The Possible Proteolytic Modification of Cellulase Components in the Aqueous Solutions of a Crude Enzyme Preparation from *Trichoderma viride*

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Changes in the cellulase components of an aqueous extract from Cellulase Onozuka, a commercial enzyme product from *Trichoderma viride*, were followed by two steps of column chromatography after its incubation during some periods. The results were essentially similar to those observed previously for the culture medium of this fungus, and their multiplicity pattern changed depending on the incubation conditions. Acid, neutral and alkaline proteases were demonstrated to exist in this crude cellulase preparation. Particularly, the neutral protease was accompanied with any cellulase fraction, and it was suggested that the proteases may cause such conversion of cellulase components to generate their multiplicity.

Introduction

The cellulase components of *Trichoderma viride* which had been purified so far were all derived from Peak Ⅱ which was one of the groups of this cellulase system and it was eluted as the second peak on Amberlite CG-50 column chromatography of the commercial cellulase preparations from this fungus (Meicelase and Cellulase Onozuka). It contained about half an amount of the original cellulase activity [1~3]. However, little has been elucidated for the nature of Peak Ⅲ components, in spite of its being nearly another half of the cellulase activity. The writer, therefore, tried to achieve the purification of this group, starting from Cellulase Onozuka.

During the fractionation for obtaining highly purified Peak Ⅲ, we found, however, that the chromatographic patterns of cellulase proteins eluted from ion-exchangers or molecular shieves gradually changed only by the storage of the obtaining fractions, and that more or less protease activities were always detected in these fractions. This changing pattern by the storage was observed

even after the fractionation by Amberlite CG-50 column chromatography. The results seemed to be similar phenomenon to the changes of cellulase components as has been observed previously on the fractionation of growing cultures of *Trichoderma viride* [4].

Therefore, these changes of multiplicity patterns of cellulase components may be due to a reflect of their possible modification by some factors including the action of contaminating proteases. For verifying this assumption, it seems necessary to isolate each of modified cellulase components by *in vitro* experiments and to compare their enzyme-chemical properties with each other. In the present work, however, some preliminary *in vitro* experiments were carried out for the elucidation of this problem.

Materials and Methods

1) *Enzyme preparations.* Cellulase preparations were obtained from Cellulase Onozuka, a commercial enzyme product from the wheat bran culture of *Trichoderma viride,* which was kindly supplied by Kinki Yakult Co. Ltd. In the ordinary experiments, a cellulase solution derived from 10% aqueous solution of the crude material was used.

In some cases, the enzyme preparations pretreated with protease inhibitor were used. They were prepared as follows. To a 2.5-fold concentrated crude extract from Cellulase Onozuka, DFP (2*mM*) was added and stood overnight at 5℃ to inactivate the DFP-sensitive protease [5]. Then, the solution was equilibrated with 0.2*M* acetate buffer, *pH* 3.5, by dialysis against the same buffer and subjected to the Amberlite CG-50 column chromatography. The resulting Peak Ⅰ, Peak Ⅱ and Peak Ⅲ were each concentrated to 10*ml,* to which EDTA (0.05*mM*) was added to inhibit the metal-requiring protease [6]. They were used for experiments as DFP-EDTA-treated enzyme preparations. Furthermore, a portion of each DFP-EDTA-treated fraction was dialyzed overnight against several changes of 0.02*M* phosphate buffer, *pH* 6.0, then either $MgCl_2$ (0.01*mM*) or $CaCl_2$ (0.01*mM*) was added. These fractions were used for experiment as DFP-EDTA-Mg^{++}- or DFP-EDTA-Ca^{++}-treated enzyme preparations.

2) *Substrates and Chemicals.* These were all the same as used previously [4].

3) *Enzyme assays.* A) Cellulase activity. (Ⅰ) CMC-saccharifying activity: The activity was determined and expressed in the same way as described previously [4], and it is called "s-CMCase activity" in this paper. (Ⅱ) Avicelase activity: The activity was assayed and expressed in the same way as described previously [4].

B) β-glucosidase (PNGase) activity. The activity was assayed and expressed in the same way as described previously [4].

C) Protease activity. The reaction mixture contained 0.5*ml* of 1% casein solution and 0.25*ml* of enzyme solution, The substrate was dissolved in 0.1*M*

phosphate buffer, *pH* 7.0, 0.1*M* MacIlvaine buffer, *pH* 8.0, or 0.1*M* acetate buffer, *pH* 2.9, for the assays of neutral, alkaline and acid proteases, respectively. After the reaction mixture was incubated at 30℃ for a suitable period, 5*ml* of 10% trichloroacetic acid was added, then the mixture was centrifuged at 3000 *rpm* for 15 *min*. A 0.5*ml* aliquot of the supernatant was analyzed for the reaction products by the method of Lowry *et al* [7]. The enzyme activity was expressed in terms of the optical density at 750*mμ*.

4) *Determination of protein.* Protein concentration of enzyme solution obtained by the column chromatography was expressed by optical density at 280*mμ*.

5) *Column chromatography.* A) Ion-exchange chromatography. Two steps chromatography on Amberlite CG-50 and DEAE Sephadex A-50 columns were performed for the fractionation of cellulase samples under the conditions described previously [4].

B) Gel-filtration. Gel-filtration chromatography was carried out a Bio-gel P-150 column (2 x 110*cm*) previously equilibrated with 0.02*M* phosphate buffer, *pH* 6.0. The eluates were collected every 5*ml*.

Results

1) *Decrease in the molecular weight of cellulase components after standing them in the cold*

A freshly prepared aqueous solution of Cellulase Onozuka was subjected to gel-filtration with Bio-gel P-150. As seen in Fig. 1A, s-CMCase activity dispersed over a range of molecular weight between *ca.* 60,000 and 15,000, forming three peaks with molecular weight of around $5 \cdot 10^4$, $4 \cdot 10^4$, and $3 \cdot 10^4$, respectively, while Avicelase was eluted in a range of higher molecular weights, forming two main peaks around $6 \cdot 10^4$ and $5 \cdot 10^4$, respectively. PNGase activity was eluted as a single peak at about $5 \cdot 10^4$.

The same enzyme solution was placed at 0℃ for 6 months, and then applied to the same gel-filtration column. The elution pattern is shown in Fig. 1B. Apparently, a long-term storage of the enzyme solution caused decreases of cellulase activities in higher molecular weight region with their concomitant increases in smaller molecular weight region. Especially noticeable phenomenon is the appearance of an entirely new Avicelase peak with molecular weight of *ca.* $3 \cdot 10^4$.

PNGase, however, showed no such shift of the molecular size at all; only a single peak with molecular weight of *ca.* $5 \cdot 10^4$ was obtained.

2) *Changes in the chromatographic patterns of crude cellulase solution with incubation period*

A freshly prepared aqueous solution of Cellulase Onozuka was fractionated

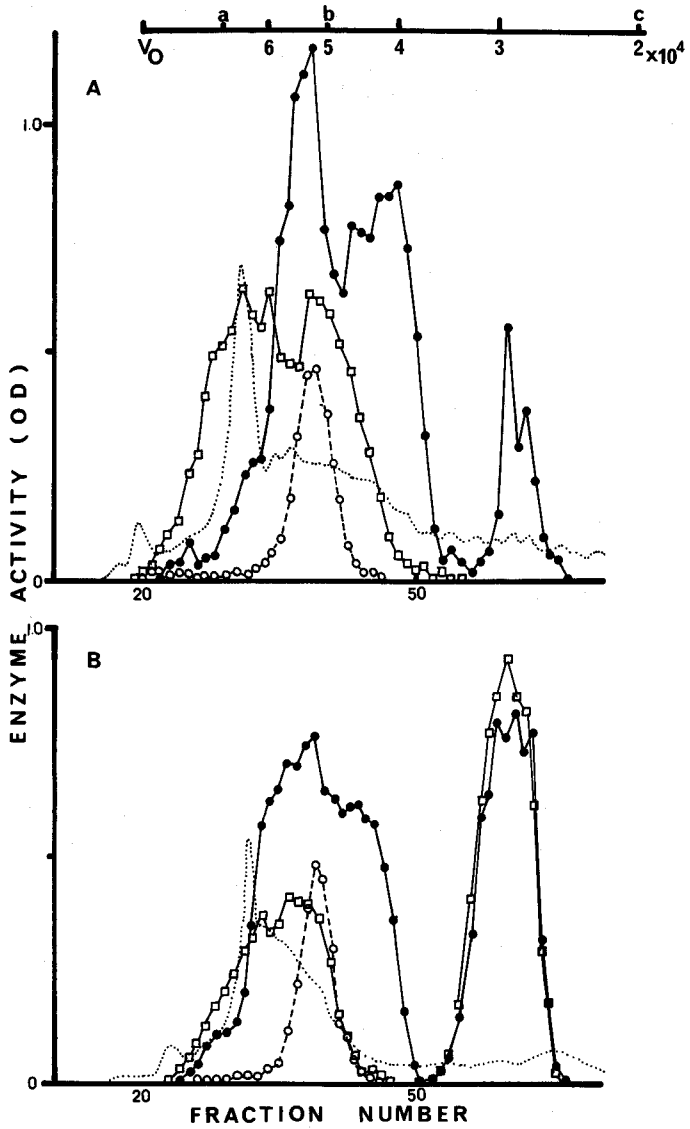

Fig. 1 Gel filtration patterns on Bio-gel P-150 of an aqueous solution of Cellulase Onozuka before (A) and after (B) storage for 6 months at 0℃.

The upper scale shows elution position-molecular weight relation calibrated by using. (a) bovine serum albumin (M. W., 6.7×10^4), (b) Taka-amylase A (M. W., 5.07×10^4) and (c) egg-white lysozyme (M. W., 1.4×10^4) as standard; void volume (V_0) was determined with Blue Dextran. Symbols; dashed line: protein (measured at 280*mμ*), closed circles: s-CMCase activity (measured at 660*mμ*) after incubation for 10*min*, squares: Avicelase activity (measured at 660*mμ*) after incubation for 20*hr*, and open circles: PNGase activity (measured at 420*mμ*) after incubation for 10 min.

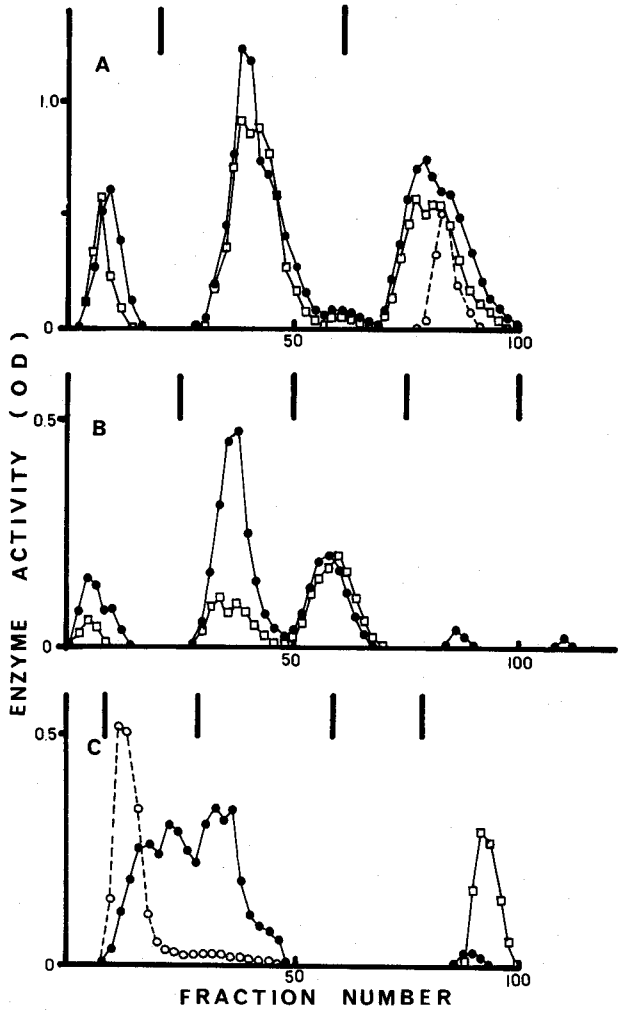

Fig.2 Elution patterns before incubation of (A) Amberlite CG-50 column chromatography of an aqueous extract from Cellulase Onozuka, (B) DEAE Sephadex A-50 column chromatography of Peak Ⅱ from (A), and (C) DEAE Sephadex A-50 column chromatography of Peak Ⅲ from (A). Symbols; closed circles: s-CMCase activity (measured at 660*mμ*) after 10 min incubation, squares: Avicelase activity (measured at 660*mμ*) after 20*hr* incubation, and open circles: PNGase actiyity (measured at 420*mμ*) after 15 min incubation.

by Amberlite CG-50 column chromatography into the three groups of cellulase, Peak Ⅰ, Peak Ⅱ, and Peak Ⅲ, as previously reported [1~4]. Elution patterns of Peak Ⅱ and Peak Ⅲ on DEAE Sephadex A-50 column chromatography are shown in Fig. 2B and 2C. Thus as already described [1~4], these two peaks were separated respectively into five subfractions, F-Ⅰ to F-Ⅴ and C-Ⅰ to C-Ⅴ. These chromatographic patterns were used as controls.

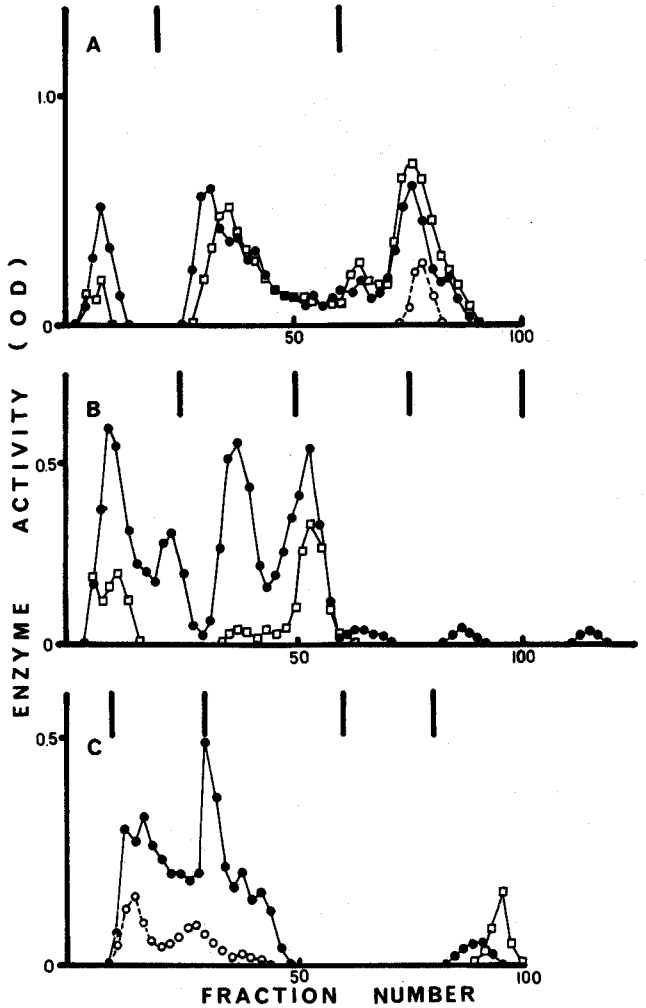

Fig. 3 Elution patterns after 20 hour incubation at 30℃ of (A) Amberlite CG-50 column chromatography of an aqueous extract from Cellulase Onozuka, (B) DEAE Sephadex A-50 column chromatography of Peak Ⅱ from (A), and (C) DEAE Sephadex A-50 column chromatography of Peak Ⅲ from (A). Symbols; closed circles: s-CMCase activity at 660*mμ* after 10*min* incubation, squares: Avicelase activity at 660*mμ* after 20 hr incubation, and open circles: PNGase activity at 420*mμ* after 15*min* incubation.

After the incubation of this crude aqueous cellulase solution at 30℃ for 20 *hrs* and 3 *days*, each was subjected to the two steps column chromatography, and the elution patterns were compared to those of the controls. Fig. 3 shows the patterns of the sample incubated for 20 *hrs*. The pattern on Amberlite column (Fig. 3A) was similar to that of the control (Fig. 2A), except that a minor peak, called Peak Ⅲ′, was detected between the Peak Ⅱ and Peak Ⅲ.

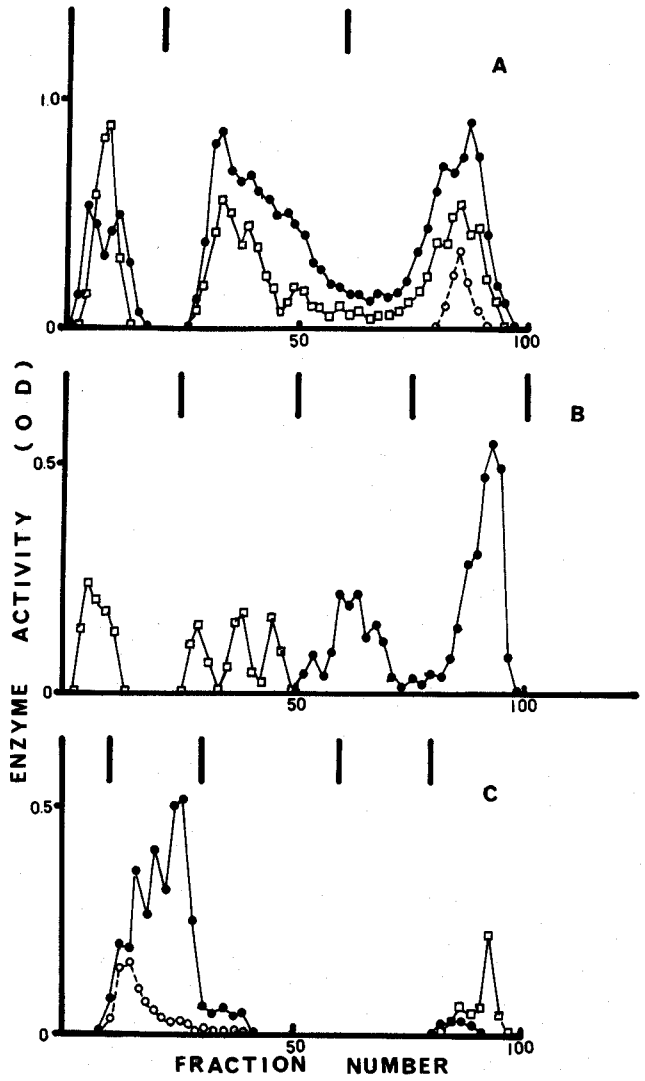

Fig. 4 Elution patterns after incubation at 30℃ for 3 days of (A) Amberlite CG-50 column chromatography of an aqueous extract from Cellulase Onozuka, (B) DEAE Sephadex A-50 column chromatography of Peak Ⅱ from (A), (C) DEAE Sephadex A-50 column chromatography of Peak Ⅲ from (A). Symbols: Closed circles represent s-CMCase activity (measured at 660*mμ*) after 10 min. Open squares indicate Avicelase activity (measured at 660*mμ*) after 20*hr* incubation. Open circles are PNGase activity (measured at 420*mμ*) after 15*min* incubation.

The pattern of Peak Ⅱ on DEAE Sephadex column (Fig. 3B) was remarkably different from that the control (Fig. 2B); *e. g.* a new s-CMCase peak was detectable between F-Ⅰ and F-Ⅱ, and s-CMCase activity in F-Ⅰ was relatively

high. Such differences in the elution pattern may indicate that increasing heterogeneity of cellulase components occurred during the incubation of the cellulase solution at 30℃.

The pattern of Peak Ⅲ on DEAE Sephadex column (Fig. 3C) indicated that certain changes also occurred in the cellulase components; *e. g.*, s-CMCase activity in C-Ⅱ decreased and that in C-Ⅰ and C-Ⅲ increased.

Fig. 4 shows the elution patterns of the sample incubated for 3 *days*. In the elution pattern on Amberlite column (Fig. 4A), increase in the activity of Peak Ⅰ, especially in Avicelase activity, was noticeable. These irregularly increasing elution peaks may suggest an advanced heterogeneity of Peak Ⅱ and Peak Ⅲ.

The elution pattern of Peak Ⅱ on DEAE Sephadex column (Fig. 4B) was conspiously different from those of the control and the cellulase solution incubated for a shorter period. Thus, Avicelase activity was detected only in the F-Ⅰ and F-Ⅱ fractions, whereas s-CMCase activity in F-Ⅲ and F-Ⅳ. In the elution pattern of Peak Ⅲ on DEAE Sephadex column (Fig. 4C), it was noticed that the s-CMCase activity of C-Ⅲ and C-Ⅳ decreased remarkably and that of C-Ⅱ increased.

3) *The protease activity in the aqueous extract from Cellulase Onozuka*

Since the protease may be thought to be a possible agent that causes the above mentioned shift of molecular and/or electric charges of cellulase proteins, its activity in the crude cellulase solution was determined at acid, neutral and alkaline *pH* regions and in the presence or absence of DFP and EDTA [5, 6]. The results are shown in Table Ⅰ.

Table. Ⅰ. Protease activity in an aqueous extract from Cellulase Onozuka and the effects of protease inhibitors.

Enzyme	pH of the reaction	Activity (OD/20 hours)			
		cotrol	DFP added (2*mM*)	EDTA added (0.05*mM*)	EDTA and DEP added
acid protease	2.9	0.057	0.046	0.031	0.013
neutral protease	7.0	0.182	0.088	0.006	0.000
alkaline protease	8.0	0.098	0.000	0.023	0.000

The activity at alkaline *pH* was inhibited completely by 2*mM* DFP, and partially by 0.05*mM* EDTA, while the activity was highly inhibited by EDTA and partially by DFP at neutral *pH;* the activity at acid *pH* was relatively resistant to both the compounds. These results indicate that this crude enzyme preparation from Cellulase Onozuka contains at least three proteases similar to those reported of other fungi: alkaline serine-protease [8], neutral metal-

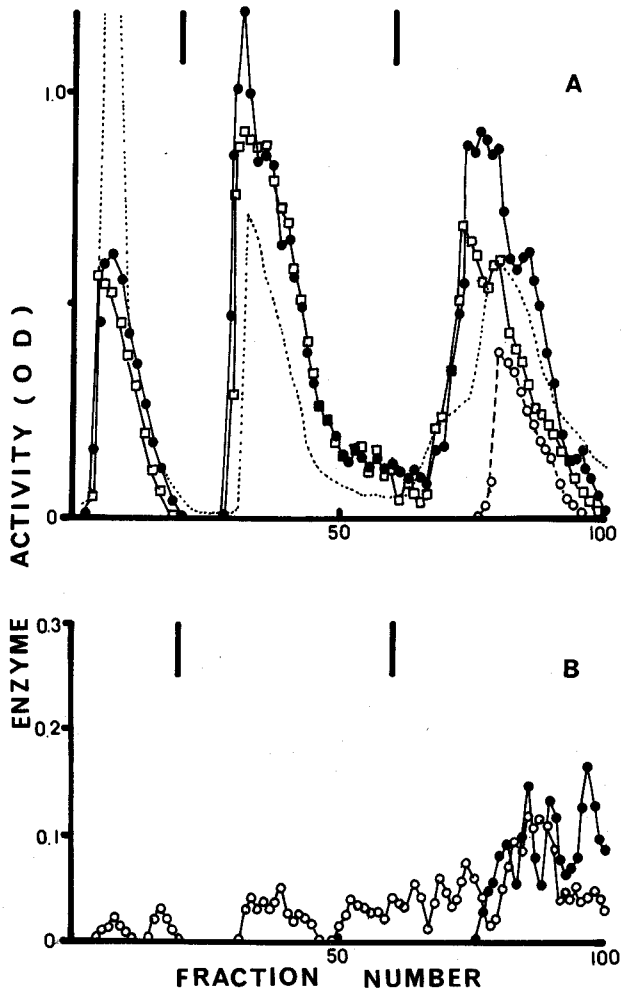

Fig. 5 (A) Elution pattern of cellulase components in an aqueous extract from Cellulase Onozuka on Amberlite CG-50 column (2 x 36 *cm*) chromatography.

Symbols: dashed line; protein (measured at 280*mμ*), closed circles; s-CMCase activity (measured at 660*mμ*) after 10*min*, squares; Avicelase activity (measured at 660*mμ*) after 20*hr* incubation, and open circles; PNGase activity (measured at 420*mμ*) after 15 min incubation. (B) Proteolytic activities in the eluates on Amberlite CG-50 column chromatography of an aqueous extract from Cellulase Onozuka. The proteolytic activity at acid *pH* could not be detected. Symbols: closed circles; alkaline protease activity (measured at 750*mμ*) after 20 *hr* incubation, and open circles; neutral protease activity (measured at 750*mμ*) after 20 *hr* incubation.

protease [9] and acid protease indifferent of both DFP and EDTA [10].

Then, the behavior of these proteases upon the Amberlite CG-50 column

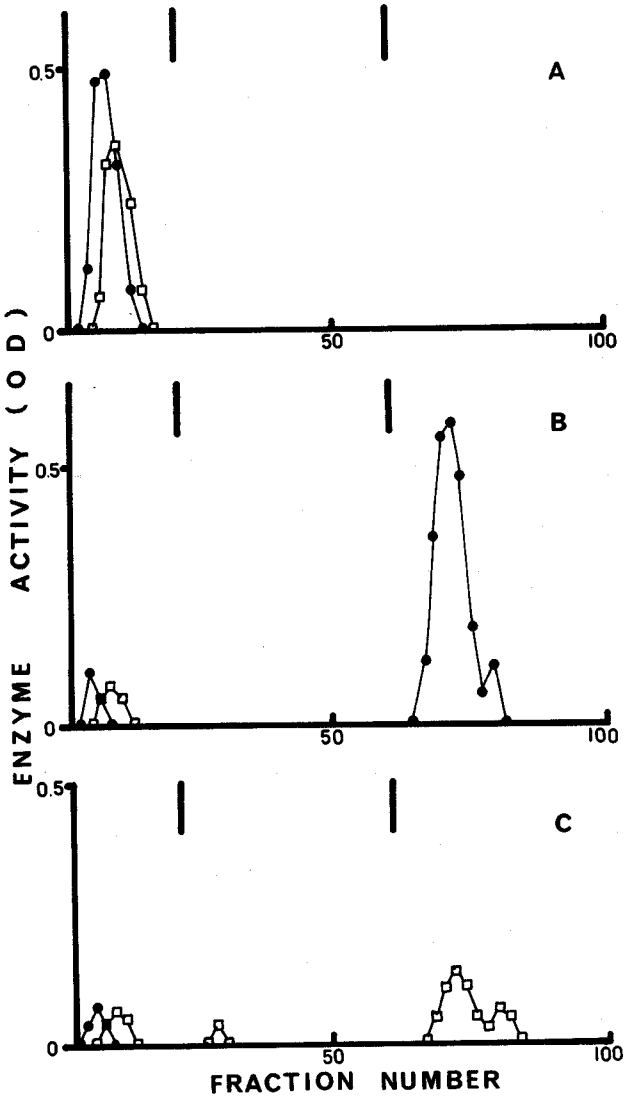

Fig. 6 Resolution patterns on Amberlite CG-50 column (1 x 44 *cm*) chromatography of (A) DFP-EDTA-treated Peak I without incubation, (B) DFP-EDTA-Mg^{++}-treated Peak I and (C) DFP-EDTA-Ca^{++}-treated Peak I after incubation at 30℃ for 3 days. CMCase activity was measured after incubation for 10*min* in (A), but for 20*hr* in (B) and (C). Avicelase activity was measured after incubation for 20 *hr* in (A), but for 3 *days* in (B) and (C). Symbols: closed circles; s-CMCase activity at 660*mμ*, and squares; Avicelase activity at 660*mμ*.

chromatography, which was first used in the two steps chromatography to separate cellulase components, was examined. As shown in Figs. 5A and 5B,

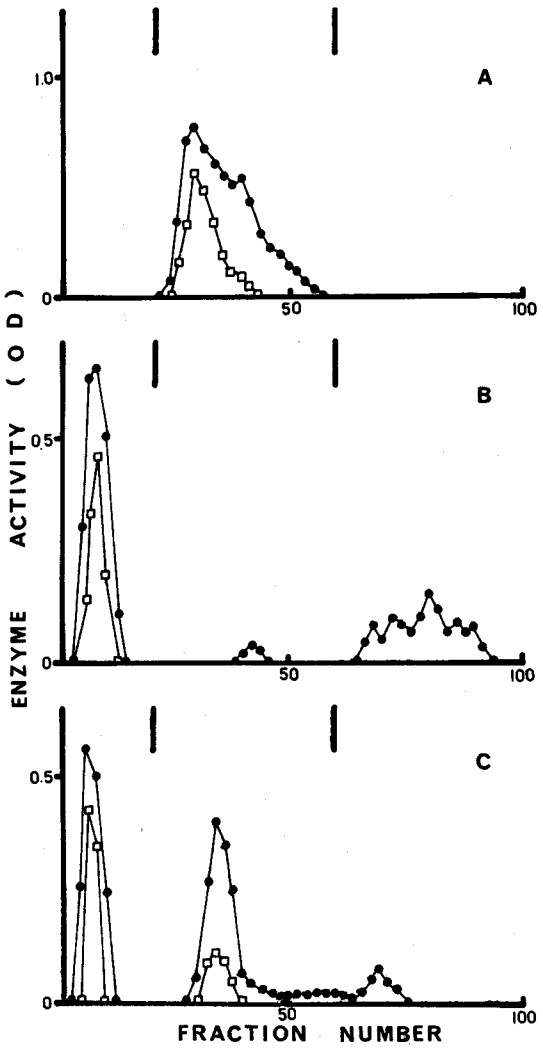

Fig. 7 Resolution patterns on Amberlite CG-50 column chromatography of (A) DFP-EDTA-treated Peak Ⅱ without incubation, (B) DFP-EDTA-Mg^{++}-treated Peak Ⅱ and (C) DFP-EDTA-Ca^{++}-treated Peak Ⅱ after incubation at 30℃ for 3 *days*. The size of column and the period of incubation for enzyme assays are the same as described in the legend for Fig. 6. Symbols; closed circles: s-CMCase activity at 660*mμ*, and squares: Avicelase activity at 660*mμ*.

neutral protease activity was mostly coincident with Peak Ⅱ and Peak Ⅲ, but alkaline protease activity was found exclusively in Peak Ⅲ. Acid protease activity could be weakly detected in a combined fractions of Peak Ⅰ, Ⅱ and Ⅲ, but not be detected in each fraction, possibly due to their very low activities.

4) *Changes in chromatographic patterns of Peak* Ⅰ, Ⅱ *and* Ⅲ *pretreated with protease-inhibitors*

As mentioned above, some neutral protease activity was accompanied with any of the three cellulase fractions, but mostly with Peak Ⅱ and Ⅲ, Then, in order to study the effect of this neutral protease on the cellulase component in each peak, Peak Ⅰ, Ⅱ and Ⅲ were pretreated with the protease inhibitors (DFP-EDTA-treated fractions) and those reactivated with Mg^{++} or Ca^{++} (DFP-EDTA-Mg^{++}-or Ca^{++}-treated fractions) as described in "Materials". Then, these treated cellulase samples were analyzed for their components by two steps chromatography after incubation at 30℃ for 3 days. Nontreated fractions were also analyzed without incubation by similar chromatography as the control experiments.

A) Patterns on Amberlite CG-50 column chromatography: The result from the Peak Ⅰ are shown in Fig. 6. The pattern of DFP-EDTA-treated Peak Ⅰ, which was not incubated, gave no other extra-peak that Peak Ⅰ with both s-CMCase and Avicelase activities (Fig. 6A). From the pattern of DFP-EDTA-Mg^{++}-treated Peak Ⅰ, which was incubated, it was found that s-CMCase and Avicelase activities at the original elution position were much decreased, whereas new s-CMCase peaks were found at the Peak Ⅲ region (Fig. 6B). In this case of DFP-EDTA-Ca^{++}-treated sample, s-CMCase activity was almost inactivated, but the weak activities of Avicelase were detected in the three peak regions, in which the highest was in Peak Ⅲ (Fig. 6C).

Fig. 7 shows the similar results with Peak Ⅱ. In this case also, the sample without incubation contained only Peak Ⅱ cellulase components (Fig. 7A), whereas the new other components were eluted at the regions of Peak Ⅰ and Peak Ⅲ after incubation in the presence of either Mg^{++} (Fig. 7B) or Ca^{++} (Fig. 7C). Moreover, it is to be noticed that a relative activity of Avicelase to that of s-CMCase in Peak Ⅱ region was more decreased in the Ca^{++}-treated sample than in the control.

The results of similar experiments with Peak Ⅲ are shown in Fig. 8. The cellulase components which were not present in the DFP-EDTA-treated Peak Ⅲ without incubation became detectable after incubation in the presence of Mg^{++} or Ca^{++}, though their amounts were very small as compared with those of Peak Ⅰ and Peak Ⅱ.

B) Patterns on DEAE Sephadex A-50 column chromatography: The cellulase components of Peak Ⅱ and Peak Ⅲ were also analyzed by DEAE Sephadex chromatography after entirely the same treatments as in Amberlite column chromatography. The results are shown in Fig. 9 and Fig. 10, respectively. The pattern of DFP-EDTA-treated and not incubated Peak Ⅱ (Fig. 9A) was similar to that of Peak Ⅱ without incubation, in which five components were present (Fig. 2B). DFP-EDTA-Mg^{++}-treated and incubated Peak Ⅱ showed only a little CMCase activity in F-Ⅱ and F-Ⅳ regions (Fig. 9B). For the sample incubated with Ca^{++}, slightly different situation was noticed (Fig. 9C).

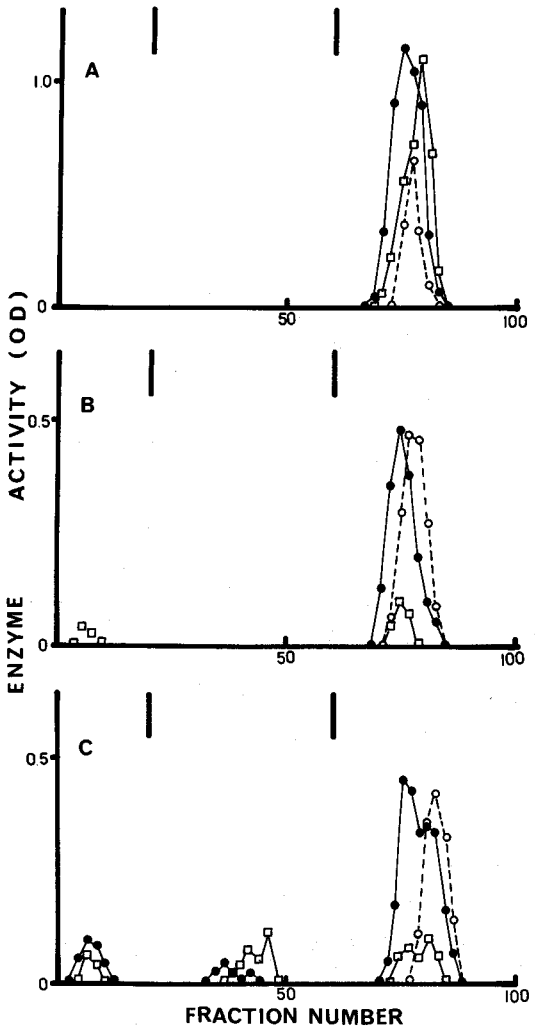

Fig. 8 Resolution patterns on Amberlite CG-50 column chromatography of (A) DFP-EDTA-treated Peak Ⅲ without incubation, (B) DFP-EDTA-Mg^{++}-treated Peak Ⅲ and (C) DFP-EDTA-Ca^{++}-treated Peak Ⅲ after incubation at 30℃ for 3 *days*. The size of column and the period of incubation for cellulase assays are same as described in the legend for Fig. 6. PNGase activity was measured after incubation for 15 *min* in (A), but for 60*min in* (B) and (C). Symbols; closed circles: s-CMCase activity at 660*mμ*, squares: Avicelase activity at 660*mμ*, and open circles: PNGase activity at 420*mμ*.

Although F-Ⅱ, as in the control, was still largest among the four s-CMCase peaks, the s-CMCase activities were slightly enhanced as a whole as compared

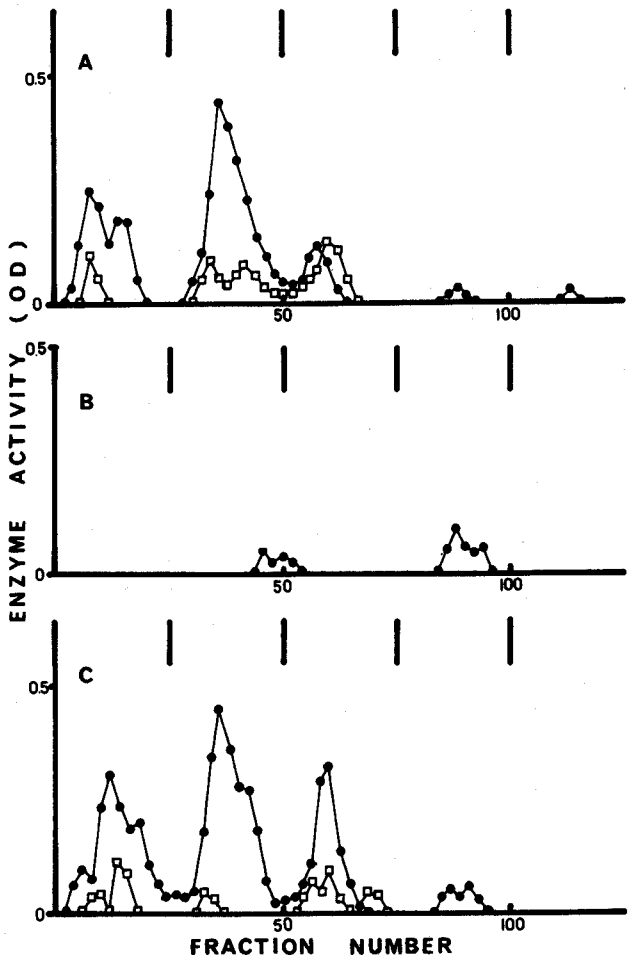

Fig.9 Resolution patterns on DEAE Sephadex A-50 column (1 x 50 *cm*) chromatography of (A) DFP-EDTA-treated Peak Ⅱ without incubation, (B) DFP-EDTA-Mg^{++}-treated Peak Ⅱ and (C) DFP-EDTA-Ca^{++}-treated Peak Ⅱ after incubation at 30℃ for 3 *daus*. The enzyme activities were determined after incubation time as specified in the legend for Fig. 6. Symbols; closed circles: s-CMCase activity at 660*mμ*, and squares: Avicelase activity at 660*mμ*.

with those of the control. In contrast, a relative Avicelase activity to s-CMCase one in this region more decreased than in the control. Thus, the patterns of Fig. 9C and Fig. 7C suggested that some proteolytic modification has occurred within Peak Ⅱ during incubation of DFP-EDTA-Ca^{++}-treated sample.

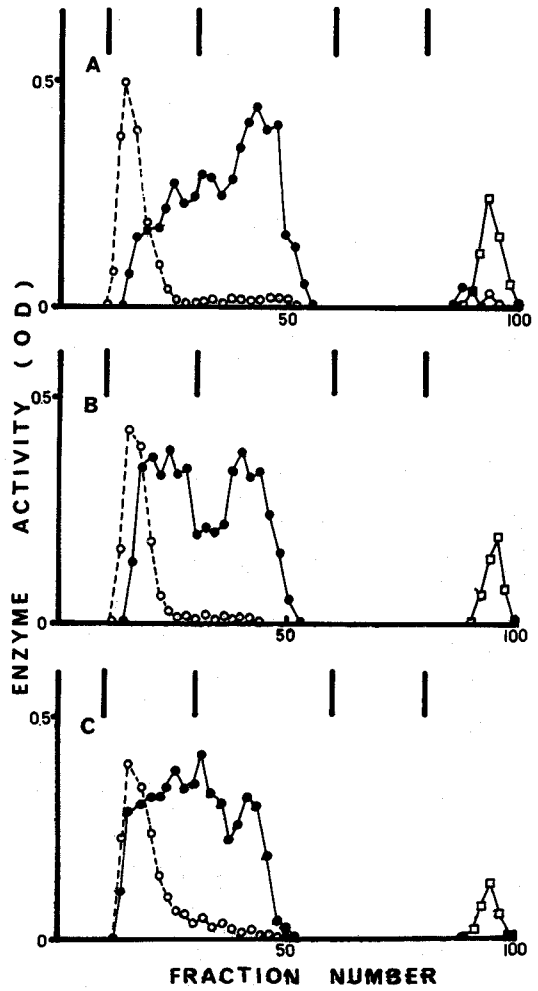

Fig. 10 Resolution patterns on DEAE Sephadex A-50 column chromatography of (A) DFP-EDTA-treated Peak Ⅲ without incubation, (B) DFP-EDTA-Mg^{++}-treated Peak Ⅲ and (C) DFP-EDTA-Ca^{++}-treated Peak Ⅲ after incubation at 30℃ for 3 *days*. The size of column and the incubation time for enzyme assays are respectively the same as described in Fig. 8. and 9. Symbols; closed circles: s-CMCase activity at 660*mμ*, squares: Avicelase activity at 660*mμ* and open circles: PNGase activity at 420 *mμ*.

The resolution pattern of DFP-EDTA-treated and not incubated Peak Ⅲ (Fig. 10A) was essentially similar to those of the untreated one (Fig. 2C), though relatively high activity of s-CMCase was detected in C-Ⅳ. Even after incubation with Mg^{++} or Ca^{++}, their elution patterns (Fig. 10B and C) not much differed from those of the control, except minor changes in the proportion among the s-CMCase activities of C-Ⅰ to C-Ⅳ.

Discussion

In the previously paper, changes of the chromatographic patterns of extracellular cellulases of *Trichoderma viride* were investigated with its cultural age [4]. and the multiplicity of cellulase components showed a tendency to increase with the culture period. These results suggested that the modification of the enzyme protein like a limited proteolysis was the possible cause for this phenomenon. In fact, at least one of the s-CMCase components in Peak Ⅱ obtained by Amberlite CG-50 column chromatography of a culture filtrate of *Trichoderma viride,* has alreaby been reported to be converted to another component in the same Peak by some agent seeming protease [10].

It was also proved that the multiple forms of cellulase in *Pseudomonas fluorescens* var. *cellulosa* have been generated from the modification of cellulase molecules one by one by the action of protease and/or carbohydrase [11]. Moreover, there are reports on the formation of isoenzyme-like protein species by proteolytic modification of some enzymes, such as yeast triose phosphate dehydrogenase [12]; bovine liver β-glucosidase [13] and rabbit liver fructose 1, 6-diphosphatase [14].

In the present work, it was revealed that the cellulase components in an aqueous extract from Cellulase Onozuka changed their chromatographic patterns by incubating it such as at 30℃ for some periods, and that the changes in electric charges of their molecules were more prominent than those in the molecular sizes. Furthermore, it was also found that this crude cellulase preparation contained acid, neutral and alkaline protease activities, among which a EDTA-sensitive, neutral protease was most potent.

In view of the changed resolution patterns on Amberlite CG-50 column chromatography after inhibition and reactivation of the neutral protease, which was contaminated in the cellulase preparation, it was suggested that there is a possibility of modification of Peak Ⅰ components into Peak Ⅲ ones or that of Peak Ⅱ components into Peak Ⅰ and Peak Ⅲ ones, whereas Peak Ⅲ components remain nearly unchanged. The latter cellulase components, therefore, seems to be the enzymic forms at the final step of several series of modification processes.

At any rate, the enhancement of irregularlity in the chromatographic patterns of cellulase components in these *in vitro* conversion experiments should be caused by the same factors as observed *in vivo* experiments as previously described [4]. It may be concluded, therefore, that the protease would be at least a probable factor participating in the modification of these cellulase components leading to the multiplicity of this enzyme.

Although nature of the original species of cellulase in *Trichoderma viride* and the precise conversion-sequence and its mechanism have been entirely unknown, evidences obtained at present work appear to support the assumptions

that some of the cellulase components of Peak Ⅱ or Peak Ⅰ may be the precursor molecule of Peak Ⅲ components and that most of Peak Ⅲ components may be the final products.

Acknowledgement

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References

1) Okada, G., K. Nisizawa, and H. Suzuki, *J. Biochem.*, **63**: 591, 1968.
2) Toda, S., H. Suzuki, and K. Nisizawa, *J. Ferment. Technol.*, **49**: 499, 1971.
3) Tomita, Y., H. Suzuki, and K. Nisizawa, *J. Ferment. Technol.*, **52**: 233, 1974.
4) Nakayama, M., 1975, *Memoirs of Osaka Kyoiku University, Ser* Ⅲ, **24**: 55, 1975.
5) Matsubara, H., and J. Feder, In: *The Enzymes*, vol. Ⅲ, 3rd ed. (Ed. Boyer, P. D.), p. 744, 1972.
6) Matsubara, H., and J. Feder, In: *The Enzymes*, vol. Ⅲ, 3rd ed. (Ed. Boyer, P. D.), p. 765, 1972.
7) Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**: 265, 1951.
8) Morihara, K., H. Tsuzuki, and T. Oka, *Arch. Biochem. Biophys.*, **123**: 572, 1968.
9) Nakanishi, K., *J. Biochem.*, **47**: 16, 1960.
10) Tomita, Y., H. Suzuki, and K. Nisizawa, *J. Ferment. Technol.*, **46**: 701, 1968.
11) Yoshikawa, T., H. Suzuki, and K. Nisizawa, *J. Biochem.*, **75**: 531, 1974.
12) Sasaki, R., E. Sugimoto, and H.Chiba, *Arch. Biochem. Biophys.*, **115**, 53, 1966.
13) Plapp, B. V., and R. D. Cole, *Biochemistry*, **6**, 3676, 1967.
14) Pontremoli, S., E. Melloni, and S. Traniello, *Arch. Biochem. Biophys.*, **147**, 762, 1971.