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Detection of Major Xylanase-Containing Cellulose-Binding Domain from *Penicillium verruculosum* by Combination of Chromatofocusing and Limited Proteolysis

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Abstract

Adsorption on microcrystalline cellulose of enzyme components of cellulase complex from *Penicillium verruculosum* was studied by chromatofocusing on a Mono P column. The most strongly adsorbed and major component was identified as xylanase (XYN) with MW 65 kDa and p*I* 4.5. The high adsorption degree of XYN on cellulose indicated the possible presence of a cellulose-binding domain in the molecular structure. Limited proteolysis of XYN with papain was carried out. Kinetics of proteolysis was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and measuring activities toward insoluble xylan and 4-methylumbelliferyl-β-D-lactoside (MUF-LAC). During the proteolysis, formation of two polypeptides with MW 51 and 14 kDa was observed. No loss of activity toward the soluble substrate was observed, whereas the activity toward xylan decreased rapidly. Adsorption distribution coefficient (K_d) of the core protein separated by gel-filtration was found to be 15 times lower than the K_d for the initial nondigested XYN (0.02 and 0.29 L/g, respectively). The activity of core protein toward insoluble xylan was close to zero, whereas the activity toward MUF-LAC was close to that exhibited by the original enzyme. The results presented indicate a bifunctional organization of XYN, where one domain acts as a binding anchor for insoluble substrates and the other, localized in the core protein, contains the active site.

Index Entries: *Penicillium verruculosum;* xylanase; cellulose-binding domain; limited proteolysis; papain; enzyme adsorption.

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Introduction

Enzymatic cellulose hydrolysis is a heterogeneous reaction catalyzed by cellulase multicomponent systems (cellulase complexes) *(1)*. As a rule, cellulase complexes also contain xylanases, enzymes involved in hydrolysis of the hemicellulose fraction of plant biomass *(2)*. Because the adsorption of cellulases and xylanases on insoluble substrates is a prerequisite stage to hydrolysis, clear understanding of the adsorption properties of the enzymes may provide essential information on the reaction mechanism.

Previous studies on cellulase complexes have used activity measurements toward several substrates to quantify the adsorption of enzyme components *(3,4)*. The measurement of activities of cellulases toward traditional substrates (carboxymethylcellulose, Avicel) did not allow clear information about the adsorption properties of individual cellulase components to be obtained. This situation was even more complicated by the fact that cellulase complexes contain multiple cellulase and xylanase components, which may display nonspecific activities. Elution profiles after chromatofocusing of an original cellulase preparation and nonbound enzyme fraction (supernatant) obtained after the adsorption of the preparation on cellulose could be used for quantitative monitoring of adsorption ability of individual enzyme components. Limited proteolysis of those protein fractions after chromatofocusing that strongly bind to cellulose could be used for an identification of enzymes with a possible bifunctional structure organization, where one domain acts as a binding anchor for insoluble substrates and the other, localized in the core protein, contains the active site *(5–7)*.

In this article, we used the combination of chromatofocusing with limited proteolysis for identification of the major xylanase from *Penicillium verruculosum*, which had the highest adsorption ability among the components of multienzyme system, and for a verification of its bifunctional structure organization.

Materials and Methods

Enzyme and Activity Assays

A laboratory cellulase preparation 3-55.1.1 produced by fungus *Penicillium verruculosum* was used *(8)*. Activities toward soluble carboxymethylcellulose (CMC) and carboxymethylxylan (CMX) were determined by incubating 0.5 mL of diluted enzyme preparation with 0.5 mL of 10 g/L CMC (medium viscosity, Sigma) or CMX (NPO Biolar, Latvia) solution in acetate buffer (0.1 *M*, pH 4.5) for 15 min at 50°C. Activity toward insoluble xylan was determined using a suspension of birchwood xylan (Sigma); 0.5 mL of diluted enzyme preparation and 0.5 mL of xylan suspension (10 g/L) in 0.1 *M* acetate buffer, pH 4.5, were incubated for 30 min at 50°C. Avicelase activity was assayed using 5 g/L suspension of Avicel cellulose (Serva, Germany) at 40°C and pH 4.5. In all cases, reducing sugars formed were measured by the Somogyi–Nelson method *(9)* using glucose as a standard.

Endoglucanase activity was assayed viscometrically at 40°C and pH 4.5 using CMC as a substrate *(10)*.

Activities toward 4-methylumbelliferyl-β-D-lactoside (Sigma) (MUF-LAC) and 4-methylumbelliferyl-β-D-xyloside (Sigma) (MUF-XYL) were assayed spectrophotometrically by measuring the initial rate of hydrolysis at 350 nm *(11)*. The initial concentration of substrates was 0.5 m*M*. The assays were carried out at 40°C and pH 4.5.

All activities were expressed in international units (IU), where 1 U corresponds to the quantity of enzyme hydrolyzing 1 μm of glucoside bonds per 1 min.

Determination of Adsorption Distribution Coefficients

Adsorption distribution coefficient (K_d) for purified homogeneous xylanase was determined with Avicel cellulose at 50°C and pH 4.5 *(11)*. K_d was calculated as the slope of a straight line in coordinates E_o/E_s against cellulose concentration, where E_o is the initial protein concentration before adsorption, E_s is the protein concentration in solution after the adsorption. Protein concentration was measured by the Lowry's method *(12)* using bovine serum albumin (BSA) as a standard.

Chromatofocusing and Monitoring of Adsorption Ability of Enzyme Fractions

Adsorption of a cellulase complex from *P. verruculosum* on Avicel cellulose (50 g/L) was carried out under agitation at 50°C and pH 5.0 (0.1 *M* acetate buffer). Initial protein loading was 16 mg/g substrate. After 15 min of incubation, the supernatant containing nonbound proteins was separated by centrifugation.

The original solution of cellulase sample and supernatant obtained after the adsorption of the enzymes on Avicel were desalted using a Bio-Gel P-6 column (Bio-Rad, Hercules, CA) equilibrated with 25 m*M* imidazole-HCl buffer (pH 7.4). Desalted enzyme solutions were separated into fractions using chromatofocusing on a Mono P HR 5/20 column (Pharmacia, Sweden) using Pharmacia FPLC system. Elution was carried out with 40 mL of diluted (1:10) Polybuffer 74-HCl (gradient of pH from 7.4 to 4.0) and then with 15 mL of the diluted Polybuffer (gradient of pH from 4.0 to 3.0). The flow rate was 0.9 mL/min. Protein concentration at the column outlet was monitored photometrically at 280 nm using an ultraviolet detector. The elution profiles of enzyme solutions before and after adsorption on Avicel were used to quantify the adsorption degree of each enzyme fraction (Fig. 1).

Limited Proteolysis of Major Xylanase (XYN)

Limited proteolysis of XYN was carried out incubating the enzyme with activated papain (Sigma) *(6)*. The ratio of XYN to papain (w/w) was 30:1; 300 μL of XYN (2 mg/mL) were incubated with 10 μL of papain (2 mg/mL, 30 BAEE U/mL) at pH 6.5 for 15 h.

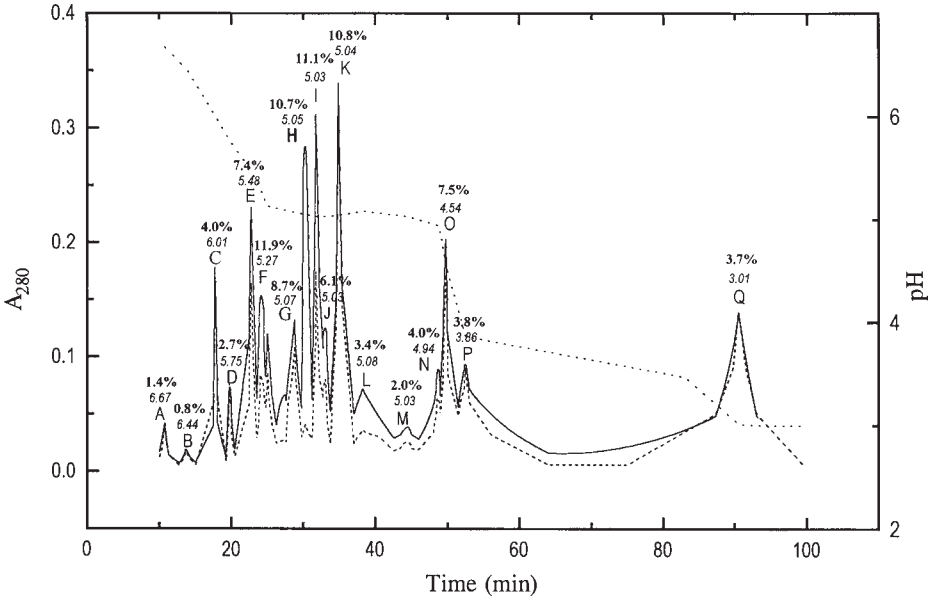

Fig. 1. Monitoring of adsorption of protein fractions of enzyme complex from *P. verruculosum* on Avicel cellulose by chromatofocusing on a Mono P HR 5/20 column. ——, protein elution profile of the original preparation (before adsorption); – – – –, protein elution profile of supernatant after adsorption; ……, pH gradient profile.

Kinetics of proteolysis was monitored by SDS-PAGE analysis (Mini Protean II, Bio-Rad) and measuring activities toward xylan and MUF-LAC.

SDS-PAGE and IEF-PAG analysis (Model 111 Mini IEF Cell, Bio-Rad) of XYN and papain-digested enzyme were carried out conventionally. SDS-PAGE was run on 12% and 15% gels under denaturing reducing conditions (1% SDS, 0.1% mercaptoethanol). Staining was carried out with Coomassie Blue.

Preparative Separation of XYN Catalytic Domain

A catalytic domain (core protein) of XYN, obtained by papain-digestion, was separated by gel-filtration on a Superose 12 HR 10/30 column (Pharmacia) using FPLC system. The column was equilibrated with 0.1 *M* acetate buffer (pH 5.0), and elution was carried out using the same buffer. Gel-filtration was carried out twice with 200-µL samples of a digested enzyme solution. Eluted fractions containing core protein were pooled and activities and K_d were measured.

Results

Evaluation of Adsorption Ability of Separated Enzyme Fractions

In order to identify enzyme components with high adsorption ability on cellulose, the multienzyme preparation was separated into fractions by chromatofocusing on a Mono P column using Pharmacia FPLC system.

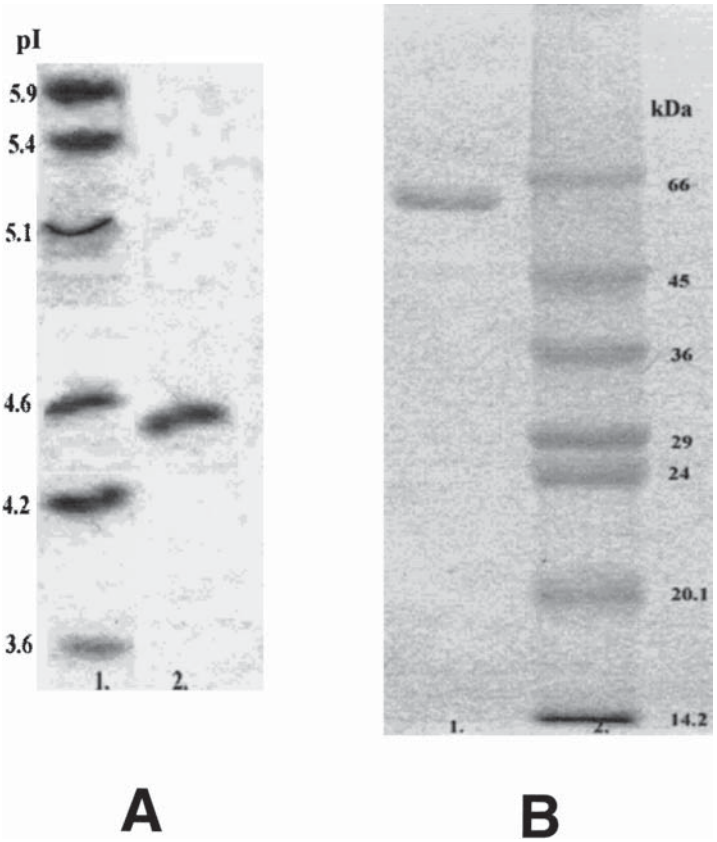

Fig. 2. IEF-PAG **(A)** and SDS-PAGE **(B)** of XYN purified by chromatofocusing. Lanes: (A) 1, IEF markers; 2, XYN. (B) 1, XYN; 2, molecular mass markers.

Two separations were carried out: one of original preparation and another of supernatant obtained after the adsorption of original preparation on microcrystalline cellulose (Avicel). This allowed quantitative monitoring of adsorption ability of individual enzyme fractions on cellulose (Fig. 1). Each fraction was marked by a letter. Numbers above peaks in Fig. 1 show a relative content (%) of the corresponding component in the crude preparation and pH, at which the fraction is eluted from the column.

Some adsorption was observed for protein fractions E, F, G, I, J, L, M, and N. The most strongly adsorbed and major component was that from the H fraction with the adsorption degree of 82%. This component represented 11% of the total protein in the cellulase complex.

Characterization of Xylanase from Fraction H

Biochemical parameters of the enzyme from the fraction H were determined by SDS-PAGE and IEF-PAG. It was a homogeneous 65 kDa protein with p*I* approx 4.5 (Fig. 2). High specific activities toward xylan and CMX as well as relatively low cellulase activities of this enzyme (Table 1) allowed

Table 1
Specific Activities (U/mg protein) of Xylanase from *P. verruculosum*[a]

CMCase	Endoglucanase	Avicelase	Xylanase[b]	CMXase	MUF-LAC	MUF-XYL
0.95 ± 0.06 (6.5)	0.27 ± 0.01 (1.8)	0.00 (0)	14.7 ± 0.7 (100)	9.4 ± 0.6 (63.9)	0.24 ± 0.01 (1.6)	0.00 (0)

[a]Relative activities of the enzyme, taking xylanase activity as 100%, are shown in parenthesis.
[b]Activity toward insoluble xylan.

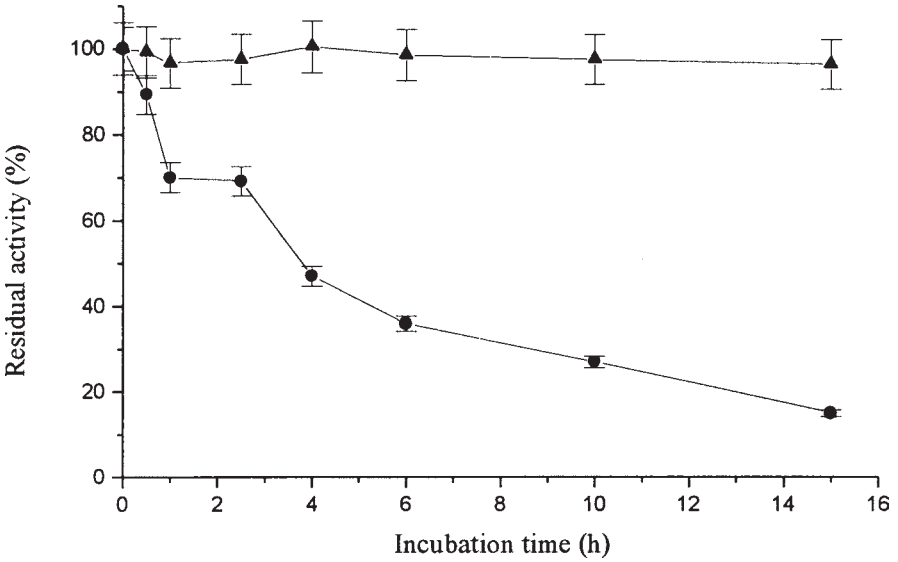

Fig. 3. Residual activities of papain-digested XYN given as a percent of the initial activity. ●, activity toward insoluble xylan; ▲, activity toward MUF-LAC.

it to be classified as xylanase (XYN). This was the first xylanase purified from *P. verruculosum* in our laboratory (previously, several endoglucanases and cellobiohydrolases have been isolated *[8]*). Nonspecific activity toward a low-molecular substrate (MUF-LAC) as well as some cellulase activity did not allow classifying XYN as a strict endo-β-1,4-xylanase.

Effects of Limited Proteolysis on Activities and Molecular Weight of XYN

Aliquots of papain-digested XYN were analyzed at time intervals for residual xylanase and MUF-LAC activities (Fig. 3). No loss of activity against the soluble low-molecular substrate was observed even after 15 h of incubation, whereas the activity toward insoluble xylan decreased rapidly.

During the proteolysis, a formation of two polypeptides with the molecular weights of approx 51 and approx 14 kDa was detected by SDS-PAGE analysis under reducing conditions (Fig. 4). Even after the extensive proteolysis (6 h), the higher molecular mass fraction (51 kDa) persisted.

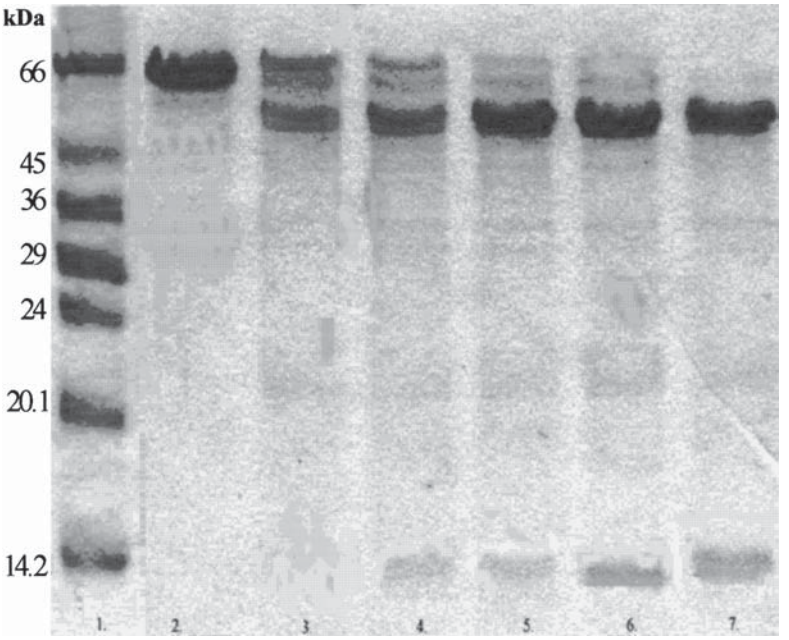

Fig. 4. SDS-PAGE of papain-digested XYN. Lanes: 1, molecular mass markers; 2, initial XYN; 3–7, papain-digested XYN at 0.5, 1, 2, 4, and 6 h, respectively.

Separation, Adsorption Ability, and Activities of XYN Core Protein

The major polypeptide (approx 51 kDa, XYN core protein) was separated by gel-filtration on a Superose-12 column. Its adsorption ability on Avicel cellulose and specific activities were studied. Adsorption distribution coefficient (K_d) of the core protein was found to be 0.02 L/g. It was 15 times lower than the K_d for the initial nondigested xylanase (0.29 L/g). The activity of the core protein toward insoluble birchwood xylan was close to zero, whereas the activity towards MUF-LAC was close to that exhibited by the original enzyme.

Discussion

The relative ease in separating the two polypeptides by papain-proteolysis suggests that these protein domains may be linked by an amino acid sequence susceptible to proteolytic attack. In the case of cellulolytic enzymes, a two-domain structure has been reported for the first time for cellobiohydrolase from *Trichoderma reesei (6)*. Since then, the bistructural–bifunctional organization, where the catalytic domain is connected to the cellulose-binding domain via a flexible peptide linker, has been reported for many cellulases and xylanases *(13,14)*. Typically, the enzymes lost the ability to bind to cellulose after a removal of cellulose-binding domains, and this was accompanied by a significant decrease or complete loss of

activity toward the insoluble substrate *(6,13)*. At the same time, the activity toward soluble substrates was conserved as a rule.

Surprisingly, in this study on *P. verruculosum* cellulase complex, the enzyme component with the highest adsorption ability on cellulose was identified as xylanase but not cellulase. The combination of data on adsorption and catalytic properties of original XYN and its core protein, presented above, allowed us to conclude that XYN represents a typical two-domain protein, where one domain has a cellulose- and xylan-binding site and the other, localized in the core protein, contains the active site.

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