

Full Length Research Paper

# Purification and properties of carboxymethylcellulase (CMCase) from a cellulose-decomposing bacterium *Sporocytophaga* sp. JL-01

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***Sporocytophaga* sp.**, a Gram-negative aerobic cellulose decomposing bacterium, is capable of gliding on the surface of solid medium. A new endoglucanase, carboxymethylcellulase (CMCase1), was purified from an aerobic bacterium *Sporocytophaga* sp. JL-01. The molecular weight of the enzyme was 82 kDa as determined by SDS Polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH was 5.0 and optimum temperature was 50°C under experiment conditions. The Km of CMCase1 was 9 mgmL<sup>-1</sup> and Vmax was 27.3 ug min<sup>-1</sup>mg<sup>-1</sup> protein. The Zn<sup>2+</sup> could inhibit the activity of this enzyme at ion concentration of 5 mM. RP-HPLC revealed that aromatic amino acid, acidic amino acid, and basic amino acid accounted for 10.5, 24.5 and 7.0%, respectively. The far-UV circular dichroism (CD) spectrum showed that the amount of  $\beta$ -sheet was decreased with the temperature changed from 25 to 75°C.

**Key words:** *Sporocytophaga*, carboxymethylcellulase, enzyme characteristics, circular dichroism spectrum.

## INTRODUCTION

Cellulose, a principal component of plant cell walls, consists of linear polymers of  $\beta$ -1, 4-linked glucose molecules that are organized into higher order fibrillar structures. As the most abundant biomass in nature, its decompositions do not only plays a key role in the carbon cycle of nature, but also provides a great potential for application, most notably for biofuel production (Lynd et al., 2002, 2005, 2008).

Microbial degradation of cellulose involves in a complex interplay between different cellulolytic enzymes. Among them, it has been widely accepted that three types of cellulases including endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and  $\beta$ -glucosidases (EC 3.2.1.21) act synergistically to convert cellulose to glucose (Lynd et al., 2002). A broad range of microorganisms causing these activities either in

separated enzymes or in multi-protein complexes has been described (Bayer et al., 2004; Steenbakkens et al., 2003; Warnecke et al., 2007).

The degradation of cellulose is exclusively carried out by anaerobic bacteria, aerobic bacteria and many fungi. Most of our understanding on cellulose degradation comes from the study of the mesophilic fungi and anaerobic thermophilic cellulolytic bacteria (Lynd et al., 2002). Relatively few aerobic cellulolytic bacteria have been reported in comparison with anaerobic cellulolytic bacteria.

*Myxobacteria* are the common organisms as they occur in large numbers in a variety of habitats: soil, rotting wood, decaying plant material and dung of herbivorous animals (Bayer et al., 2004; Berg et al., 1972). *Sporocytophaga* is a typical representative of *Myxobacteria*. In our recent studies, a novel cellulolytic gliding bacterium *Sporocytophaga* sp. JL-01 was isolated from corn growth soil in Jilin province, China. This organism could decompose the ball milled cellulose CF11 and filter paper, and glide on the cellulose CF11 solid

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medium surface. It has a complex life cycle including two main morphologies, filamentous bacillus and microcyst, and it is able to utilize crystalline cellulose as sole carbon and energy source (Liu et al., 2003; Grace, 1951). Although *Sporocytophaga* has been found to be an efficient aerobic degrader of crystalline celluloses such as filter paper, cellulose CF11 and cotton wool (Stanier, 1942), detection of cellulases including endoglucanase, exoglucanase, and  $\beta$ -glucosidase in the extracellular supernatant suggests that this bacterium can produce a low extracellular CMCCase activity (Chang and Thayer, 1977; Xie et al., 2007). The CMCCase characteristics might give us some novel proofs. In this paper, we will introduce the purification and some properties of CMCCase1 from *Sporocytophaga* sp. JL-0.1.

## MATERIALS AND METHODS

### Organism and cell culture

The cellulose-decomposing bacteria were isolated from soil sample of Jilin province, China, on bilayer cellulose powder CF11 plates. The up-layer medium was prepared with nutrient salts medium, 1% cellulose powder CF11 (milled by glass ball) and 1.5% agars, but the bottom medium were water and 2% agar. The nutrient salts medium contained (g/L):  $\text{NaNO}_3$ , 2.0;  $\text{K}_2\text{HPO}_4$ , 1.5;  $\text{MgSO}_4$ , 0.3;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.0016;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0014 and  $\text{CoCl}_2$  0.0005, the pH was adjusted to 7.0-7.2 (Mandels, 1975). One pure culture, *Sporocytophaga* sp. JL-01, was obtained for the present study.

### The production of CMCCase

*Sporocytophaga* sp. JL-01 was inoculated on a filter paper medium (Whatman No.1, 10 × 80 mm on nutrient salts medium with 1.5% (w/v) agar) in tubes 37°C for 4 days, and then it was washed with sterilized nutrient salts medium 10 ml. The suspension was inoculated in 250 ml flask, which contained 100 ml sterilized nutrient salts medium with 1% filter paper (5 × 5 mm). The flasks were incubated at 37°C for 1, 2, 3, 4, 5 and 6 days, on a shaker at 120 rpm. The samples were harvested and centrifugated at 14000 g, for 20 min, at 4°C. The supernatant was used as source of extracellular enzymes and the deposit used as intact cell was washed by diluted water twice. CMCCase activity was determined.

### Assay methods

CMCCase activity was measured by mixing 1 ml appropriately diluted enzyme solution with 1 ml 1% (w/v) viscosity CMCNa<sub>2</sub> solution in 50 mM acetate buffer, pH 5.0, and incubating at 50°C for 30 min. The reducing sugar released in the reaction mixture was measured by the dinitrosalicylic acid method (DNS) (Miller, 1959). 2 ml DNS dilution reagent was added and incubated in boiled water for 15 min, and diluted with water. One unit of enzyme activity (IU) was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  glucose per minute.

### Preparation of crude enzyme

Strain JL -01 was inoculated in nutrient salts medium with 1% filter paper, in 250 ml flasks containing 100 ml medium, and incubated at

30°C on a rotary shaker at 120 rpm for 96 h. The samples were harvested and centrifugated at 14000 g, for 20 min, at 4°C. The supernatant was used as source of extracellular enzymes and stored at 4°C for further purification.

### Purification of CMCCase

The supernatant was concentrated using PM 30 membrane (30, 000Da cut off, Pall Filtration Co. USA). All manipulations were carried out at 4°C. The concentrated filtrate was brought into 30% saturation with solid ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ). The precipitate was removed by centrifugation at 12000×g for 30 min. And the supernatant was added to 70%  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was harvested by centrifugation at 12000×g for 30 min, and then it was diluted with 20 mM acetate-buffer. The DEAE-SephadexG50 previously equilibrated with 20 mM acetate buffer (pH5.5). The crude enzyme was eluted with a linear gradient of 0.01-0.8 M NaCl in the same buffer at a low of 40 ml/h, collecting time 12 min per tube. The fractions with CMCCase activity were collected and dialyzed against 10mM acetate buffer (pH5.5) at 4°C for next separation process. The sample was applied onto a Sephadex G-100 column pre-equilibrated with 20 mM acetate-buffer, pH5.5 containing 0.5 M  $(\text{NH}_4)_2\text{SO}_4$ . The protein was eluted with the same buffer until the washing showed no absorbance at 280 nm. Gel filtration was carried out on Sephadex G-100 in a 2×95 cm column, and the proteins were eluted with 20 mM acetate buffer, pH 5.0, at a flow rate of 11 ml/h and a fraction volume of 4 ml. Continuous fractions with CMCCase activity were pooled together and concentrated. One CMCCase fraction, CMCCase1, was separated and stored at -20°C (Osmundsvag and Goksoyer, 1975; Pere et al., 1995).

### Sodium Dodecyl-Sulfate polyacrylamide gel electrophoresis SDS-PAGE

Purity and molecular mass were determined by SDS-PAGE using the 12% (w/v) polyacrylamide gel, using a discontinuous system with 5% stacking gel (Tanio et al., 1982). Gel was stained with Coomassie brilliant blue R-250 and estimated with molecular mass markers from 14.4 to 97.4 kDa (Electrophoresis LMW Calibration Kit, Pharmacia Co. USA).

### Effect of pH and temperature on CMCCase activity

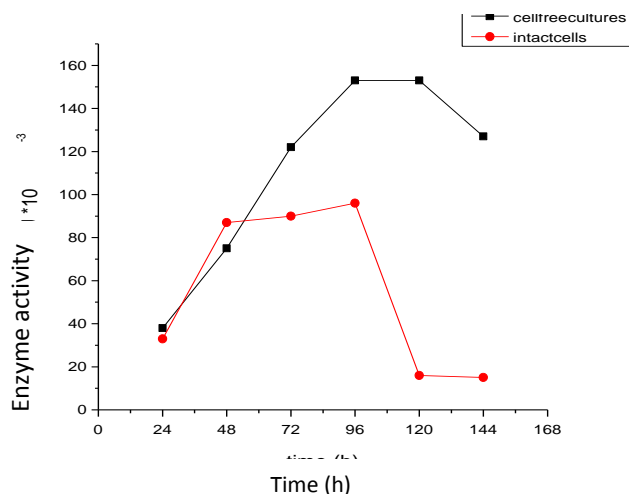
The effect of pH on CMCCase1 activity was determined by standard assays over the pH range of 4.0-8.0 at 50°C, and the buffer systems were acetate-buffer (pH 4.0-5.6) and phosphate-citrate buffer (pH 5.6-8.0). To determine the optimum temperature, the reaction was carried out at the range 4-90°C.

### Effect of metal ions

The CMCCase1 activity was assayed after incubation with 5 mM various metal ions for 15 min at 50°C.  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  were examined for their effects on the activity of the purified CMCCase.

### Enzyme Kinetic

The enzymatic kinetic parameters,  $K_m$  and  $V_{max}$ , of the CMCCase1 were obtained from the Lineweaver-Burke double reciprocal analysis. 1% CMCNa<sub>2</sub> solution was diluted to a range of different concentrations (0.05–1%) as the substrate. The hydrolysis of CMCNa<sub>2</sub> was initiated by the addition of 1 ml of 0.15 mg/ml



**Figure 1.** The production of CMCase of cell-free cultures and intact cells.

CMCase1 solution at pH 5.0 into 1 ml substrate. CMCase activity was measured by the dinitrosalicylic acid method (DNS).

#### Amino acid analysis of the CMCase1

The enzyme was hydrolyzed at 110°C by 6 M HCl for 24 h. The hydrolyzed products were analyzed by Reversed-phase high-performance liquid chromatography (RP-HPLC) (Bartolomeo and Maisano, 2006). RP-HPLC was performed on a Nova-pak C-18 column (3.9×150 mm) using Waters Series 600 HPLC system. Standard amino acids were diluted by water. And samples were prepared by solvent A, 0.2 M Acetate buffer. The elution was performed with a linear gradient from 0 to 100% B (water/acetonitrile (H<sub>2</sub>O: CAN=1:4)) for 30 min, at a flow rate of 1 ml/min and the elution was monitored by Water 2487 photodiode array at 248 nm.

#### Analysis of the temperature effect on CMCase1 structure by Circular dichroism

The far-UV circular dichroism (CD) spectra of the CMCase1 were measured at 25°C on a JASCO J-810 spectra polarimeter (JASCO Corporation, Tokyo, Japan) (Baker et al., 2001). The enzyme solution, concentration on 450 µg/mL, was incubated at 25 and 75°C for 10 min, and then it was placed in a 1 mm path-length cell and scanned in the amide band (190-250nm) under constant nitrogen purge. The scanning speed was 50 nm/min with a 1 nm bandwidth and a 1 ms response. The spectra analysis was made by the Spectra Manager™ software associated with the J-810 system.

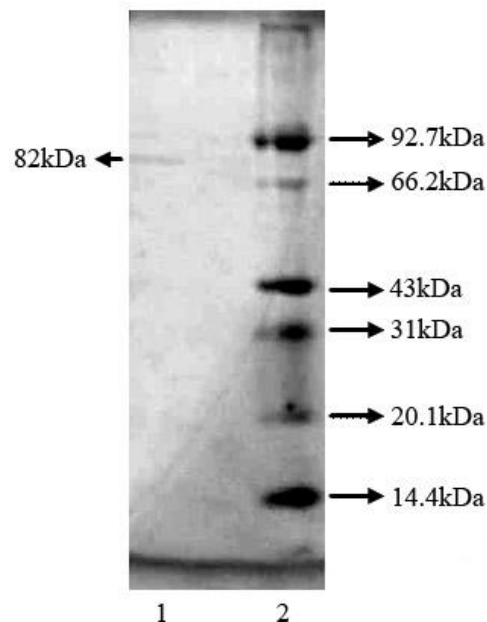
## RESULTS

### The production of CMCase

The production characteristics of CMCase of *S. sp.* JL-01 grown on filter paper as a sole source of carbon was shown in Figure 1. The results showed that CMCase was present in cell-free cultures and intact cells. In general, CMCase activity was associated with the culture time. This strain produced maximum CMCase activity

**Table 1.** Purification of CMCase from *Sporocystophaga sp.* JL-01.

	Total protein (mg)	Total activity (IU)	Yield (%)
Culture supernatant	800	93.63	100
Ultrafiltration	127.4	19.25	20.56
DEAE-SephadexG50	46.8	10.47	11.18
SephadexG-100	26.3	6.23	6.65



**Figure 2.** SDS-PAGE of CMCase1. Lane 1 was purified CMCase1, and Lane 2 was molecular weight standards.

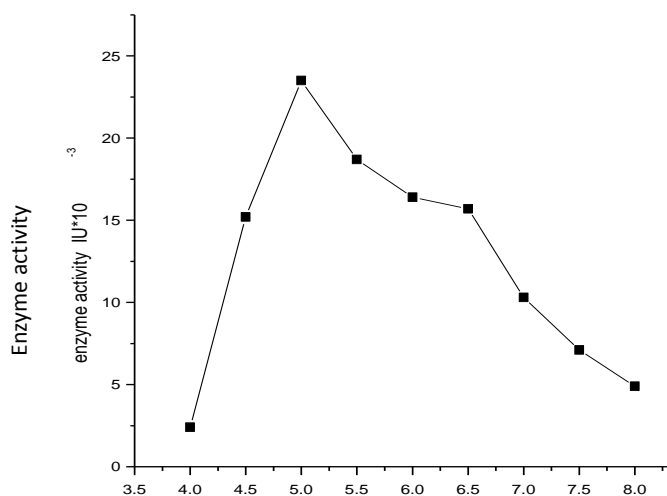
(153×10<sup>-3</sup> IU/ml and 92×10<sup>-3</sup> IU/ml) in supernatant and on intact cell surface on 96 h. Figure 1 showed that the enzyme activity of cell free cultures decreased from 120 h, and the activity of intact cells decreased greatly.

### Enzyme purification

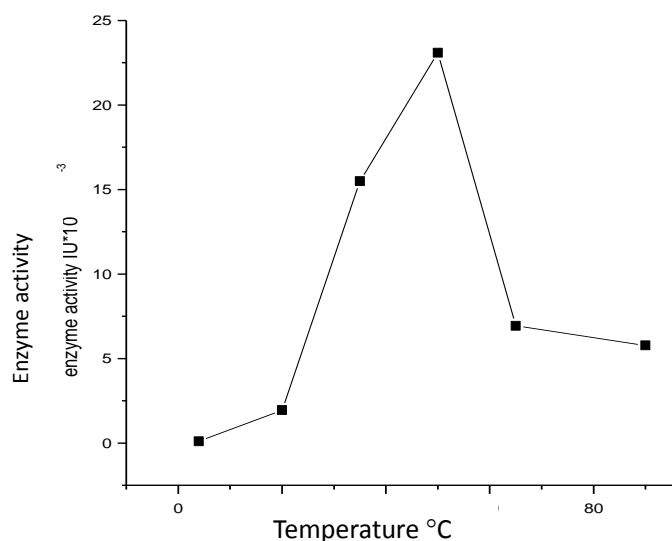
A summary of the purification of CMCase from *S. sp.* JL-01 was shown in Table 1. The crude enzyme was concentrated by ultrafiltration first and then precipitated by saturated ammonium sulfate. And the concentrated enzyme was loaded on column of DEAE-SephadexG50 followed by Sephadex G-100 (1.6~90 cm). A main peak with CMCase activity was eluted and named CMCase1. The yield of CMCase was only 6.65%.

### Relative molecular mass

The enzyme named CMCase1 showed a single protein



**Figure 3.** Effect of pH on the activity of CMCCase1 at 50°C.



**Figure 4.** Effect of temperature on the activity of CMCCase1 under pH 5.0.

band which displayed CMCCase activity in non-denaturing PAGE. The purified CMCCase1 also showed a single band of protein with a molecular weight of 82 kDa after running of SDS-PAGE (Figure 2).

#### Influence of pH and temperature on enzyme activity

The results for the temperature and pH dependence of activity of the purified enzyme were shown in Figures 3 and 4. The CMCCase1 showed maximal activity at pH 5.0 under the assay conditions used. And the optimum temperature was 50°C at pH 5.0.

**Table 2.** Effect of metal ions on the purified CMCCase1 activity.

Ions	Enzyme activity (IU 10 <sup>-3</sup> )	Residual activity (%)
Control	126	100
Zn <sup>2+</sup>	51	40.76
Fe <sup>2+</sup>	141	111.7
Mg <sup>2+</sup>	129	102.1
Ca <sup>2+</sup>	125	99.1

#### Effect of metal ions

The effect of several metal ions on the relative activity of CMCCase1 was assayed at ion concentration 5 mM (Table 2). The addition of Fe<sup>2+</sup> increased the hydrolysis rate slightly, whereas the addition of Zn<sup>2+</sup> clearly inhibited CMCCase activity. It was similar that the enzyme activity was significantly inhibited by Zn<sup>2+</sup> (Huang and Monk, 2004).

#### Enzyme kinetic

The kinetic features of the activity of the CMCCase1 were obtained from enzymatic hydrolysis of CMCNa<sub>2</sub> by a Lineweaver-Burke analysis. The Km of CMCCase1 was 9 mgml<sup>-1</sup> and Vmax was 27.3 ug min<sup>-1</sup>mg<sup>-1</sup> protein from Lineweaver-Burke blot over a CMC concentration range from 0.05 to 1.0% (w/v).

#### Amino acid composition

Analysis of the hydrolysed purified protein by HPLC revealed that Aspartic acid, Glutamic acid, and Threonine were the major components that were more than 10%, and the composition of Glycine, Serine, Proline, Valine, Phenylalanine and Leucine were over 5%. More than 17 normal amino acids were found in the CMCCase1 and their content shown in Table 3. Aromatic amino acid was about 10.5%. Acidic amino acid was about 24.54; this might be the reason of the optimum pH 5.0. And basic amino acid is just 7.0%. No Trp was found in this assay method.

#### The effect of temperature on CMCCase1 conformation

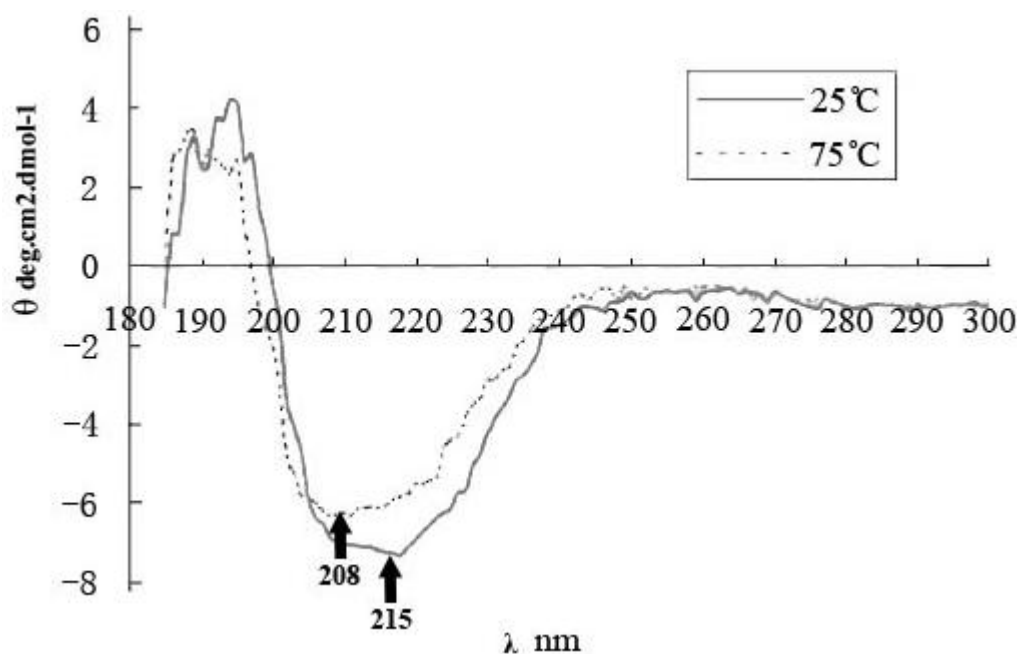
The CD spectroscopy was shown in Figure 5. The temperature ramps were performed in 50 mM HAC-NaAC buffer (pH 5.0). The protein concentration was 0.5 mg/ml (cell path 1 cm) for CD measurements in the near- and far-UV regions, respectively. At 25°C, there was a negative peak at 215 nm; while at 75°C the peak position was at 208 nm, indicating a decreased in the β-sheet.

#### DISCUSSION

The CMCCase production of *Sporocytophaga* sp. JL-01

**Table 3.** Amino acid contents of the CMCase1.

Name	MWs	Concentration (%)
Asp	133.60	14.13
Ser	105.06	8.01
Glu	147.08	10.19
Gly	75.05	9.78
His	155.09	0.90
Arg	174.40	2.53
Thr	119.18	10.22
Ala	89.06	4.96
Pro	115.08	6.40
Cys	121.11	0.66
Tyr	181.09	4.19
Val	117.09	5.25
Met	149.15	0.78
Lys	146.13	4.39
Ile	131.11	3.91
Leu	131.11	6.56
Phe	165.09	6.23
Trp	204.11	---

**Figure 5.** The CD spectrum of CMCase1 under different temperature. Line was under 25°C, and dot was under 75°C.

was higher at 96 to 120 h, that was contacted with the cell shapes changing during the fermentation and the cysts were formed from filamentous shapes on this time (Liu et al., 2003; Grace, 1951). So when cyst is formed, the CMCase production might decrease.

In the present study, molecular weight of CMCase1

from *Sporocytophaga* sp. JL-01 was 82 kDa, which was different from two extracellular cellulases reported by Osmundsvag et al. (1975). Osmundsvag et al. (1975) separated two extracellular cellulases, with molecular weights of 52 and 46 kDa, acting on carboxymethylcellulose, separated from the culture

supernatant of *Sporocytophaga myxococcoides*. Therefore, we considered it as one of isoenzymes from *Sporocytophaga*.

As a mesophilic bacteria, the optimum pH of CMCCase1 was almost 5.0 and the optimum temperature was about 50°C in many bacteria (Rastogi et al., 2009; Li et al., 2003), and the higher CMCCase activity of *Sporocytophaga* sp. JL-01 was at this condition. But it was sensitive to temperature, while the enzyme activity was 40% at 65°C for 5 min. Connected with CD spectrum results, the data showed that  $\beta$ -sheet was decreased when temperature was from 25 to 75°C. Integrated with the change of enzyme activity,  $\beta$ -sheet might be key molecular structure conformation, which was similar to other fungi cellulases (Xu et al., 1995; Tormo et al., 1996).

The characteristics of CMCCase1 showed that it was a typical endoglucanase. The reason that *Sporocytophaga* sp. JL-01 could decompose fiber paper quickly needed more proofs. The physical contact with fiber might be the key influence on cellulose degradation. But we need more evidence to prove it.

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