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https://doi.org/10.5109/23966

出版情報：九州大学大学院農学研究院紀要. 36 (1/2), pp.37-44, 1991-10. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 36 (1•2), 37-44 (1991)

Purification of Neutral Cellulase from a Mixture of Thermophilic Bacilli

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(Received June 17, 1991)

Neutral cellulase was purified from a culture of mixed thermophilic bacilli. After purification, its mol weight was found to be 42 KDa, composed of two subunits of 22 KDa and 20 KDa, by SDS-PAGE. The activity of this neutral cellulase was optimum at pH 7 and 60°C and stable at a pH range of 5 to 10 and up to 65°C. The cellulase could hydrolyze some insoluble celluloses although the activity was not so powerful.

INTRODUCTION

There are many microorganisms which have been classified as cellulolytic and some comprehensive reviews on cellulase production have been published, such as that of a recent paper by Kosaric et al. (1983). It was also known that thermophilic microorganisms often produce thermostable enzymes. When a mixture of 11 kinds of thermophilic bacilli (isolates from a sewage sludge compost) was cultured on a medium containing municipal sewage and filter paper, it was found that two types of thermophilic cellulases and three types of proteases appeared in the culture liquid. Some properties regarding the above cellulases (Kume and Fujio, 1991) and proteases (Fujio and Kume, 1991) were already reported by using crude enzyme solution.

The present paper is concerned with the purification and some properties of a neutral cellulase from the above mentioned mixed culture of 11 thermophilic bacilli.

MATERIALS AND METHODS

Cellulase source

Crude cellulase was obtained from the culture liquid of 11 bacteria by the same methods described by Kume and Fujio (1991), wherein a neutral and 3 alkaline cellulases, and 3 types of proteases have previously been identified.

Purification of cellulase

The crude cellulase was applied on a Sephacryl S-200 column (1.8 × 100 cm), equilibrated with potassium phosphate buffer (0.05M, pH 7.5). Then, the obtained cellulase fraction was re-applied on a hydroxylapatite column (2.6 × 30 cm), and eluted stepwisely with 0.02, 0.08, 0.16 and 0.40 M phosphate buffers (pH 7). The fractions with cellulase activity was collected and subsequently purified on a Sephadex G-100 column (1.8 X 100 cm), equilibrated with potassium phosphate buffer (0.05M, pH 7.5).

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Cellulase assay

Cellulase activity was determined on CMC as substrate by the assay of reducing sugar released from CMC by the method of Horikoshi et al. (1983). The activites on various substrates, i. e. filter paper (as FPase), Avicel (as Avicelase), laminaran and cotton were also measured by the methods described by Mandels et ***al.*** (1976). On those substrates, unless otherwise stated, cellulase activity was assayed at pH 7.0 (potassium phosphate buffer, pH 7, 0.1M) and at 60°C for 10 min.

Dependencies on **pH** and temperature

The following buffer (0.1~) systems were used to measure activity dependency on pH; CH_3COOH-CH_3COONa, pH 4-6; KH_2PO_4-K_2HPO_4, pH 6-8; KH_2PO_4-$Na_2B_4O_7$, pH8-9; and $Na_2B_4O_7$-NaOH, pH 9-12. For optimum pH measurements, cellulase activity was assayed at a desired pH (0.05M), ranging from 5-11. In the case of pH stability, 4 *ml* of cellulase solution was added into 4 *ml* of buffer (0.1~) at a desired pH and placed at 4°C for 24 h. Thereafter, cellulase activity was determined at pH 7 and 60°C for 10 min.

The optimum temperature of cellulase was determined at pH 7 at a desired temperature range of 40~75°C. In the case of temperature stability, the cellulase solution at pH 7 was placed at a desired temperature ranging from 40-75°C for 20 min. Thereafter, cellulase activity was assayed at pH 7 and 60°C.

Homogeneity and molecular weight

The homogeneity and molecular weight of purified cellulase was estimated with PAGE or SDS-PAGE (Weber and Osbone 1969). Protein bands were detected by staining with coomassie blue or silver stain (Merril, et ***al.*** 1981).

Dependencies on metal ions

A 0.1 *ml* of metal salt solution and 0.5 *ml* CMC solution (1%, w/v) in buffer (phosphate buffer, pH 7, 0.05~) were mixed with 0.1 ***ml*** cellulase solution (final metal concentration was adjusted to 25 mM) for KCl, MaCl and to 1 mM for $ZnSO_4$, $CuCl_2$, $CaCl_2$, $BaCl_2$, and PCMB). The cellulase activity was assayed with the desired metal ion.

Substrate specificity of purified neutral cellulase

In accordance with cellulase activity assay, the hydrolysis of various substrates (CMC, filter paper, Avicel, cotton and laminaran) by the neutral cellulase were determined.

Protein assay

Protein concentration was estimated by using Bio-Rad protein assay system (Bio-Rad Laboratories Japan) with gamma-globulin as a standard.

Chemicals and reagents

All chemicals and reagents were of analytical grade purchased from Nakarai Chemicals Co. Ltd. (Osaka, Japan).

RESULTS

Purification of cellulase

It has been already found by Kume and Fujio (1991) that the crude cellulase preparation contained two types of cellulases with optimum pHs of 7 and 10. Then, the activity profiles were determined at both pHs. Figure 1 shows an elution profile of the crude cellulase preparation by Sephacryl S-200 column chromatography. In accordance with the chromatogram, the cellulases were collected as Fraction I (tubes, 25-60) having both cellulase activity at pH 7.0 and 10.0 and Fraction II (tubes, 70-80) with the activity at pH 10.

Hydroxylapatite column chromatography of fraction I

Figure 2 shows the elution profile of fraction I by hydroxylapatite column chromatography, eluted stepwisely with 0.02, 0.08 and 0.16M, pH 7.0 phosphate buffer.

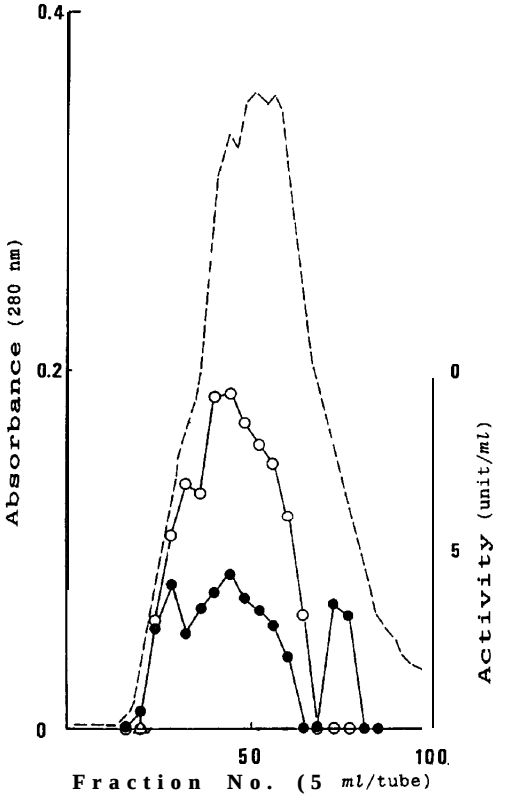

Fig. 1. Sephacryl S-ZOO gel filtration of crude cellulase

Crude cellulase was applied on a Sephacryl S-200 column (1.8 × 100 cm) equilibrated with 0.05 *M* phosphate buffer (pH 7.5) at a flow rate of 15ml/h. Cellulase activity was determined on CMC as substrate at 60 °C for 10min using 0.01 *M* phosphate buffer (pH 7 : **0)** or 0.01 *M* boric acid-NaOH buffer (pH 10 : ●).

The fraction (neutral cellulase fraction) eluted with 0.08 M phosphate buffer shows only one cellulase with the pH 7 activity, while other fractions (eluted with 0.04 and 0.16M of the above buffer) contained both pH 7 and 10 activities.

Sephadex G-100 column chromatography of pH 7 fraction

The neutral cellulase fraction was subjected to Sephadex G-100 column chromatography and Fig. 3 shows the elution profile. The fraction with pH 7 activity gave a single peak with the same protein peak. This peak was collected as the purified neutral cellulase.

Homogeneity and molecular weight of pH 7 cellulase

The homogeneity of neutral cellulase was confirmed with PAGE as shown by a single protein band obtained (Fig. 4-A, b). But subjecting the neutral cellulase to SDS-PAGE, two protein bands were obtained from it (Fig. 4-B, d). Comparing with

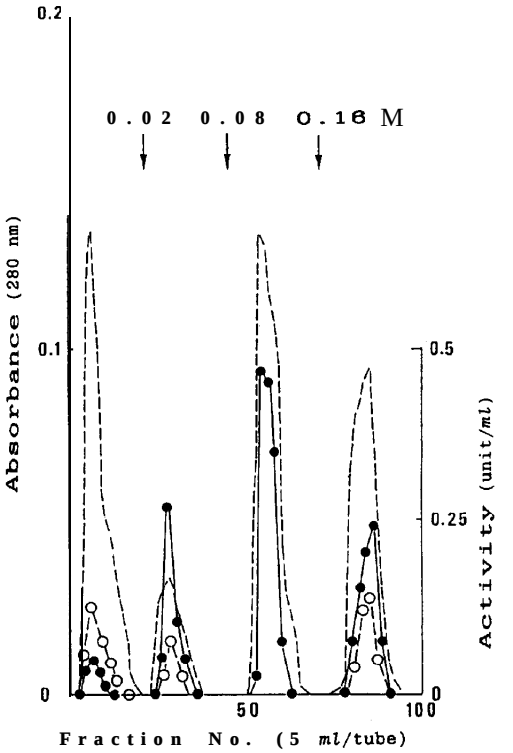

Fig. 2. Hydroxylapatite column chromatography

Hydroxylapatite column (2.6 × 30 cm) was washed with 0.02, 0.08, 0.16 and 0.40 *M* phosphate buffer (pH 7) at a flow rate of 10ml/h. Cellulase activity was determined on CMC as substrate at 60°C for 10min using 0.01 *M* phosphate buffer (pH 7 : ●) or 0.01 *M* boric acid-NaOH buffer (pH 10 : ○). Broken line indicates absorbance at 280 nm.

standard proteins of known molecular weight, the neutral cellulase gave a molecular weight of 42 KDa may be composed of two subunits of 22 KDa and 20 KDa.

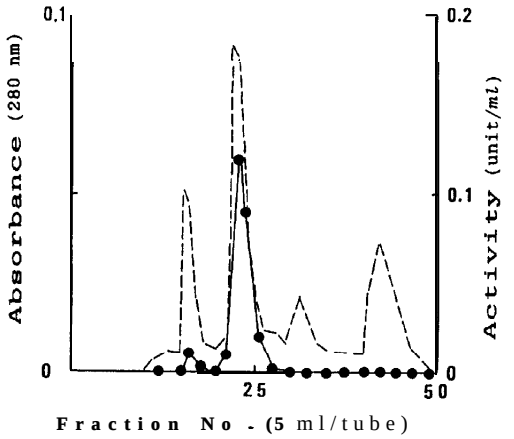

Fig. 3. Sephadex G-100 gel filtration of 0.08 *M* fraction of hydroxylapatite chromatography

Sephadex G-100 (1.8 **X** 100 cm) equilibrated with 0.05 *M* phosphate buffer (pH 7.5) at a flow rate of 15ml/h. Cellulase activity was determined on CMC as substrate at 60°C for 10min using 0.01 *M* phosphate buffer (pH 7: ●). Broken line indicates absorbance at 280 nm.

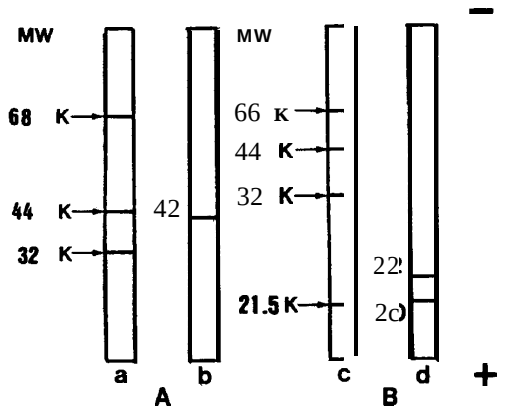

Fig. 4. PAGE and SDS-PAGE of purified cellulase

PAGE (A) containing 7.5% polyacrylamide was done on lane a (standard proteins of BSA, 68 ; ovalbumin, 44 ; carbonic anhydrase, 32 ; and soybean trypsin inhibitor, 21, 5 KDa) and b (purified cellulase).

SDS-PAGE (B) containing 11% polyacrylamide and 0.1%

SDS was done on lane c (the same standard proteins as lane a) and d (purified cellulalse).

Summary of pH 7 cellulase purification

Table 1 summarizes the purification results regarding neutral cellulase. Based on the specific activity, the cellulase was purified by 16 folds based on the crude cellulase.

Effect of pH and temperature on neutral cellulase activity

Figure 5 (A and B) shows the pH and temperature dependencies of the neutral cellulase (purified). The optimum reaction pH and temperature were at pH 7 and at 60°C, respectively while the pH and temperature stabilities were at a pH range of 5–10 and up to 65°C, respectively.

Effect of metal ions on the activity and substrates specifity of neutral cellulase

Table 2 summarizes the effect of various metal ions on the activity of neutral cellulase. The cellulase activity was increased with Ca^{+2} (+12%), Ba^{2+} (+17%), Na^{+} (+29%) and K^{+} (+10%) but was decreased with Hg^{2+} (−21%), Zn^{2+} (-19%) and Pb^{2+} (− 16%).

Table 3 shows the neutral cellulase activities on various substrates. The cellulase could hydrolyze every substrate used. As shown in Table 3, neutral cellulase could decompose solid cellulose. However, the activity on cotton was considerably low comparing with that on CMC or laminaran as soluble cellulolytic substrates.

DISCUSSION

Eleven bacilli were isolated from a sewage sludge compost (Kume and Fujio, 1990). As a result of a survey of sewage sludge Iytic enzymes, a neutral and 3 alkaline cellulase, and 3 type of proteases were produced in culture liquid from 11 thermophilic bacilli (Kume and Fujio, 1991 ; Fujio and Kume, 1991). Among those cellulases, only neutral cellulase could be purified as a single protein and characterized in this paper. Comparing this neutral cellulase with other cellulases (crude cellulase) from mixed

Table 1 Summary of neutral cellulase purification

Step	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)
Culture liquid	1,000	1,280	330	0.26
Ethanol ppt dialyzate	270	500	149	0.30
Sephacryl s-200	100	34.4	26	0.76
Hydroxyl-apatite	45	1.8	8	4.50
Sephadex G-100	15	1.2	5	4.25

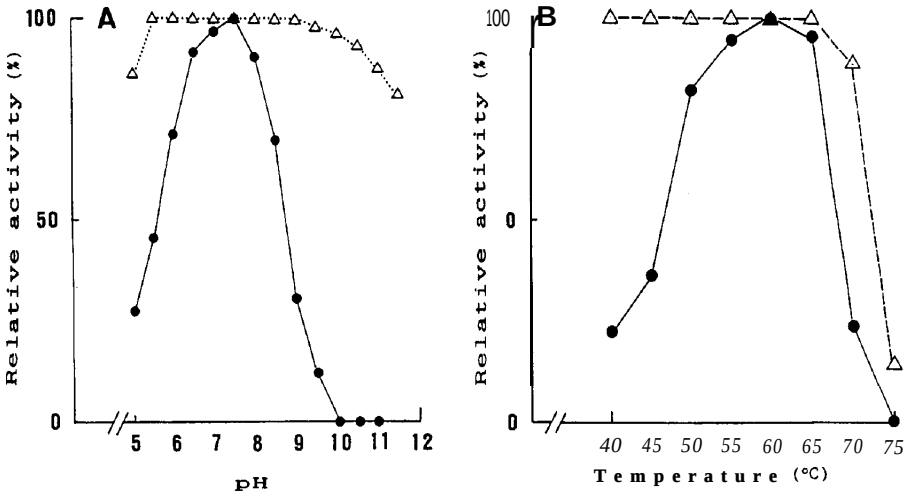

Fig. 5. Effects of pH (A) and temperature (B) on purified cellulase activity

(A) : Cellulase activity (●) was determined on CMC as substrate using 0.05 *M* phosphate buffer (pH 6-8), 0.05 *M* KH_2PO_4-$Na_2B_2O_7$ buffer (pH 8-9) and 0.05 *M* $Na_2B_4O_7$-NaOH buffer (pH 9912). Cellulase activity (△) was determined after holding at 4 °C for 24 h in the above buffers.

(B) : Cellulase activity (●) was determined on CMC as substrate at the indicated temperature for 10 min.

Cellulase stability (△) was determined after holding at the indicated temperature for 20 min.

Table 2 Effect of metal ions on neutral cellulase activity

Metal ion	Concentration (mM)	Relative neutral Cellusase activity (%)
None		100
KC1	25	110
NaCl	25	129
ZnCl	1	81
Pb $(CH_3COO)_2$	1	84
$CuCl_2$	1	116
$CaCl_2$	1	112
$BaCl_2$	1	117
$MgCl_2$	1	98
PCMB'	1	79

1) p-Chloromercurricbenzenesulphonic acid.

bacterial culture (Kahn, 1977 ; Kahn et al. 1981), it seems that the present cellulase could be more tolerable to higher temperature. In comparison with other bacterial cellulases (Beguin *et al.*, 1977 ; Reynolds et *al., 1986* ; Sissons et *al.*, 1987), the present neutral cellulase shows some similarity to that of *Clostridium thermocellum* or

Table 3 Purified neutral cellulase activity on various substrates

Substrate	Activity at pH 7 (unit)	Activity/CMC activity (%)
CMC'	69.6	100
Filter paper	0.27	0.39
Avicel	0.16	0.23
Cotton	0.03	0.04
Laminaran	0.42	0.60

1) Carboxylmethylcellulose
1 unit = 1μmol glucose released per h

Cellulomonas with regards to their optimum pH and temperature while the characteristics of any other bacterial cellulases are considerably different.

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