Purification and Properties of the Endo-1,4- β -glucanase from *Bacillus subtilis*

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Carboxymethylcellulase (endo-1,4- β -glucanase; EC 3.2.1.4) was purified from the culture filtrate of *Bacillus subtilis* AU-1 by $(\text{NH}_4)_2\text{SO}_4$ precipitation, Avicel affinity chromatography, DEAE Sephadex G-75 chromatography and Sulphopropyl Sephadex C-50 chromatography. The enzyme was purified 36-fold and had an M_r of 23000 as determined by gel filtration on a Sephadex G-75 column. The pH optimum of the purified enzyme was 5.5; the enzyme was stable at 65 °C. Activity of the purified enzyme was significantly reduced by Cu^{2+} , Pb^{2+} , Sn^{2+} , Ag^+ , Hg^{2+} and Fe^{2+} , but was increased by 139.5% in the presence of Co^{2+} . Inhibition studies indicated that the purified enzyme was either a metalloprotein or required certain metal ions for activation/stabilization; that iron was not a prosthetic group of the enzyme; that a tryptophanyl group was not involved in enzyme action; and that reduced thiol groups were required for enzyme activity and involved in the active site of the enzyme. The K_m of the purified enzyme for carboxymethylcellulose was 4 mg ml⁻¹, and the $V_{\max}$ for carboxymethylcellulose hydrolysis was 0.42 mg D-glucose min⁻¹ (mg protein)⁻¹.

INTRODUCTION

Cellulase has been used for industrial saccharification of cellulosic materials (Linko, 1977; Ryu & Mandels, 1980). Although many cellulolytic micro-organisms have been studied as potential sources of cellulase, attention has focused on the fungus *Trichoderma reesei* (Mandels, 1982), the thermophilic cellulolytic species of *Clostridium* (Lee & Blackburn, 1975; Ng *et al.*, 1977; Shinmyo *et al.*, 1979) and *Thermomonospora* (Moreira *et al.*, 1981), and the mesophilic species of *Cellulomonas* (Langsford *et al.*, 1984), *Cytophaga* (Chang & Thayer, 1977) and *Acetivibrio* (Saddler & Khan, 1980). Most of the information on the mechanism of cellulase activity has come from studies on purified cellulase from these micro-organisms (Beguín & Eisen, 1978; Shoemaker & Brown, 1978; Halliwell, 1979; Ng & Zeikus, 1981; MacKenzie & Bilous, 1982).

Many species of the bacterial genus *Bacillus* produce extracellular enzymes of industrial importance (Priest, 1977), including cellulase (Knösel, 1971; Fogarty & Griffin, 1973; Zemek *et al.*, 1981); there is, however, little information about the properties of the purified cellulases produced by these species. The purpose of the present study was to purify and characterize a major endo-1,4- β -glucanase (carboxymethylcellulase) of *Bacillus subtilis* AU-1.

METHODS

Organism and culture conditions. *Bacillus subtilis* AU-1 is a mutant obtained by treating *B. subtilis* T-14 with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The parent strain, *B. subtilis* T-14, was originally isolated from cotton waste compost, and produces carboxymethylcellulase (CMCase) when grown in a liquid mineral medium containing yeast extract and D(+) raffinose as the major carbon source (Au & Chan, 1986).

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Abbreviations: CMCase, carboxymethylcellulase; DNS, dinitrosalicylic acid.

B. subtilis AU-1 was grown at 50 °C in a liquid medium with the following composition: D(+)-raffinose, 2 g; NH₄Cl, 1 g; NaCl, 0.6 g; MgSO₄·7H₂O, 0.8 g; K₂HPO₄, 5 g; KH₂PO₄, 10 g; CaCl₂·2H₂O, 0.15 mg; MnSO₄·4H₂O, 0.5 mg; CoCl₂·6H₂O, 2 mg; FeSO₄·7H₂O, 0.1 mg; CuSO₄·5H₂O, 0.2 mg; ZnSO₄·7H₂O, 0.5 mg; yeast extract, 5 g; Casamino acids, 5 g; proteose peptone, 5 g; and 1000 ml distilled water (pH 6.0).

Enzyme assays. CMCase (EC 3.2.1.4) was assayed according to Stewart & Leatherwood (1976). The reaction mixture contained 1 ml 1% (w/v) carboxymethylcellulose (Sigma) in 0.05 M-potassium phosphate buffer (pH 6.0) and 1 ml of clarified culture supernatant. The mixture was incubated at 50 °C for 30 min, and the reaction was stopped by the addition of 2 ml 3,5-dinitrosalicylic (DNS) acid reagent (Miller, 1959). The tubes were covered and placed in boiling water for 10 min, and the absorbance values at 575 nm were then read against blanks containing equivalent amounts of boiled enzyme. Glucose equivalents were obtained by reference to a standard curve. One unit (U) of CMCase activity was defined as the amount of enzyme required to liberate 1 µg reducing sugar as D-glucose in 30 min.

Avicelase (EC 3.2.1.74) was also assayed by the DNS method. The reaction mixture was the same as that for the CMCase assay except that the substrate used was 1 ml of 1% (w/v) Sigmacell type 20 (Sigma). One unit (U) of enzyme activity was defined as the amount of enzyme which released 1 µg reducing sugar in 30 min.

Protein determination. Protein was determined by the Lowry method with bovine serum albumin as a standard.

Enzyme purification. *B. subtilis* AU-1 was grown at 50 °C in the above culture medium with shaking for 20 h. The culture was centrifuged at 10000 g for 15 min at 4 °C, and the supernatant containing CMCase activity was collected. Solid (NH₄)₂SO₄ was slowly added to the supernatant and finally the protein precipitate was collected by centrifugation at 9000 g for 30 min. The proteins were resuspended in the minimum volume of 20 mM-ammonium acetate buffer (pH 5.0). The enzyme solution was then dialysed against the same buffer for 24 h at 5 °C.

An Avicel affinity column (18 × 800 mm) was prepared according to Gum & Brown (1976); it contained Sigmacell type 20 (Sigma) equilibrated in 50 mM-citric acid/100 mM-Na₂HPO₄ buffer (pH 5.0). Lyophilized fractions from the (NH₄)₂SO₄ precipitation (30% saturation) in the same buffer were applied to the column and eluted at 5 °C with the same buffer at a flow rate of 8 ml h⁻¹. The eluted fractions containing CMCase activity were pooled and dialysed against distilled water for 12 h. The dialysed enzyme solution was then applied to a column (1.0 × 50 cm) of DEAE Sephadex G-75 at 5 °C. The gel was equilibrated with 50 mM-citric acid/100 mM-Na₂HPO₄ buffer (pH 5.0) containing 0.03% sodium azide. At a flow rate of 4 ml h⁻¹, fractions (1.5 ml) were collected, and those fractions with high CMCase activity were combined and dialysed against distilled water for 20 h. This concentrated enzyme solution was applied to a Sulphopropyl Sephadex C-50 column (2.5 × 30 cm) that was eluted with a continuous gradient of NaCl (0 to 0.4 M) in 50 mM-sodium citrate buffer (pH 3.5) at 5 °C. A constant flow rate of 14 ml h⁻¹ was used, and 2 ml fractions were collected. After dialysis against distilled water for 20 h, the enzyme fraction was subjected to purity determination by PAGE.

Electrophoresis under nondenaturing conditions was done in a 7.5% (w/v) polyacrylamide slab gel with 1.5 M-Tris/HCl buffer (pH 6.8). The enzyme (20 µg) was subjected to electrophoresis in the presence of 0.1% SDS at a current of 30 mA. The gels were stained for protein with Coomassie brilliant blue R-250 and destained in methanol/acetic acid/water (5:2:5, by vol.).

Determination of *M_r*. The *M_r* of the CMCase was estimated by gel filtration on a Sephadex G-75 column (1.0 × 50 cm) according to the method of Andrews (1964). The *M_r* standards used were haemoglobin (BDH; *M_r* 64500), α-amylase (Sigma; *M_r* 48500), subtilisin (Sigma; *M_r* 26700), and lysozyme (Merck; *M_r* 14300).

Chromatography of hydrolysis products. The purified CMCase was incubated with Whatman no. 1 filter paper (20 mg ml⁻¹), Sigmacell type 20 (20 mg ml⁻¹), or 0.2% carboxymethylcellulose at 50 °C for 30 min. Then 0.1 ml of the enzyme digest was added to 1 ml ice-cold ethanol/acetone (1:2, v/v) to precipitate protein. The supernatant was obtained by centrifugation at 10 000 g for 10 min, and residual ethanol and acetone were removed under vacuum. Then the hydrolysis products were analysed by ascending chromatography of 10 µl samples on precoated Silica Gel 60 plates (Merck), using an ethyl acetate/methanol/acetic acid/water (12:3:3:2, by vol.) solvent system. Reference sugars used were D-glucose, D(+)-cellobiose and D-fructose. The chromatogram was developed with 4-aminobenzoic acid reagent (2 g 4-aminobenzoic acid, 1.64 ml 88%, w/v, phosphoric acid and 36 ml acetic acid in 40 ml distilled water).

Substrate specificity of the purified CMCase. This was determined according to a modification of the method of Shepherd *et al.* (1981). Each substrate (see Table 2) was preincubated with 0.9 ml 0.05 M-citric acid/0.1 M-Na₂HPO₄ buffer (pH 6.0) at 50 °C for 10 min, and then 0.1 ml of enzyme in the same buffer was added and incubation was continued for another 30 min before CMCase activity was assayed.

Effects of pH and temperature on the purified CMCase. The optimum pH for CMCase activity and the effect of temperature on activity were determined by adding 0.1 ml purified enzyme solution to 0.25 ml each of the following buffers: 0.1 M-citric acid/0.2 M-Na₂HPO₄ (pH 3.0 to 8.0), and 0.2 M-glycine/0.2 M-NaOH (pH 8.0 to 10). Then 0.25 ml of 20 mg carboxymethylcellulose ml⁻¹ in deionized water was added; the mixture was incubated at between 20 and 80 °C for 30 min, and enzyme activity was assayed.

The pH stability of the enzyme was studied by preincubating 0.1 ml enzyme solution with 0.1 ml buffer (0.1 M-citric acid/0.2 M-Na₂HPO₄ buffer for pH 3.0 to 8.0, and 0.2 M-glycine/0.2 M-NaOH buffer for pH 8.0 to 10) at 50 °C

for 10 min. Then 0.8 ml of 20 mg carboxymethylcellulose ml^{-1} in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer (pH 6.0) was added in order to assay for residual CMCase activity at 50 °C.

Temperature stability of the purified CMCase was evaluated by incubating 0.1 ml enzyme solution in 0.1 ml 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer (pH 6.0) at various temperatures for 60 min; the residual CMCase activity was then assayed.

Effects of cations and various reagents on enzyme activity. Individual cations (see Table 3) and reagents (see Table 4) in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer (pH 6.0) were added to a reaction mixture containing purified CMCase and carboxymethylcellulose in the same buffer, and the CMCase activity was assayed.

RESULTS

Initial $(\text{NH}_4)_2\text{SO}_4$ fractionation showed that 92% of the enzyme activity was recovered between 10 and 40% $(\text{NH}_4)_2\text{SO}_4$ saturation, and highest CMCase activity (3724.8 U) was associated with the 30 to 40% saturated $(\text{NH}_4)_2\text{SO}_4$ pellet; therefore only pellets from the latter range were used for chromatography on the Avicel affinity column. The affinity chromatography produced one major protein peak which contained 70.6% of the CMCase activity (Fig. 1). Two peaks of Avicelase activity were also observed; however the activities of these two peaks were relatively low (Fig. 1).

The pooled fractions containing the CMCase activity from the Avicel affinity column were applied to the DEAE Sephadex G-75 column; this yielded one major peak (peak C) and two minor peaks (peaks A and B) of CMCase activity (Fig. 2). The major peak contained 87%, and the two minor peaks respectively 10% and 7% of the total CMCase activity applied to the column. Only fractions comprising peak C were pooled and further purified by Sulphopropyl Sephadex C-50 chromatography. Two protein components were obtained by elution with a linear gradient of NaCl, and the major CMCase activity coincided with the smaller protein peak (Fig. 3). The CMCase activity contained in the other protein peak was too low for further purification. Fractions of the major CMCase peak were pooled and subjected to PAGE, which revealed a single band of protein. A 36-fold purification of CMCase was achieved in triplicate experiments (Table 1).

Kinetic study of the purified CMCase showed that the K_m for carboxymethylcellulose was 4 mg ml^{-1} , and the $V_{\max}$ for carboxymethylcellulose hydrolysis was 0.42 mg D-glucose min^{-1} (mg protein) $^{-1}$ as determined from the Lineweaver-Burk plot.

The M_r of the purified enzyme was estimated to be 23 000.

Fig. 1. Elution profile of CMCase and Avicelase on an Avicel affinity chromatography column. The column (18 × 800 mm) was equilibrated with 50 mM-citric acid/100 mM- Na_2HPO_4 buffer (pH 5.0). Lyophilized fractions from the $(\text{NH}_4)_2\text{SO}_4$ precipitation in the same buffer were applied to the column, which was then eluted with the equilibration buffer at a flow rate of 4 ml h^{-1} . Fractions (1.5 ml) were collected and assayed: ●, protein; □, CMCase activity; ○, Avicelase activity.

Fig. 2. Separation of the CMCase activities on a DEAE Sephadex G-75 column. Dialysed fractions containing CMCase activity obtained after Avicel affinity chromatography were applied to the column (1.0×50 cm) which was equilibrated with 50 mM-citric acid/100 mM- Na_2HPO_4 buffer (pH 5.0) containing 0.03% sodium azide. Fractions (1.5 ml) were collected and assayed: ○, CMCase activity; ●, protein.

Fig. 3. Separation of CMCase peak C components on a Sulphopropyl Sephadex C-50 column. The column (2.5×30 cm) was equilibrated with 50 mM-sodium citrate buffer (pH 3.5). The dialysed fractions of CMCase peak C obtained after DEAE Sephadex G-75 column chromatography were applied to the column, which was eluted with a continuous gradient of 0 to 0.4 M-NaCl (□) in 50 mM-sodium citrate buffer (pH 3.5). Fractions (2 ml) were collected and assayed: ○, CMCase activity; ●, protein.

Table 1. *Purification of the CMCase from B. subtilis AU-1*

Procedure	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Yield (%)	Purification (-fold)
Cell-free supernatant	100	4174.35	41.75	100	—
(NH_4) ₂ SO ₄ precipitation	62	3724.8	60	89	1.4
Affinity chromatography	29.5	2947	100	71	2.4
DEAE Sephadex G-75 chromatography	2.7	2223.5	823.5	53	19.7
Sulphopropyl Sephadex C-50 chromatography	1.1	1654.5	1504	39.6	36

Table 2. *Substrate specificity of CMCase*

Each substrate was preincubated with 0.9 ml 0.05 M-citric acid/0.1 M- Na_2HPO_4 buffer (pH 6.0) at 50 °C for 10 min before addition of 0.1 ml enzyme in the same buffer. The mixture was further incubated for 30 min before CMCase activity was assayed.

Substrate	Concn (mg ml ⁻¹)	CMCase activity (U ml ⁻¹)
Carboxymethylcellulose	6	79.5
Sigmacell type 20	20	0.74
α -Cellulose	20	1.56
Cellulose-azure	20	8.1

Table 3. *Effect of cations on CMCase activity*

Each compound, dissolved in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer (pH 6.0), was added to the standard assay system and assayed at 50 °C. 100% activity is equivalent to 184 U ml⁻¹.

Cation	Concn (mM)	Relative activity (%)	Cation	Concn (mM)	Relative activity (%)
Control		100	Ca^{2+} (CaCl_2)	10	132
NH_4^+ (NH_4Cl)	200	12.96	Cu^{2+} (CuSO_4)	1	23.8
Ag^+ (AgNO_3)	0.1	48.91	Pb^{2+} (PbCl_2)	1	48
Na^+ (NaCl)	100	137.33	Zn^{2+} (ZnSO_4)	1	75
K^+ (KCl)	100	140	Mg^{2+} (MgSO_4)	10	143
Hg^{2+} (HgCl_2)	0.1	51.6	Mn^{2+} (MnCl_2)	1	164
Fe^{2+} (FeSO_4)	1	50	Co^{2+} (CoCl_2)	1	239.5
Sn^{2+} (SnCl_2)	0.1	56.5			

Substrate specificity and hydrolysis products of the purified CMCase. The purified enzyme displayed highest activity towards carboxymethylcellulose and significantly lower activity toward α -cellulose and cellulose-azure (Table 2). Notably, the enzyme was five times more active towards dyed cellulose than towards α -cellulose. In addition, the enzyme showed negligible activity towards Avicel (Sigmacell type 20).

Hydrolysis of carboxymethylcellulose by the action of the purified CMCase resulted in the formation of glucose and cellobiose. No soluble sugars were detected as hydrolysis products from Whatman no. 1 filter paper and Sigmacell type 20.

Effects of temperature and pH on the activity and stability of purified CMCase. The CMCase was active up to 70 °C, and was stable for 60 min up to 65 °C; above this temperature activity fell rapidly.

The optimum pH for the purified enzyme was 5.5; at 50 °C the purified enzyme was stable at pH 5.0 and over 95 % of activity was retained at pH 5.5 to 8.0.

Effects of cations and various reagents on the activity of purified CMCase. Significant inactivation of the enzyme was observed with Cu^{2+} , Pb^{2+} , Sn^{2+} , Ag^+ , Hg^{2+} and Fe^{2+} (Table 3). On the other hand, the addition of Co^{2+} increased the enzyme activity by 139.5% over that of the control (no added cations).

Among the various reagents tested (Table 4) the reducing agent dithiothreitol, and the amines, polyols and histamine, stimulated enzyme activity while all three thiol-group-binding agents and sodium sulphite inhibited CMCase activity. In addition, EDTA inhibited enzyme activity by 17 % and sodium azide did not affect enzyme activity.

DISCUSSION

It is generally agreed that microbial cellulases are difficult to purify (Tong *et al.*, 1980; Ng & Zeikus, 1981). Fractionation of the crude cellulolytic activity produced by *B. subtilis* AU-1 into as many different components as possible was not the objective of the present study. Rather, the purification scheme was designed for the isolation of one homogeneous endo-1,4- β -glucanase

Table 4. *Effect of various reagents on CMCase activity*

Each reagent dissolved in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer (pH 6.0) was added to the standard assay system and assayed at 50 °C. 100% activity is equivalent to 192 U ml⁻¹.

Reagent	Concn (mM)	Relative activity (%)
Control		100
Reducing agent		
Dithiothreitol	5	128.8
Thiol-group binding agents		
Mercuric chloride	0.1	51.6
Sodium arsenite	0.1	58.5
Disulphide bond cleaving agent		
Sodium sulphite	0.1	72
Amines		
Glycine	50	170.9
L-Histidine	50	132.6
Polyols		
Arabitol	50	151.7
Erythritol	50	157
Glycerol	50	139
Tryptophanyl group binding agent		
Histamine	20	124
Metallo-enzyme inhibitors		
EDTA	50	83.4
Sodium azide	5	99.3

(CMCase) from the crude enzyme preparation. In order to avoid loss of activity, the purification processes did not include DEAE cellulose chromatography. Since highest CMCase activity was found to associate with the 30 to 40% saturated $(\text{NH}_4)_2\text{SO}_4$ pellet, only enzyme from this fraction was collected and used for further purification. A similar observation has been reported for the CMCase isolated from *Thermoascus aurantiacus* (Tong *et al.*, 1980). The high percentage (70.6%) of protein recovery from the Avicel affinity chromatography was probably due to the fact that the CMCase, as the major component of the enzyme preparation, has little affinity for Avicel (Sigmacell type 20). The observation of a single peak of high CMCase activity and two peaks of relatively low Avicelase activity supports the view that CMCase is the major component of the cellulase system in *B. subtilis* AU-1.

Chromatography on DEAE Sephadex G-75 revealed three peaks (A, B and C) of CMCase activity, and subsequent effort was spent to purify the CMCase from peak C which comprises 87% of the total CMCase activity applied to the column. Sulphopropyl Sephadex C-50 column chromatography further separated peak C activity into two protein components, of which the peak containing almost all the CMCase activity was confirmed to comprise a single protein species as evidenced by its appearance as a single protein band on PAGE.

The CMCase of *B. subtilis* AU-1 has a smaller M_r than other purified bacterial CMCases (Beguin & Eisen, 1978; Ng & Zeikus, 1981; Groleau & Forsberg, 1983). The K_m (4 mg ml⁻¹) for carboxymethylcellulose of the purified CMCase is similar to that of *Thermoascus aurantiacus* (Tong *et al.*, 1980), and higher than those of *Trichoderma reesei* (Shoemaker & Brown, 1978) and *Cytophaga* spp. (Chang & Thayer, 1977).

In general, cellulases have high temperature optima when compared with other enzyme systems. The purified CMCase of *B. subtilis* AU-1 remained totally active up to 70 °C, which is higher than reported for *Clostridium thermocellum* (Ng & Zeikus, 1981), *Aspergillus niger* (Hurst *et al.*, 1977) and *Humicola lanuginosa* (Olutiola, 1982), and is comparable to temperature optima of cellulases from the thermophiles *Thermoascus aurantiacus* (Tong *et al.*, 1980) and *Thermomonospora* sp. (Hägerdal *et al.*, 1980).

The hydrolysis of carboxymethylcellulose by the purified CMCase produced glucose and cellobiose. This differs from the report by Chang & Thayer (1977) on *Cytophaga* cellulase, which

produced only glucose from carboxymethylcellulose, and from *Pseudomonas* cellulase, which produced galactose, mannose, fucose and glucosamine (Yamane *et al.*, 1970).

Our results suggest that reduced thiol groups are required for CMCase activity in *B. subtilis* AU-1, and that these groups are involved in the active site of the enzyme. Similar results have been reported for the cellulase of *Acetivibrio cellulolyticus* (MacKenzie & Bilous, 1982) and *Humicola lanuginosa* (Olutiola, 1982). It is conceivable that the inhibition of CMCase activity by sodium sulphite is due to partial damage or alteration of the molecular configuration of the enzyme by cleavage of the disulphide linkages present in the enzyme molecule. It has been reported that inhibition of protein activity by compounds containing a protonated imidazole ring such as histamine was at least partially due to the formation of a charge-transfer complex with the tryptophanyl group of the proteins (Shinitzky *et al.*, 1966). The fact that histamine did not inhibit CMCase activity might therefore imply that a tryptophanyl group is not involved in the enzyme action. In addition, our results on the effects of EDTA and sodium azide on the purified enzyme imply that (1) iron is not a prosthetic group of the enzyme, and (2) the enzyme is a metallo-protein or it requires certain metal ions for activation and/or for stabilization. The CMCase purified in the present study represents only a portion of the total endoglucanase activity of *B. subtilis* AU-1.

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