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Full Length Research Paper

Pseudo-affinity chromatography of rumen microbial cellulase on Sepharose- Cibacron Blue F3GA

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Pseudo affinity adsorption of bioproducts on Sepharose-cibacron blue F3-GA was subjected to rumen microbial enzyme evaluation through batch binding and column chromatography of cellulase. The results showed that homogenizing method had better performance in the release of enzyme, so that the amount of enzyme in rumen liquor approximately doubled. Among the preconcentration methods, it was shown that freeze drying and precipitation of enzyme using ammonium sulphate were the best. Based on equilibrium adsorption data, the best temperature, contact time and elution agent were 30°C, 15 min and 0.5 M NaCl (pH 6.5), respectively. Microbial cellulase was purified from rumen liquor to 1.2 and 14.2- fold by ammonium sulphate fractionation and Sepharose-CB column chromatography, respectively. The generic sample preparation and application of the sorbent for the adsorption and separation of cellulase is discussed.

Key words: Cellulase, pseudo-affinity chromatography, purification, sepharose-cibacron blue F3-GA.

INTRODUCTION

There is a great deal of interest in using enzyme preparations containing high levels of cellulase activities for improving feed utilization, milk yield and body weight gain by ruminants (Eun and Beauchemin, 2007; Pinos-Rodríguez et al., 2002; Stokes et al., 1995; Beauchemin et al., 1995; Cowan., 1996; Galante et al., 1998b; Graham and Balnave, 1995). In addition to their established use as animal feed additives, cellulases have a wide range of applications in food, paper and pulp, textile, fuel and chemical industries (Chauynarong et al., 2008; Gilbert and Hazlewood, 1993; Beguin and Aubert, 1994; Bhat and Bhat, 1997). Affinity chromatography has proven to be the most effective technique for the purification and separation of proteins from complex mixtures (Labrou and Clonis, 1994). Synthetic affinity ligands, such as reactive chlorotriazine dyes, have become an integral part of affinity-based protein purification methods for a number of reasons. The dyes are inexpensive, chemical immobilization of the dyes to the matrix is easy and the resultant dye-adsorbents are resistant to chemical or biological degradation, the protein binding capacity is high and far exceeds the binding capacity exhibited by biological ligands (Denizli and Pikin, 2001; Clonis, 2006). Dye ligand chromatography offers the convenience and high capacity of ion-exchange chromatography in combination with unique selectivities that can allow purification of some proteins difficult to purify by any other means (Labrou, 2003). In the present study, the separation of cellulase from rumen content using homogenization and sonication methods as well as the concentration and purification of extract through precipitation and immobilized Sepharose-Cibacron blue F3-GA column chromatography was investigated.

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Abbreviations: CMC, Carboxymethylcellulose sodium salt; **TCA,** trichloroacetic acid; **EDTA,** ethylene dinitrilo-tetraacetate.

MATERIALS AND METHODS

Ammonium sulphate, acetic acid, bovine serum albumin, citric acid , potassium sodium tartrate, di-potassium hydrogen phosphate anhydrous, sodium hydroxide, sodium chloride, sulfuric acid, Tris, Triton x 100, d (+)-glucose anhydrous, ethanol absolute, phenol and trichloroacetic acid were purchased from Merck (Darmstadt, Germany).

3, 5-Dinitrosalicylic acid was purchased from Sigma Chemical Company (St. Louis MO, USA). Carboxymethylcellulose sodium salt (CMC, medium viscosity, Acrose Organics, New Jersey, USA) and Centrifuge (Sigma, 3K30, Ostrode am Harz, Germany). Freeze dryer (Christ, Alpha 1-4 LD, Germany) and Spectrophotometer (Varian, Cary 50, Palo Alto, CA, USA).

Sample preparation

Rumen samples were collected from slaughterhouse and transported to the laboratory in thermos flasks. Sample was filtered using six layers cheesecloth to separate the undigested matter. The filtrate was centrifuged at 10,000 × *g* for 30 min to separate the cell mass. The supernatant was filtered using Whitman filter #4 under suction to remove any micro particles. The cell-free extract was stored at 70°C until use.

Enzyme assay

Cellulase activity was assayed with carboxymethylcellulose (15 mg/mL) as substrates by determining the amount of reducing sugar released, using the dinitrosalicylic acid method of Miller (1959). The assay mixture (1 mL) contained 100 µl of enzyme solution and 5 mM substrate dissolved in 0.1 M citrate-phosphate buffer (pH 6.5) and appropriate diluted enzyme, was incubated at 50°C for 30 min; glucose was used as the standard. One unit of enzyme activity is defined as the amount of enzyme required to produce one µmol of reducing sugar per minute under assay conditions (Ghose, 1987).

Protein content

Protein concentration was determined according to the procedure described by Bradford (1976) using bovine serum albumin as the standard.

Homogenization and sonication

Solid substance of rumen content (40 g fresh weight) was suspended in 60 mL rumen liquor. In homogenization and sonication process, the sample was blended 1 min and sonicated for 30 s with 30 s intervals, respectively. Protein content and cellulase activity of resulted was determined for each method.

Ammonium sulphate precipitation

The proteins in the crude preparation were precipitated by the addition of solid ammonium sulphate to 90% saturation. The precipitate was allowed to form at 4°C for 24 h, and was collected by centrifugation at 10,000 × *g* in a cold centrifuge at 4°C for 15 min. The precipitate was redissolved in 10 mL of 0.1 M citrate phosphate buffer, pH 6.5.

Trichloroacetic acid (TCA) precipitation

An equal volume of 20% TCA was added to the enzyme solution. The mixture was incubated overnight on ice and centrifuged at 10,000 × *g*, 4°C for 15 min. The supernatant was removed and 100 mL of 90% ice-cold acetone were added to wash the pellet. The sample was incubated on ice for 15 min and centrifuged as above. The acetone-containing supernatant was removed and the pellet was air dried.

Ethanol precipitation

Nine volumes of cold ethanol (–80°C) were added into the enzyme solution and kept for 2 h on –80°C. The sample was centrifuged at 10,000 × *g*, 4°C for 15 min, the supernatant was removed and the pellet was air dried. Finally, the pellets were redissolved in a small volume of 0.1 M citrate phosphate buffer (pH 6.5).

Fractionation with ammonium sulphate

Ammonium sulphate was added to reach 20% saturation. After stirring on a stir plate for 10 min, the sample was left for 1 h at 4°C and centrifuged at 10,000 × *g*, at 20°C for 15 min. The supernatant was transferred to another tube and more ammonium sulphate was added to reach 35% saturation. The mixture was treated as above. The enzyme in the supernatant were further fractionated with 50, 65 and 80% ammonium sulphate saturation .Each of the five pellets was washed with ice-cold 90% acetone, dried in the air and suspended in 5 mL of sample buffer.

Immobilization of dyes on sepharose CL-6B

Cibacron blue was immobilized on SepharoseCL-6B according to the method of Stellwagen (1990). Sepharose CL-6B (100 mL Settled gel) was exhaustively washed with distilled water and sucked moist on a three-necked round bottom flask provided with a paddle stirrer. Gel was suspended in 100 mL water and, while stirring, heat to 60°C using a heating mantle. Cibacron blue F3GA solution (1 g dissolved in 30 mL water) was added to gel suspendsion and stirred for 30 min 60°C slowly. 15 g NaCl was added to reaction mixture and stirred for 1 h at 60°C. Temperature of reaction mixture was increased to 80°C and sodium carbonate (1.5 g) was added to the gel suspension and heated for 2 h at 80°C. The dyed gels were thoroughly washed with distilled water and then stored as moist gels in distilled water containing 0.02% NaN_3 at 4°C.

Batch method

Optimization of sorption time

Sepharose-CB (2 mL) was shaken with 10 mL of rumen liquor containing cellulase enzyme at 4°C for different length of time (5, 15, 30 and 60 min) under optimum condition. After filtration of the sorbent, the protein content in solution was determined.

Optimization of sorption temperature

Sepharose-CB (2 mL) was mixed with 10 ml of rumen liquor containing cellulase enzyme at 4, 20, 30 and 40°C for 15 min by means of a magnetic stirrer and then an aliquot portion of the filtrate was applied to determine protein content.

Elution agents

The degree protein desorption at optimum condition was determined by batch equilibration technique. Sepharose-CB (2mL) was mixed with 10 ml of rumen liquor containing cellulase enzyme at 4°C for 15 min by means of a magnetic stirrer and then sorbent residue on filter paper was mixed with 10 mL of different treatments including sodium chloride (1, 0.5 and 0.1 M), ethylene dinitrilotetraacetate (EDTA) (0.1 and 0.05 M) and pH (4, 6 and 8) for 15

Table 1. Influence of different treatments on cellulase liberation.

Treatment	Protein (mg/mL)	Cellulase Activity*	Cellulase Specific Activity**
Control	0.561 ± 0.029[c]	0.573 ± 0.037[c]	10.222 ± 0.662[a]
Homogenizing	0.999 ± 0.015[a]	1.021 ± 0.152[a]	10.226 ± 1.52[a]
Ultrasonication	0.668 ± 0.023[b]	0.682 ± 0.040[b]	10.208 ± 0.6[a]

Values are presented as mean + standard deviation (n=5); values with different super scripts along a vertical column are statistically different ($p<0.05$). *Expressed as micromoles of reducing substance per minute per milliliter; **expressed as micromoles of reducing substance per minute per milligram of protein.

Table 2. Influence of different time of homogenizing on cellulase liberation and activity.

Treatment	Protein (mg/mL)	Cellulase specific activity*
Control	0.565 ± 0.012[c]	10.792 ± 0.22[a]
1 min	0.929 ± 0.005[b]	10.795 ± 0.52[a]
1.5 min	1.087 ± 0.053[a]	8.076 ± 0.42[b]
2 min	1.191 ± 0.021[a]	7.734 ± 0.36[b]

Values are presented as mean + standard deviation (n=5); values with different super scripts along a vertical column are statistically different ($p<0.05$). *Expressed as micromoles of reducing substance per minute per milligram of protein.

Table 3. Comparison of different enzyme concentrating method on cellulase activity.

Treatment	Protein (mg/mL) recovery	Cellulase specific activity*
Control	1.086 ± 0.048[a]	10.662 ± 0.711[a]
TCA precipitation	0.512 ± 0.012[c]	1.755 ± 0.401[c]
Ammonium sulphate precipitation (90% Saturation)	0.877 ± 0.041[b]	11.951 ± 0.617[a]
Ethanol precipitation	1.022 ± 0.015[a]	9.140 ± 0.342[b]
Freeze drying	1.093 ± 0.02[a]	10.470 ± 0.528[a]

Values are presented as mean + standard deviation (n=5); values with different super scripts along a vertical column are statistically different ($p<0.05$). *Expressed as micromoles of reducing substance per minute per milligram of protein.

min. An aliquot portion of the filtrate was applied to determine protein content.

Column method

Affinity chromatography on cibacron blue 3GA

The enzyme was further purified with an affinity column (1.4 × 12 cm) of Cibacron Blue F3GA previously equilibrated with 0.1 M sodium acetate (pH 5.0). The enzyme was eluted with a 5 column volume of 0.5M NaCl (pH 6.5) at a flow rate of 18 mL/h. The combined active fractions were pooled.

RESULTS AND DISCUSSION

The results of application of homogenizing and sonication methods are presented in Table 1. It showed that homogenizing method had better performance in the liberation of enzyme, so that the amount of enzyme in rumen liquor approximately doubled. To determine the best time length for the maximum release of cellulase from solid substance, various periods time including 1, 1.5 and 2 min were experimented. According to this experiment, 1 min turned out to be the best (Table 2).

To attain a high level of concentration of cellulase obtained from rumen liquor, various procedures were applied. The results indicate that freeze drying and precipitation of enzyme using ammonium sulphate were best methods, while TCA precipitation turned out to be the most inappropriate method due to improper effect on enzymatic activity (Table 3). Fractionation with ammonium sulfate was used to achieve the initial purification of cellulase and the results are summarized in Table 4. It indicates that the precipitation obtained from 35 to 50 saturation percent using ammonium sulfate brought about the highest degree of purity ($p<0.05$).

According to the results of batch method tests presented in Tables 5, 6 and 7, protein contents in rumen

Table 4. Enzyme fractionation by different percentage of ammonium sulphate saturation.

	Control	0-20	20-35	35-50	50-65	65-80
Protein(mg/mL)	0.931	0.342	0.212	0.103	0.073	0.0433
Cellulase specific activity*	5.825± 0.414^{c}	3.098± 0.343^{e}	4.516± 0.381^{d}	14.820± 0.224^{a}	13.175± 0.371^{b}	6.696± 1.126^{c}

Values are presented as mean + standard deviation (n=5)
Values with different super scripts along a row are statistically different
*Expressed as micromoles of reducing substance per minute per milligram of protein

Table 5. Kinetic of protein absorption by sepharose-cibacron blue at different time interval.

Parameter	5 min	15 min	30 min	60 min
Absorbed protein (mg/mL)	0.230 ± 0.058^{a}	0.354 ± 0.039^{a}	0.276 ± 0.062^{a}	0.28 ± 0.043^{a}

Values are presented as mean + standard deviation (n=3); values with different super scripts along a row are statistically different ($p<0.05$).

Table 6. Comparison of protein absorption by sepharose-cibacron blue at different temperature.

Parameter	4°C	20°C	30°C	40°C
Absorbed Protein (mg/mL)	0.178 ± 0.039^{b}	0.135 ± 0.065^{b}	0.295 ± 0.029^{a}	0.275 ± 0.062^{a}

Values are presented as mean + standard deviation (n=3); values with different super scripts along a row are statistically different ($p<0.05$).

liquor are rapidly adsorbed to the sorbent and there is no significant difference among various periods ($p<0.05$). However, due to high numeric value, the amount of 15 min was considered as the minimum time required for binding of protein to sorbent. As the maximum adsorption rate of protein was obtained at temperature of 30 and 40°C, the column chromatography test was carried out at ambient temperature. In addition, 0.5 M sodium chloride (pH 6.5) was applied as the best condition ($p<0.05$) for the elution of the enzyme in the experiment (Table 7).

Figure 1 demonstrates the column chromatography test results. Fractions number from 15 to 35 turned out to have cellulase activity. The final results of cellulase purification are summarized in Table 8 which indicates that the proposed column chromatography procedure with Sepharose-CB can purify the cellulase about 14-fold.

It has been demonstrated that microbial enzymes are bound to compounds containing fibers using scaffoldin (Doi et al., 2003; Bayer et al., 1995; Bayer et al., 2007). Therefore many extra cellular enzymes are bound to solid substance. Hence, homogenizing and sonication approaches were employed for the liberation of cellulase from ruminal solid substances. Although Table 2 shows that the amounts of 1.5 and 2 min homogenizing resulted in higher level of protein release, the assay of specific activity of cellulase revealed that the most amount is attributed to plant non-enzymatic source. Therefore the best time of homogenizing chosen was 1 min. During TCA precipitation, a cellulase activity loss took place, most likely due to improper effect on enzymatic activity. The ethanol precipitation, a relatively easy to perform concentration, resulted in a good recovery of protein but less enzyme activity. Precipitation with ethanol requires large organic solvent volumes (at least nine fold of sample volume) and it is inconvenient to perform if the original sample volume is large. Quantitative ammonium sulfate precipitation also resulted in an efficient precipitation of protein and good enzyme activity although it usually requires two steps, precipitation with ammonium sulfate and removal of ammonium sulfate with dialysis. Freeze drying was the best method of concentrating; however, this method is cost and time consuming as the last stage being used to concentrate. Thus, the method of choice is precipitation with ammonium sulfate, where costs, enzyme activity and possibility of initial purification are important factors. It shows that Sepharose-CB resulted in the adsorption of non-cellulase enzyme protein. Also, the enzyme is weakly bound to sorbent so that it can be separated out of column following the unbound protein through buffer washing process. Moreover, fractions number from 15 to 35 turned out to have cellulase activity, whereas fractions which were separated out after applying elution buffer appeared to contain a large amount of protein and low level of enzyme specific activity.

Conclusion

A method for the separation and purification of cellulase

Table 7. Comparison of different agents for sepharose-cibacron blue elution.

Parameter	NaCl		EDTA			pH		
	1 M	0.5 M	0.1 M	0.1 M	0.05 M	4	6	8
Released protein (mg/mL)	0.175±0.016[a]	0.185±0.019[a]	0.100±0.003[c]	0.032±0.003[e]	0.039±0.003[e]	0.039±0.004[e]	0.136±0.016[b]	0.1445±0.012[b]

Values are presented as mean + standard deviation (n=3); values with different super scripts along a row are statistically different ($p<0.05$).

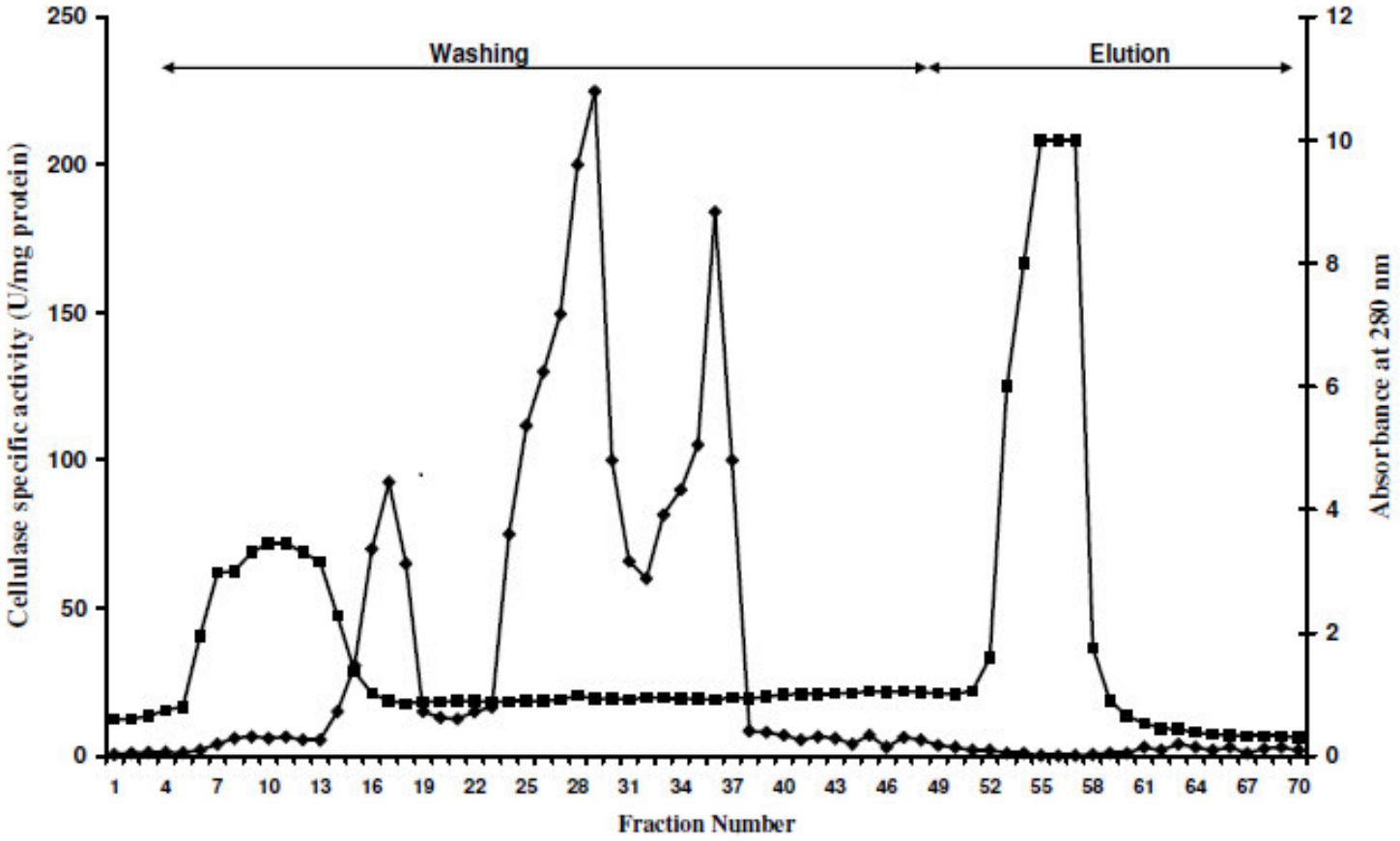

Figure 1. Chromatographic profile of cellulose using sepharose CL-6B-immobilized dye cibacron Blue F3GA. cellulase specific activity(-♦-). Absorbance at 280 nm (-■-).

Table 8. Summary of purification of rumen microbial cellulose.

Purification step	Volume (mL)	Protein (mg)	Activity (µmol min^{-1})	Specific activity [µmol min^{-1} (mg protein)$^{-1}$]	Recovery (%)	Purification (fold)
Crude extract	40	37.1	302	8.14	100	1
$(NH_4)_2SO_4$30-50%	5	3.83	41.21	10.76	13.64	1.2
Cibacron Blue F3GA	12	0.0736	9.34	126.9	3.1	14.24

from rumen content was described. Specific adsorption of the proteins on the modified resin was registered. The protein adsorption was due to immobilized Cibacron blue which has interactions with some specific proteins and enzymes. Based on different liberation methods, homogenizing method can be an efficient method for liberation of rumen microbial cellulase. Due to the growing interest in the application of affinity chromatography, the descrybed procedure might be useful for the development of pseudo-affinity for other enzymes. To the best of our knowledge, the current study is one of the first one have demonstrated potential for affinity adsorption of rumen cellulase content upon such matrices and deserves further study.

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