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Purification of alternanase by affinity chromatography

Abstract The enzyme alternanase, produced by *Bacillus* sp. NRRL B-21195, hydrolyzes alternan, a polysaccharide produced by certain strains of *Leuconostoc mesenteroides* that consists of glucose linked by alternating $\alpha(1 \rightarrow 6)$, $\alpha(1 \rightarrow 3)$ linkages. The main product of enzymatic hydrolysis by alternanase is a novel cyclic tetrasaccharide of glucose that also has alternating linkages between the glucose moieties. An improved purification scheme for alternanase has been developed that incorporates the use of isomaltosyl units linked to agarose for selectively binding the alternanase enzyme. Bound enzyme was eluted with 0.5 M sodium chloride and was nearly pure after this procedure. When followed by preparative isoelectric focusing, a single band of 117 kDa was measured when the purified protein was analyzed by HPLC size-exclusion chromatography/ multiangle light scattering. The purification procedure can be scaled to permit large quantities of enzyme to be purified in high (36%) yield.

Keywords Affinity chromatography · Alternan Alternanase · Cyclic tetrasaccharide · *Leuconostoc*

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Introduction

Alternan is an extracellular polysaccharide produced by several strains of *Leuconostoc mesenteroides* consisting mainly of alternating α-1,3-D-glucopyranosyl and α-1,6-D-glucopyranosyl residues [9]. This repeating, alternating linkage arrangement produces a structure that is rather resistant to enzymatic hydrolysis [5]. In 1994, Biely et al. [4] described the purification and properties of the novel enzyme alternanase from bacterial strain NRRL B-21195; this enzyme was the first glucanase shown to hydrolyze alternan in endo fashion. This soil-derived bacteria was tentatively identified as a *Bacillus* sp. based on the formation of heat-tolerant spores and other diagnostic features [16]. Strain NRRL B-21195 constitutively produces alternanase, unlike other similar strains that require alternan in the medium for enzyme production. One of the products of the hydrolysis of alternan by alternanase is a cyclic tetrasaccharide with a unique structure that maintains the alternating α-1,3 and α-1,6 linkages of the native polysaccharide [7,10]. Practical applications of this novel cyclic oligosaccharide are being evaluated, requiring significant amounts of the material for testing purposes. Therefore, there is a need for a consistent supply of alternanase in order to produce the cyclic tetrasaccharide for further research and evaluation. The method of purifying alternanase described by Biely et al. [4] produced very pure enzyme, but included techniques that are not amenable to large scale enzyme production, such as HPLC-SEC (size-exclusion chromatography), and cutting slices from SDS-PAGE gels for electroelution of the enzyme from the gel slice. Their reported yield of 5% was also a factor that stimulated our interest in developing a method of processing the enzyme to produce high purity material while significantly increasing the quantity and yield of the enzyme from the culture supernatant.

Recent studies of the specificity of this enzyme toward a variety of substrates revealed that alternanase is inhibited by isomaltose (D-glucopyranosyl-α-

1,6-D-glucopyranose), which is one of the reaction products in addition to the cyclic tetrasaccharide [2,6,10]. This finding suggested that there may be a binding site on the enzyme for isomaltose, which could be exploited by utilizing immobilized isomaltose to bind the enzyme. Immobilized isomaltose is available commercially and is used as an affinity ligand for concanavalin A. This report describes the use of immobilized isomaltose as an affinity ligand during the purification of alternanase to enhance yields of this enzyme. This method is also readily scalable for the purification of significant quantities of alternanase from strain NRRL B-21195. Current pilot-scale production of the cyclic tetrasaccharide in Japan emphasizes the need for improved methods for the purification of the enzyme. This and similar cyclic saccharides are currently under evaluation as precursors for pharmaceutical and specialty chemical applications as a complexants, chelators, cryoprotectants [11,12], charge control agents [3], and noncaloric food additives [8,12].

Materials and methods

Bacterial growth and preliminary enzyme isolation

Lyophilized samples of *Bacillus* sp. NRRL B-21195 were obtained from the bacterial culture collection maintained at the National Center for Agricultural Utilization Research (Peoria, Ill.). The strain was grown at 30°C on a rotary shaker in a medium containing 1.7% Bacto tryptone, 0.3% Bacto Soytone (both from Difco, Detroit, Mich.), 0.5% NaCl, 0.25% K_2HPO_4, and 0.25% maltose; the pH of the medium was adjusted to 7.0 prior to autoclaving [4,16].

For production of larger quantities of alternanase, 2.8 l Fernbach flasks containing 1 l of the standard tryptone/soytone medium with maltose were inoculated from starter cultures and grown with shaking at 30°C for up to 4 days, at which point assays of the culture fluid (described below) showed no further increases in hydrolytic activity. Bacteria were removed by centrifugation (17,000 g, 30 min at 4°C) and the supernatant was pooled and stored at 4°C for further processing. The cell-free supernatant was concentrated approximately 30-fold by ultrafiltration with a 10 kDa molecular-weight cut-off (MWCO) membrane (Millipore; Bedford, Mass.). Solid granular ammonium sulfate was added to concentrated enzyme solution to 50% saturation and stirred for 30 min at 0°C. The precipitated protein was isolated by centrifugation (17,000 g for 30 min at 4°C). The pelleted protein was dissolved in 25 mM sodium MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer, pH 7.0, and transferred to 10 kDa MWCO dialysis tubing and dialyzed for 48 h against 25 mM sodium MOPS, pH 7.0, containing 0.02% sodium azide.

Protein and enzyme assays

Alternan was prepared from sucrose enzymatically from alternansucrase from *L. mesenteroides* NRRL B-21297 [9]; for the synthesis of RBB-alternan (RBBA) it was derivatized with Remazol brilliant blue as described by Rinderknecht et al. [13]. Protein content was measured using the bicinchoninic acid assay (BCA assay; Pierce, Rockford, Ill.) using bovine serum albumin as the protein standard [14].

Alternanase activity was measured by one of two methods previously described [4]. One method utilizes RBBA as a substrate and is based on the formation of blue, ethanol-soluble alternan

oligosaccharides at 40°C by the action of alternanase. A second assay is based on the formation of total reducing sugar and is quantitated by an automated reducing sugar analyzer (AutoAnalyzer II; Bran and Luebbe Analyzing Technologies; Elmsford, N.Y.). This latter method underestimates activity because the major product of the reaction is a nonreducing cyclic tetrasaccharide, and hence is not detected by this method. One unit is defined here as the amount of enzyme that liberates the equivalent of 1 μmol D-glucose per minute at 40°C. The assay was performed with 2 mg/ml alternan in 50 mM sodium MOPS, pH 7.0, containing 1 mM $CaCl_2$ and 0.02% NaN_3 for varying times at 40°C. Reactions were stopped by adding 4% $NaCO_3$ and measuring the reducing sugar concentration on the AutoAnalyzer, which was calibrated with D-glucose.

Determination of binding capacity of alternanase on immobilized isomaltose

Isomaltose immobilized on 4% beaded agarose was obtained from Sigma (St. Louis, Mo.). It has a spacer arm of three atoms and is provided as a lyophilized powder. Dried resin (1 g) was rehydrated in water and washed with water several times to remove residual lactose. The swollen resin was resuspended in 50 mM sodium MOPS pH 7.0 and sufficient material was transferred to a small calibrated column to produce a bed of 1.0 ml. Individual 1.0 ml aliquots of crude enzyme from the ammonium sulfate treatment were passed over the column and the eluent was collected and assayed for alternanase activity by the reducing sugar assay. After elution of 15 ml the amount of enzyme in the eluent was equivalent to the activity level of the starting preparation, indicating that the binding sites on the column for alternanase were saturated. The column was washed with 15 ml (i.e., 15 column volumes) 50 mM sodium MOPS pH 7.0. The alternanase bound to the column was displaced with 1.0 ml aliquots of 50 mM sodium MOPS containing 0.5 M NaCl and the column eluent was collected and assayed for alternanase activity as described above. The active fractions were pooled and the protein concentration was determined by the BCA protein assay.

Preparative affinity chromatography on immobilized isomaltose

Dried isomaltose-agarose resin (3 g) was rehydrated in water and washed several times with water to remove residual lactose. It was transferred to a glass column with a 25 mm internal diameter and had a bed depth of approximately 25 mm. The resin was washed with several volumes of 25 mM sodium MOPS, pH 7.0, containing 0.02% sodium azide.

The concentrated alternanase solution from the ammonium sulfate precipitation was divided into four portions containing 7.8 mg total protein as measured by BCA assay. Each portion was added individually to the immobilized isomaltose column and the eluent was monitored at 280 nm. After a portion was passed over the column, the column was washed with 25 mM sodium MOPS, pH 7.0 until the absorbance of the eluent had returned to the baseline value. The column was then washed with 25 mM sodium MOPS containing 0.5 M NaCl and 0.02% sodium azide and collected in 2 ml fractions. The collected fractions were assayed for alternanase activity using the reducing sugar assay. After the column was re-equilibrated with the 25 mM sodium MOPS buffer, the remaining portions were processed in a similar fashion.

Preparative isoelectric focusing

Active fractions from the four individual immobilized isomaltose chromatography runs were pooled separately and dialyzed as described above. The volume of each dialyzed enzyme fraction was adjusted to 50 ml with deionized water and 1 ml of pH 3–10 ampholyte added (Bio-Rad; Hercules, Calif.). The mixture was

loaded in the preparative isoelectric focusing cell of a Rotofor preparative isoelectric focusing apparatus (Bio-Rad) and constant power (12 W) applied for 4 h; water at 0°C was circulated through the ceramic cooling core. After the 4 h period, fractions were vacuum-aspirated out of the Rotofor cell into 20 sample tubes and assayed for alternanase activity using the reducing sugar assay. Active fractions were pooled and dialyzed against 25 mM sodium MOPS, pH 7.0, containing 0.02% sodium azide, and stored at 4°C.

Assessment of protein purification

SDS-PAGE was performed on precast polyacrylamide gels (10×10 cm) using 3–8% gradient Tris-acetate buffered gels calibrated with the Mark12 protein standards (Novex NuPage gels and reagents; Invitrogen; San Diego, Calif.). Precast analytical IEF gels with a pH gradient from 3–10 were also from Novex; pH 3–9 pI standards were from Sigma. The purified alternanase preparation was analyzed by HPLC using a Shodex OH-pak KB806 M column (8×300 mm; Showa Denko, Ichihara, Japan) and 50 mM sodium nitrate containing 0.02% sodium azide flowing at 0.5 ml/min; the column temperature was maintained at 30°C. The column eluent was monitored with a MALS detector (DAWN EOS; Wyatt Technology, Santa Barbara, Calif.) and an interferometric refractometer (Optilab DSP; Wyatt Technology). The molecular mass was determined by the ASTRA software (version 4.73) supplied with the DAWN EOS detector (Wyatt Technology).

Results and discussion

The results of the purification of alternanase are summarized in Table 1. Examination of the degree of purity at each stage (Fig. 1) revealed that virtually all of the protein after affinity chromatography appeared to be contained in two bands that migrated at molecular masses of 140 and 116 kDa, the latter being close to the molecular mass reported by Biely et al. [4] of 110 kDa for alternanase from this strain. The 140 kDa protein was removed by preparative isoelectric focusing. Examination of the final concentrate by SDS-PAGE showed a single band of molecular mass 117 kDa (Fig. 1, lane 5) indicating that the Rotofor had efficiently separated the two proteins and that the alternanase was now electrophoretically pure. Analytical isoelectric focusing showed a protein band with a pI of 4.5 after the final purification step (data not shown). Activity staining verified that this was alternanase. The lack of appreciable increase in specific activity after preparative isoelectric focusing despite the obvious increase in purity (Table 1, Fig. 1) suggests significant denaturation during this stage. The overall yield of 36%
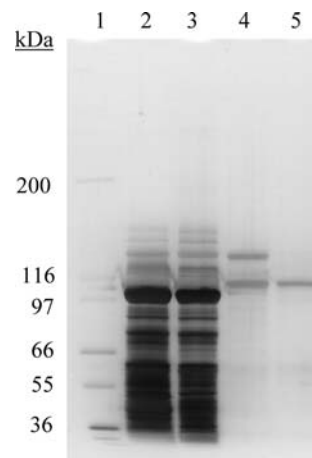

Fig. 1 Analytical SDS-PAGE on Tris-acetate buffered 3–8% acrylamide gradient gels followed by silver staining. Lanes: *1* Novex Mark12 protein standards, *2* B21195 crude cell-free concentrate, *3* ammonium sulfate precipitate after dialysis, *4* pooled and concentrated sample after immobilized isomaltose chromatography, *5* pooled and concentrated sample after preparative isoelectric focusing with a pH 3–10 gradient

compares favorably with the 5% yield previously reported by Biely et al. [4].

The molar mass of the purified enzyme was also analyzed by HPLC-SEC with multiangle light scattering (MALS) detection [15]. The single peak was homogeneous, with a calculated molecular mass of 117 kDa. This is consistent with the value of 116 kDa as determined by SDS-PAGE, and is reasonably close to that of 110 kDa determined by Biely et al. [4] under slightly different conditions.

The use of immobilized isomaltose as an affinity ligand in the purification of alternanase is a marked improvement over the previously described purification method, resulting in significantly higher yields. The method is easy to perform and is reasonably scalable so that significant quantities of purified alternanase can be produced. For many applications the level of purity obtained after the affinity chromatography step alone would be sufficient, eliminating the problematic denaturation observed during isoelectric focusing. The alternanase obtained from affinity chromatography is useful for the production of the cyclic tetrasaccharide from alternan. The identity of the 140 kDa protein that adsorbs to the immobilized isomaltose column and

Table 1 Summary of thepurification of alternanase from NRRL B-21195

Step	Volume ml	Protein		Alternanase activity		Specific activity U/mg	Purification degree	Yield %
		concentration mg/ml	amount mg	U/ml	U			
Culture fluid concentrate	250	1.26	315.0	0.03	7.50	0.02	1	100
(NH$_4$)$_2$SO$_4$ precipitate	35	0.89	31.2	0.17	5.95	0.19	9.5	79
Immobilized isomaltose	70	0.15	10.5	0.12	4.98	0.47	23	66
Rotofor	45	0.12	5.5	0.06	2.70	0.49	25	36

co-elutes with alternanase in the 0.5 M NaCl wash was initially unknown, but recent results from our laboratory indicate that it is a glucosyltransferase capable of hydrolyzing α-1,4-linked glucosaccharides and forming α-1,6-linkages (M. Kitaoka et al., personal communication). Studies are currently underway to characterize this novel transferase and to discover the role of both enzymes in the metabolism of starch by this organism. Recent work in Japan has shown that this enzyme may be part of a complex that certain bacteria have evolved for the metabolism of starch, providing them with a unique adaptive advantage [1].

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