties of CMCase components

Enzymes	Substrates	Incubation time(hrs.)	Products G_1	G_2	G_3	G_4
F-I-Ia	G_3	12	+	±		
	G_4	12	+	±	+	
	G_5	12	+	±	±	±
	G_6	12	+	±	+	±
F-I-Ib	G_3	12	+	±		
	G_4	12	±	±	+	
	G_5	12	±	±	±	+
	G_6	12		±	±	±
F-II-Ia	G_3	12				
	G_4	12		±	+	
	G_5	12		±	±	+
	G_6	12		±	+	+

medium, and that avicelase was not formed by this fungus. After 12-hour incubation, CMCase activity reached its maximum and showed a plateau thereafter. This result suggests that catabolite repression might be caused by glucose produced by CMCase and β-glucosidase (Nisizawa *et al.* 1972). It is also possible that cellobiose produced by CMCase might inhibit the enzyme activity (Yoshikawa *et al.* 1974).

At least three CMCase components were separated from crude enzyme preparation by Bio-Gel P-150 column chromatography (Fig.1). Among these components, F-I and F-II were further purified by consecutive column chromatography on DEAE-Sephadex A-50 and Sephadex G-100 and F-I-Ia, F-I-Ib and F-II-Ia were prepared (Figs. 2, 3, 4 and 5).

The three CMCase components were similar in their pH-optima. But, optimal temperature and thermal stability of F-II-Ia were quite different from those of the other two components (Figs. 7 and 8).

Generally, hydrolysis products formed from various substrates have been analyzed by paper chromatography (Nisizawa *et al.* 1959, Hirayama *et al.* 1978, Okada *et al.* 1968). In the present experiments, liquid chromatography was proved to be an excellent method for the analysis of cellooligosaccharides. As illustrated in Fig. 11, each cellooligosaccharide was separated completely within half an hour.

Since any of the three CMCase componeuts could not hydrolyze G_2 (Fig.10), it is clear that they needs at least three glucosyl residnes to show their activities.

F-I-Ia and F-I-Ib hydrolyzed G_3 and produced G_1 and G_2, while F-II-Ia did not split G_3 into G_1 and G_2 (Fig. 11). But, it is not clear in the present work which β-1, 4-glycosidic linkage of the two is attacked more easily by F-I-Ia and F-I-Ib.

It is likely that G_2 might be produced from G_3 via some other way than a simple hydrolysis, because the amount of G_2, formed by CMCase seems somewhat greater than that of G_1.

In the hydrolysis of G_4 by these cellulases (Fig. 12), F-II-Ia showed much less activity than the other two components and did not produce G_1 at all, while the others produced small amounts of G_1. However, G_1 was detected only after prolonged incubation even in the cases of F-I-Ia and F-I-Ib. This result supports the view that G_1 was not produced directly from G_4. Therefore, it seems that G_4 was not split into G_1 and G_3 but into G_2 and that G_3 was formed by some other mechanism. It is also likely that G_1 was produced slowly from G_3.

In the cases of G_5 hydrolysis (Fig. 13), F-II-Ia was the most active component of the three. All the CMCase components produced G_2, G_3 and G_4 but not G_1 at the early stages of hydrolysis. F-I-Ia began to produce G_1 after 6-hour incubation and F-I-Ib produced only a trace amount of G_1 after 12-hour incubation. G_1 is thought to be formed by the same way

as in the previous cases.

G_6 was readily attacked by the three components and G_2, G_3, G_4 and a trace amount of G_5 were produced after 3-hour incubation. The results obtained at the late stage of incubation were almost the same as in the cases of G_5.

The most puzzling problem generated by the results shown in Figs. 10-14 is that cellooligosaccharide of DP (n-1) was produced from cellcoligosaccaride of DP (n) without G_1 formation. This phencmenon might be related to transglucosylation and transcellobiosylation capacities of cellulases obtained from *T. viride* (Okada *et al.* 1975) and frcm *Irpex lacteus* (Nisizawa *et al.* 1959). CMCase can split G_4 into G_2 and might be able to form a small amount of G_6 from G_2 and G_4. Since G_6 is readily attacked by CMCase, G_3 can be accumulated and easily detected in the reaction mixture, while G_6 was not detected at all. G_5 is thought to be split first into G_2 and G_3, and then G_4 can be released from G_6 which might have been formed from G_3 by the action of CMCase. In case of G_6, a trace amount of G_5 seems to be produced from G_2 and G_3 which have been split off from G_6 previously. However, there is no credible evidence for these assumptions. So, it is necessary to use labelled ccmpounds to investigate the mechanisms of such phenomena in detail.

As seen in above data, F-II-Ia showed quite different properties in optimal temperature, thermal stability and substrate specificity from those of the other two components. But, several properties of F-I-Ia and F-I-Ib resembled each other.

摘 要

Aspergillus nidulans FGSC 159의 균사를 CMC minimal 액체 배지에 접종 배양하여 얻은 여과액을 Bio-Gel P-150, DEAE-Sephadex A-50와 Sephadex G-100 column chromatography로 3개의 CMCase components 즉 F-I-Ia, F-I-Ib 그리고 F-II-Ia를 얻어내었다. 이 효소의 성질을 살펴보면 다음과 같다.

1) 이들은 avicel과 PNPG를 가수분해하지 못하였다.
2) 이들은 pH 5.0에서 최대의 역가를 나타내었다.
3) F-I-Ia는 45°C, F-I-Ib는 40°C 그리고 F-II-Ia는 50°C에서 최고의 활성을 보였다.
4) F-II-Ia는 다른 두 component들보다 높은 열저항성을 보였다. 즉 60°C에서 30분간 처리한 경우 F-I-Ia와 F-I-Ib는 모든 활성을 상실했지만, F-II-Ia는 50%의 활성을 계속 유지하였다.
5) Liquid chromatography에 의하여 분석한 결과에 의하면, F-II-Ia는 F-I-Ia, F-I-Ib와는 매우 다른 기질 특이성을 보여 주었다. 즉, F-I-Ia와 F-I-Ib는 G_3를 공격하여 G_1과 G_2를 생성한 반면, F-II-Ia는 G_3를 공격하지 못하였으며 G_4에 대해서도 매우 느리게 작용하였다. 또한 기질이 어느 것이든 간에 F-II-Ia는 G_1을 생성하지 못하였다.
6) 이 실험의 결과는 적어도 두 가지 이상의 CMCase components가 *Aspergillus nidulans* FGSC 159에서 생성된다는 것을 말해준다.

REFERENCES

Berghem, L.E.R., L.G. Pettersson, and U.B.A. Fredriksson, 1976. The mechanism of enzymatic cellulose degradation; Purification and some properties of two different 1,4-β-glucan glucanohydrolases from *Trichoderma viride*. *Eur. J. Biochem.*, **61**, 621–630.

Eriksson, K.E., and B. Petterson, 1975. Extra-

cellular enzyme system utilized by the fungus *Sporolrichum pulverulentum* (*Chrysosporium lingorum*) for the breakdown of cellulose. *Eur. J. Biochem.*, **51**, 213–218.

Halliwell, G., 1961. The action of cellulolytic enzyme from *Myrothecium verrucaria*. *Biochem. J.*, **79**, 185–192.

Hirayama, T., S. Horie, H. Nakayama, and K. Matsuda, 1978. Studies on cellulases of a phytophathogenic fungus, *Pu ularia oryzae* Cavara. *J. Biochem.*, **84**, 27–37.

Kanda, T., K. Wakabayashi, and K. Nisizawa, 1976. Xylanase activity of endo-cellulose of CMCase type from *Irpex lacteus*. *J. Biochem.*, **97**, 989–995.

Kanda, T., K. Wakabayashi, and K. Nisizawa, 1976. Purification and properties of an endo-cellulase of Avicelase type from *Irpex lacteus*. *J. Biochem.*, **79**, 977–988.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biochem.* **193**, 265–275.

Miller, G.L., J. Dean, and R. Blum, 1960. A study of methods for preparing oligosaccharides from cellulose. *Arch. Biochem. Biophys.*, **91**, 21–26.

Nakayama, M., Y. Tomita, H. Suzuki, and K. Nisizawa, 1976. Partial proteolysis of some cellulase components from *Trichoderma viride* and the substrate specificity of of the modified products. *J. Biochem.*, **79**, 955–966.

Nisizawa, K., and Y. Hashimoto, 1959. Cellulose-splitting enzymes. VI. *Arch. Biochem. Biophys.*, **81**, 211–222.

Nisizawa, T., Suzuki, and K. Nisizawa, 1972. Catabolite repression of cellulase formation in *Trichoderma viride*. *J. Biochem.*, **71**, 999–1007.

Okada, G., T. Nisizawa, and K. Nisizawa, 1966. Cellulases of a marine mollusc, *Dolabella* sp. *Biochem. J.* **99**, 214–221.

Okada, C., K. Nisizawa, and H. Suzuki, 1968. Cellulase components from *Trichoderma viride*. *J. Biochem.*, **63**(5), 591–607.

Okada, G., and Nisizawa., 1975. Enzymatic studies on a cellulase system of *Trichoderma viride*; Transglycosylation properties of two cellulase components of random type. *J. Biochem.*, **78**, 297–306.

Okada, G., 1975. Enzymatic studies on a cellulase system of *Trichoderma viride*. *J. Biochem.*, **77**, 33–42.

Somogyi, M., 1952. Notes on sugar determination. J. Biol. Chem., **195**, 19–23.

Storvick, W.O., E.E. Cole, and W. King, 1963. Mode of action of a cellulase component from *Cellvibrio gilvus*. *Biochemistry*, **2**, 1106–1110.

Tanaka, M., S. Takenawa, R. Matsuno, and T. Tamikubo, 1977, Purification and properties of cellulases from *Pellicularia filamentosa*. *J. Ferment. Technol.*, **55**(2), 137–142.

Tanaka, M., S. Takenawa, R. Matsuno, and T. Kamikubo, 1978, Some factors affecting cellulose degradation with *Pellicularia filamentosa* cellulases. *J. Ferment. Technol.*, **56**(2), 108–113.

Toda, S., H. Suzuki, and K. Nisizawa., Some enzymatic properties and substrate specificities of *Trichoderma* cellulases with special reference to their actvity toward xylan. *J. Ferment. Technol.*, **49**, 499–521

Wood, T. M., 1969. The cellulase of *Fusarium solani;* Resolution of the enzyme complex. *Biochem. J.*, **155**, 457–464.

Wood, T.M., 1971. Thecellulase of *Fusarium solani;* purification and specificity of the β-1,4-glucanase and the β-D-glucosidase components *Biochem. J.*, **121**, 353–362.

Wood, T.M., and S. McCrae, 1972. The purification and properties of the C_1 component of *Trichoderma koningii* cellulase. *Biochem. J.*, **128**, 1183–1192.

Yamane, K., H. Suzuki, M. Hirotani, H. Ozawa, and K. Nisizawa, 1970. Effect of nature and Supply of carbon sources on cellulase formation in *Pseudomonas fluorescens* var. *cellulosa*. J. Biochem., **67**, 9–18.

Yoshikawa, T., H. Suzuki, and K. Nisizawa, 1974. Biogenesis of multiple cellulase components of *Pseudomonas fluorescens* var. *cellulosa* *J. Biochem.*, **75**, 531–540.