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Purification and properties of a protein from *Lantana camara* activating *Cuscuta reflexa* cellulase

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Abstract

A high molecular weight protein from *Lantana camara* leaf and petiole was purified 59-fold with 12% recovery by heat treatment, ammonium sulfate fractionation and subsequent chromatography on DEAE-cellulose and Sephadex G-200. It has been designated as cellulase stimulator (C_s) in view of its ability to stimulate/activate *Cuscuta reflexa* cellulase catalysis. The purified protein gave a single C_s-activity and protein peak on chromatography, a single band on PAGE at various pH values and a single peak on FPLC. The purified C_s-protein alone did not show cellulolytic activity with CM-cellulose but in recombination experiments with *C. reflexa* CM-cellulase, it exhibited activation effect both in terms of saccharifying and liquefying activity. The purified protein had a molecular mass 230 kDa and Stokes' radius of 62.5 Å.

Keywords: *Lantana camara*; *Cuscuta reflexa*; Cellulase; Protein; Cellulase activator

1. Introduction

Plant cellulases (endo-1,4-β-D-glucanases, EC 3.2.1.4) catalyze the hydrolysis of the internal 1,4-β-Iinkages of cellulose and cellulose derivatives, such as carboxymethyl cellulose (CMC). Cellulase activity in different tissues of plants is closely related to various physiological aspects of plant growth. Cellulases have earlier been studied from a number of plant sources (Awad & Lewis, 1980; Byrne, Christou, Verma & Maclachlan, 1975; Edwards, Dea, Bulpin & GrantReid, 1986; Hatfield & Nevins, 1986; Koehler, Lewis, Shannon & Durbin, 1981; Lew & Lewis, 1974; Nakamura & Hayashi, 1993; Sexton, del Campillo, Duncan & Lewis, 1990; Truelsen & Wyndaele, 1991; Wang, Craker & Mao, 1994). It has been suggested to be involved in the cell wall degradation and subsequently facilitating the penetration of the parasite (*Cuscuta reflexa*) haustoria into an angiosperm host (Nagar, Singh & Sanwal, 1984). We have detected for the first time a protein from *Lantana camara* (a susceptible host to *C. reflexa*) which activates cellulase from *C. reflexa*. The purified protein fraction from *L. camara* has been designated as cellulase stimulator (C_s) in view of its ability to stimulate/activate cellulase activity from *C. reflexa*. In addition to activation function of C_s, it has also been partially characterized.

2. Results

2.1. Purification of *C. reflexa* carboxymethyl cellulase

Ammonium sulfate fractionation of the initial extract followed by chromatography steps resulted in 42-fold purification of *C. reflexa* carboxymethyl cellulase (CMCase) with about 6% recovery. The purified enzyme was free from β-glucosidase, activity. The purified enzyme was almost homogeneous as judged by

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Table 1
Purification of C_s-protein from *L. camara* leaf and petiole

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1}) ($\times 10^{-3}$)	Fold enrichment	Recovery of activity (%)
Supernatant	735	4.05	1275	3.2	–	100
Heat-treatment	815	3.58	997	3.6	1.1	88
$(NH_4)_2SO_4$ (30–75%)	38	2.26	298	7.6	2.4	56
DEAE-cellulose	172	1.41	18.6	75.8	23.7	35
Sephadex G-200	62	0.47	2.5	188	58.8	12

native PAGE and antigen–antibody cross reactivity (Chatterjee, Chauhan & Sanwal, 1997).

2.2. Purification of C_s-protein

The summary of purification of C_s-protein from *L. camara* leaf and petiole is given in Table 1. Heat-treatment and $(NH_4)_2SO_4$ fractionation of the initial extract followed by chromatography on DEAE-cellulose and Sephadex G-200 resulted in 59-fold purification of C_s-protein with 12% recovery from the initial extract. In 10 different experiments the average purification achieved was 58 ± 5 with recovery in the range 10 to 15% from the initial extract. Only one major and one minor peak of C_s-protein were found on chromatography on DEAE-cellulose. A single peak for C_s-protein was obtained on Sephadex G-200 chromatography. C_s-protein after Sephadex G-200 chromatography showed a single band on PAGE using 6% gel concentration at pH 6, 7 and 8, respectively (Fig. 1). The purified preparation gave a single peak on FPLC using protein pak glass 300 SW column (figure not shown).

The purified C_s-protein alone did not show any saccharifying or liquefying activity with CMC as a substrate as determined by reducing sugar or viscometric method, respectively. The purified preparation had no β-glucosidase activity. Progress of purification of C_s-protein, with removal of β-glucosidase and CMCase contamination is also shown in Table 2.

The fluorescence spectrum of the native C_s-protein dissolved in 0.02 M sodium phosphate buffer, pH 7.0 shows a peak of maximum emission at 360 nm, the wavelength near which tryptophan fluorescence dominates.

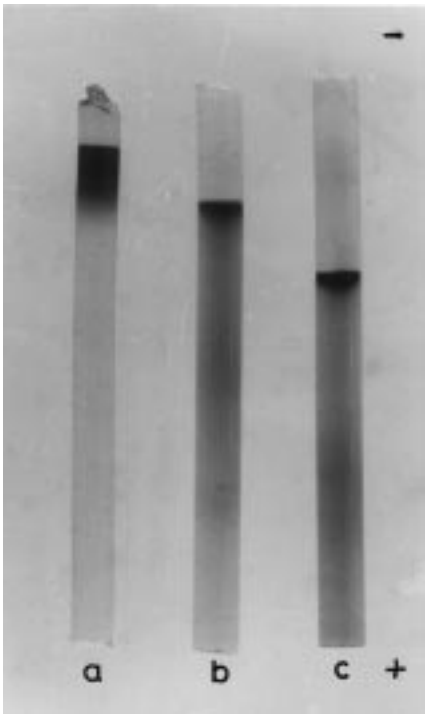

Fig. 1. Disc gel native PAGE of purified *L. camara* C_s-protein. The electrophoresis was carried out in 0.05 M phosphate buffer at pH 6.0 (lane a), pH 7.0 (lane b) and pH 8.0 (lane c). Amount of protein applied was 40–80 μg and the gel was stained with Coomassie brilliant blue.

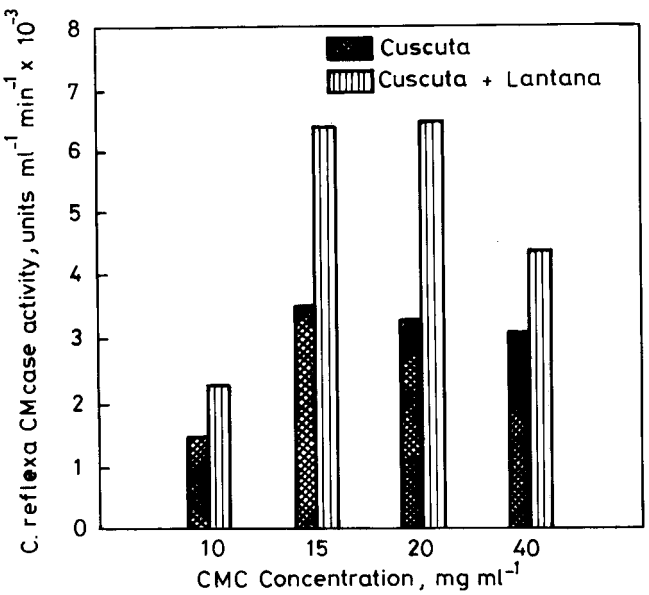

Fig. 2. Effect of C_s-protein from *Lantana* on *C. reflexa* CMCase activity at various substrate concentration. The total volume of the assay system was 2 ml with concentrationns of CMCase and C_s-protein at 12.7 μg protein each.

Table 2
Removal of *L. camara* CMCase and β-glucosidase (β-gs) with purification of C_s-protein

Fraction	Activity (units ml^{-1} min^{-1})			Ratio (CMCase/C_s)	Ratio (β-gs/C_s) ($\times 10^3$)
	CMCase ($\times 10^{-3}$)	C_s ($\times 10^{-3}$)	β-gs		
Supernatant	52.2	5.5	1033	9.49	188
Heat treatment	22.2	4.4	812	5.05	185
$(NH_4)_2SO_4$ (30–75%)	22.2	52.2	1568	0.43	30
DEAE-cellulose	0.0	7.2	75	0.00	10
Sephadex G-200	0.0	6.7	0	0.00	0

2.3. Molecular weight and Stokes' radius

The M_r of C_s-protein was found 230 kDa from the plot of V_e/V_0 and log M_r of standard proteins. A plot of $(-\log K_{av})^{1/2}$ and Stokes' radius of standard proteins gave a linear curve (figure not shown) from which Stokes' radius of 62.5 Å was found for C_s-protein.

2.4. Activation properties of the purified C_s-protein

The experiments were carried out at pH 5.0 under conditions of enzyme concentration and time linearity. Homogeneously purified CMCase from *C. reflexa* was used for activation studies in combination with the purified C_s-protein. The influence of varying CMC concentration (10–40 mg/ml) on the individual activities of CMCase and C_s-protein and on the respective combinations is shown in Fig. 2. The hydrolysis rate of the *C. reflexa* CMCase increased when CMC concentration was raised upto 15 mg/ml and subsequently decreased slightly. C_s-protein from *L. camara* did not show any hydrolytic activity as measured by reducing sugar assay. When CMCase and C_s-protein in the assay system were taken together, the observed hydrolytic activity was higher than the sum of activities of the individual CMCase and C_s at each substrate concentration. Activation of CMCase activity of *C. reflexa* by C_s-protein, was maximal at 20 mg/ml CMC concentration and subsequently decreased when the substrate concentration was increased to 40 mg/ml.

In a separate experiment, purified C_s-protein was added to the extract of *L. camara* containing CMCase activity to determine if C_s-protein enhanced its own cellulase activity. No activation was observed in such combinations.

2.5. Effect of C_s-protein on Trichoderma viridae cellulase using different substrates

Since C_s-protein was enhancing the hydrolytic activity of CMCase from plant source (*C. reflexa*), it appeared interesting to find out the effect of C_s-protein on microbial cellulase from *T. viride*. The substrates used were CMC, swollen- and insoluble-cellulose. Activation of microbial cellulase was not observed on incorporation of *L. camara* C_s-protein but in contrast, inhibition was observed (Table 3).

2.6. Nature of action of C_s-protein

C_s-protein alone did not show any cellulolytic activity, either saccharifying or liquefying but in combination with *C. reflexa* CMCase, it stimulated significantly the ability of *C. reflexa* CMCase to release a greater number of large oligosaccharide chains with reducing ends. Cellulase activity judged by formation of reducing sugars was almost similar with and without C_s-protein until the initial 6 h (figure not shown), whereas cellulase activity judged by viscosity was constantly increased by C_s-protein (Fig. 3). It indicated that initially CMC was hydrolysed into large frag-

Table 3
Inhibition of *T. viride* cellulase by *L. camara* C_s-protein. Cellulase activity was determined for 2 h only

Substrate	Cellulase activity (units ml^{-1} min^{-1})			Decrease (%)
	T. viride[a]	*L. camara*[b]	*T. viride* + *L. camara*[c]	
CM-cellulose	4.82	0.00	1.38	71.4
H_3PO_4 swollen cellulose	1.23	0.00	0.62	49.6
Insoluble cellulose	0.27	0.00	0.19	29.6

[a] 0.1 ml (0.45 units) *T. viride* cellulase.
[b] 0.8 ml (34 μg) *L. camara* C_s-protein.
[c] 0.1 ml *T. viride* cellulase + 0.8 ml *L. camara* C_s-protein.

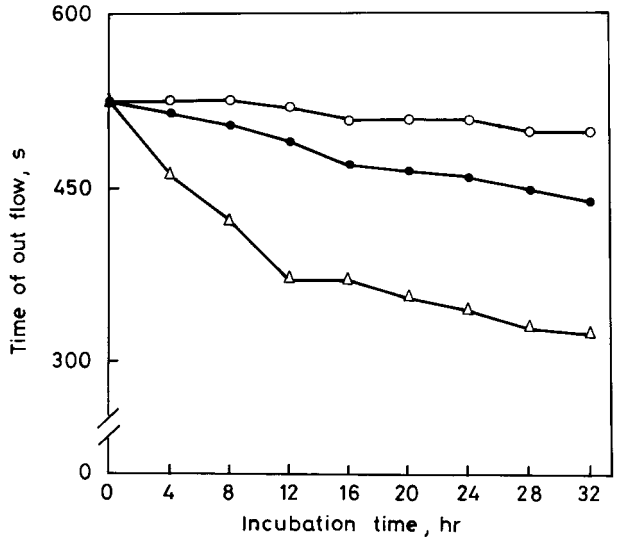

Fig. 3. Effect of C_s-protein on liquefying activity of *C. reflexa* CMCase. (○–○), C_s-protein alone; (●—●) CMCase alone; (△–△), C_s-protein and CMCase in combination.

ments and hence large decrease in viscosity with formation of less number of reducing ends.

3. Discussion

Studies concerning cellulases from higher plants are mostly involved with fruit ripening and senescence. A high molecular weight protein, designated as C_s-protein has been purified and partially characterized from an angiosperm plant *L. camara*, a susceptible host to *C. reflexa*. Activation obtained on recombination experiments of purified C_s-protein from *L. camara* with purified CMCase from *C. reflexa* is being reported for the first time. *C. reflexa* CMCase alone was active on CMC as determined by liquefying as well as saccharifying mode of measurements. Purified preparation of C_s-protein alone from *L. camara* did not show any detectable activity on CMC as determined by reducing sugar as well as by viscometric method. C_s-protein, however, when combined with *C. reflexa* CMCase was able to increase the ability of CMCase to release more reducing sugar and to cause more increase in fluidity of CMC. The activation of CMCase by C_s-protein increased when CMC concentration was increased until the number of chain ends is enough for the reaction to go to completion, i.e. 20 mg ml^{-1}. Increasing the concentration of CMC above 20 mg ml^{-1} led to decreased activation, probably because all the binding sites, for which CMCase and C_s appear to compete, might be saturated.

No activation was obtained on the recombination of purified C_s fraction with crude as well as partially purified CMCase from *L. camara*. The absence of activation may be attributed to the fact that C_s-protein might be lacking the binding sites for *L. camara* CMCase to cause the accumulation of CMCase molecules on the surface of CMC. Quite interesting are the results of recombination experiments of C_s-protein with CMCase from *T. viride* on hydrolysis of CMC, swollen-cellulose and insoluble cellulose. Decrease in hydrolytic activity of *T. viride* cellulase in the presence of C_s-protein is in agreement with the observations of Albersheim and Anderson (1971) that plants have within their walls, proteins that can inhibit specifically the wall-degrading enzymes secreted by microbes.

In conclusion, it can be said that C_s-protein under in vivo conditions may have molecular interaction with the CMCase from an angiosperm parasite during haustorial development. The high molecular weight protein from *Lantana* coordinates with *Cuscuta* CMCase in facilitating cell wall degradation and thus in establishment of haustoria on the host tissue.

4. Experimental

4.1. Plant tissue material

Fresh tissue material of *L. camara* L. and *C. reflexa* Roxb. vines, infecting the host *L. camara* L. were collected from the departmental garden.

4.2. Substrate preparation

CMC and Whatman cellulose powder were purified by precipitation from 20% (w/v) suspensions with two vols of chilled alcohol at 4°. Phosphoric acid-swollen cellulose was prepared according to Wood (1988). All substrates were prepared in 0.1 M Na0Ac, pH 5.0, to a final concentration of 1.5% (w/v). PNPG was dissolved in 0.1 M Na0Ac buffer, pH 5.0, to a final concentration of 1 mM.

4.3. Protein determination

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951)as modified by Khanna, Mattoo, Viswanathan, Tewari and Sanwal (1969)on TCA ppt. with BSA as the standard.

4.4. CMCase of C. reflexa

CMC-saccharification activity was used as the standard assay of cellulase and was carried out according to Chatterjee et al. (1997). The released reducing groups were measured by dinitrosalicylic acid assay (Bernfeld, 1955). A unit of cellulase has been defined as the amount of enzyme that produces an A_{540} of 0.05 min^{-1} under the assay conditions. CMC-liquefac-

tion activity was measured by viscometry using Ostwald viscometer as described (Chatterjee et al., 1997).

4.5. C_s activity from L. camara

The activity was assayed by measuring the activation of purified *C. reflexa* cellulase activity upon addition of C_s-protein. The assay of in a total of 2 ml contained 1 ml of 1.5% CMC and 1 ml of protein and water. The protein in set 1 was 0.2 ml *C. reflexa* cellulase, in set II 0.2 ml C_s-protein and in set III 0.2 ml cellulase and 0.2 ml C_s-protein. The control and experimental of each set were incubated at 37° for 15 h; after layering a drop of toluene. CMC-saccharification activity of each set was determined by measuring the appearance of reducing end groups as described in CMCase assay. One unit of the C_s-protein activity has been defined as the amount of protein that causes 50% enhancement of the CMCase activity of *C. reflexa* min^{-1} under the assay conditions. Specific activity was defined as units mg^{-1} protein.

4.6. β-Glucosidase

Glucosidase was assayed as described earlier (Chatterjee et al., 1997).

4.7. Purification of C. reflexa CMCase

CMCase was extracted and purified from *C. reflexa* as described earlier (Chatterjee et al., 1997).

4.8. Purification of C_s-protein from L. camara

In a typical experiment 225 g of fresh harvested leaves and petioles from *L. camara* were homogenized in a Waring blender for 2 min with 500 ml of chilled homogenizing medium consisting of 0.025 M sodium metabisulfite and 0.05% hexadecyltrimethylammonium bromide in 10% NaCl. The homogenate was filtered through 4 layers of muslin, brought to pH 8.0 with chilled 1 N NaOH and the vol. was made up to 750 ml. After 30 min, the filtered homogenate was centrifuged at $15{,}000 \times g$ for 30 min to get clear supernatant, which contained almost all the C_s-protein activity of the homogenate. The supernatant, dialyzed overnight against water, was heated in a boiling water bath for 3 min and centrifuged at $15{,}000 \times g$ for 25 min. To the supernatant, solid $(NH_4)_2SO_4$ was added and the protein precipitating between 30–75% sat. collected. The ppt. was suspended in 10 ml of 0.02 M Na-Pi buffer, pH 7.0 (buffer A) dialyzed overnight against buffer A and centrifuged at $15{,}000 \times g$ for 10 min. The supernatant was loaded onto a DEAE-cellulose column (25×3.2 cm), previously equilibrated with buffer A. The column was washed with 3 bed vols of the above buffer and the C_s-protein eluted using a linear NaCl gradient (0–1 M). The active fraction eluted between 0.44 to 0.84 M NaCl constituted a major peak with a minor peak eluted between 0.35 to 0.43 M NaCl. The total pooled fraction of the major peak was concentrated by precipitation with $(NH_4)_2SO_4$ and then subjected to gel filtration through Sephadex G-200 column (1.6×66 cm) that had been equilibrated with buffer A. Elution was carried out with the same buffer. The active fractions constituting a single peak were collected and pooled.

4.9. Polyacrylamide gel electrophoresis

PAGE was carried out according to the method of Davis, Schlisefield, Wolf, Leavitt and Krebs (1967).

4.10. Molecular weight and Stokes' radius

The M_r of C_s-protein was determined by gel filtration on a Sephadex G-200 column (1.6×66 cm) by the method in Andrews (1964). Void volume (V_0) of the column was determined with dextran blue. Stokes' radius of C_s-protein was determined according to Laurent and Killander (1964).

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References

Albersheim, P., & Anderson, A. J. (1971). *Proceedings of National Academy of Sciences, USA*, *68*, 1815.
Andrews, P. (1964). *Biochemical Journal*, *91*, 222.
Awad, M., & Lewis, L. N. (1980). *Journal of Food Science*, *45*, 1625.
Bernfeld, P. (1955). *Methods in Enzymology*, *1*, 149.
Byrne, H., Christou, N. V., Verma, D. P. S., & Maclachlan, G. A. (1975). *Journal of Biological Chemistry*, *250*, 1012.
Chatterjee, U., Chauhan, H. O. S., & Sanwal, G. G. (1997). *Indian Journal of Biochemistry and Biophysics*, *34*, 354.
Davis, C. H., Schlisefield, L. H., Wolf, D. P., Leavitt, L. A., & Krebs, E. G. (1967). *Journal of Biological Chemistry*, *242*, 4824.
Edwards, M., Dea, I. C. M., Bulpin, P. V., & GrantReid, J. S. (1986). *Journal of Biological Chemistry*, *261*, 9489.
Hatfield, R., & Nevins, D. J. (1986). *Plant Cell Physiology*, *27*, 541.
Khanna, S. K., Mattoo, R. L., Viswanathan, P. N., Tewari, C., & Sanwal, G. G. (1969). *Indian Journal of Biochemistry and Biophysics*, *6*, 21.
Koehler, D. E., Lewis, L. N., Shannon, L. M., & Durbin, M. L. (1981). *Phytochemistry*, *20*, 409.

Laurent, T. C., & Killander, J. (1964). *Journal of Chromatography*, *14*, 317.

Lew, F. T., & Lewis, L. N. (1974). *Phytochemistry*, *13*, 1359.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biological Chemistry*, *193*, 265.

Nagar, R., Singh, M., & Sanwal, G. G. (1984). *Journal of Experimental Botany*, *35*, 1104.

Nakamura, S., & Hayashi, T. (1993). *Plant Cell Physiology*, *34*, 1009.

Sexton, R., del Campillo, E., Duncan, D., & Lewis, L. N. (1990). *Plant Science*, *67*, 169.

Truelsen, T. A., & Wyndaele, R. (1991). *Journal of Plant Physiology*, *139*, 129.

Wang, Y., Craker, L. E., & Mao, Z. (1994). *Plant Physiology and Biochemistry*, *32*, 467.

Wood, T. M. (1988). *Methods in Enzymology*, *160*, 19.