C-Terminal domain of β-1,3-glucanase H in *Bacillus circulans* IAM1165 has a role in binding to insoluble β-1,3-glucan

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Received 17 April 1998; revised version received 3 July 1998

Abstract The deduced amino acid sequences of 72-kDa β-1,3-glucanase from *Bacillus circulans* WL-12 (GlcA) and 91-kDa enzyme from *B. circulans* IAM1165 (BglH) are highly homologous, except that the latter has an additional long C-terminal region composed of 192 amino acid residues. Two mutant enzymes (BglH deprived of the C-terminal region and GlcA with the C-terminal region added) were constructed. The enzymes possessing the C-terminal region bound more abundantly to pachyman (insoluble β-1,3-glucan) and *Aspergillus oryzae* cell wall than those not possessing the region. This indicates that the C-terminal region participated in binding of the enzymes to insoluble β-1,3-glucan.

Key words: *Bacillus circulans*; Laminarinase; β-1,3-Glucanase; Sequence homology

1. Introduction

In recent years, genes encoding β-1,3-glucanases have been cloned and sequenced from some bacteria [1–3]. We have cloned the gene for the β-1,3-glucanase from *Bacillus circulans* IAM1165 or WL-12 and sequenced its nucleotides [4,5]. Multiple alignment of the deduced amino acid sequence of β-glucanases from *B. circulans* and other β-glucanases showed conserved blocks [3]. The region is well conserved in bacterial β-1,3-1,4-glucanases. The deduced amino acid sequence of mature protein shows 84.6% homology to the BglH from *B. circulans* IAM1165 and the GlcA from *B. circulans* WL-12. These enzymes are similar to each other in biochemical characters except for the activity of the BglH at 70°C, which is higher than that of GlcA. The BglH has an extra C-terminal region of 193 amino acid residues, which has not been found in other β-1,3-glucanases or β-1,3-1,4-glucanases. In this paper, we describe the construction and purification of the hybrid enzymes and the comparison of the enzymes in terms of their biochemical characteristics. We also studied their affinities to insoluble β-1,3-glucans.

2. Materials and methods

2.1. Bacterial strain, plasmids and culture media

The *Escherichia coli* strain used in this study was JM101 (Takara Shuzo Co). The vectors used were pHSG399 (Takara Shuzo Co) and pBluescript SK+ (Stratagene). Full-length *bglH* (encodes BglH) and *glcA* (encodes GlcA) were encoded by pBG200 and pNT003, respectively [6,7]. The organism was grown in LB medium [8]. If necessary, the medium was solidified with 1.5% (w/v) agar. Ampicillin (100 μg/ml) or chloramphenicol (30 μg/ml) was used to select cells harboring one of the plasmids.

2.2. General recombinant DNA techniques

Preparation of plasmid DNAs, restriction enzyme digestion, ligation and transformation were performed as described [8].

2.3. Purification of β-1,3-glucanases

Recombinant β-1,3-glucanases were purified as described previously [6]. *E. coli* cells harboring plasmid were grown in LB [8] supplemented with 0.2% (w/v) glucose and ampicillin (100 μg/ml). The cells were harvested by centrifugation (8000×*g*, 5min).

2.4. Enzyme assay

β-1,3-Glucanase was assayed with 2% (w/v) substrate in 200 μl of enzyme solution and piperazine-*N*,*N*-bis-(2-ethanesulfonic acid) (PIPES)-NaOH buffer (0.1 M, pH 7.0). The mixed solutions were incubated for 10 min at 40°C. The reaction was stopped by addition of 1 ml 3,5-dinitrosalicylic acid and the amount of reducing sugar (as glucose equivalents) was measured as described previously [9]. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that released reducing saccharides equivalent to 1 μmol of glucose per min under these condition.

2.5. Binding assay

The enzymes (approximately 1 U) were incubated in 300 μl of 0.2 M PIPES-NaOH buffer (pH 7.0) containing polysaccharide (2 mg) with mild agitation. After 1 h, the mixture was centrifuged at 1000×*g* for 5 min at 4°C. The enzyme left in the supernatant was measured by standard assay. The amount of the enzyme bound to the polysaccharide was estimated by subtraction of the activity recovered from the original activity.

2.6. Polysaccharides

Laminarin produced by *Laminaria digitata* and lichenan produced by *Getraria islandica* were purchased from Sigma. Curdlan by *Alcaligenes faecalis* was purchased from Wako Pure Chemicals (Osaka, Japan). Chitin from crab shells was purchased from Nacalai Tesque (Kyoto, Japan). Pachyman was prepared from commercial fruiting bodies of the basidiomycete *Poria cocos*. Colloidal pachyman was prepared from Pachyman as described previously [7]. The cell wall preparation of *Aspergillus oryzae* used was a kind gift from K. Horikoshi.

3. Results and discussion

There is a common *Eco*RV recognition site on the DNA corresponding to the common sequence. Hybrid genes were constructed from the two genes cleaved on this *Eco*RV site (Fig. 1). The resulting plasmid pSKHΔC contained a novel gene encoding a truncated β-1,3-glucanase BglHΔC. A mature form of this enzyme consisted of 647 amino acid residues. The C-terminal amino acid region (Glu-648 to Glu-839) of the BglH was truncated and Gly-647 was converted to a novel C-terminal Gln-647. This terminal amino acid was derived from Gln-644 of GlcA. Alternatively, the GlcA C-terminus was annexed to the C-terminal region (Glu-648 to Glu-839)

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PII: S0014-5793(98)00881-3

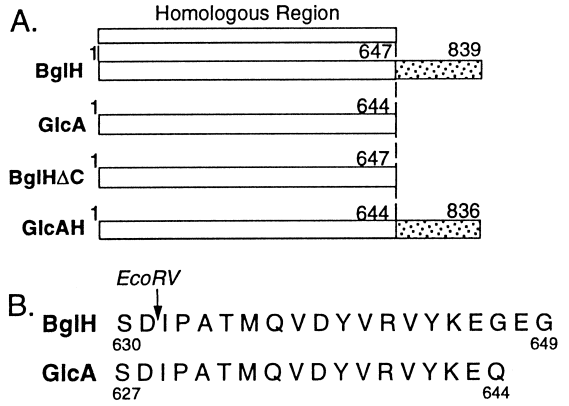

Fig. 1. Schematic comparison of mature BglH, GlcA and chimera enzymes. A: Open boxes represent GlcA, and a region of BglH homologous to GlcA. Dotted boxes represent the C-terminal regions found only in BglH and a chimera enzyme GlcAH. The amino acids of each protein are numbered from N-termini of the mature protein and shown above the boxes. B: Map of BglH/GlcA junctions in chimeric proteins. Junctions between the BglH and GlcA were made by the *Eco*RV restriction site, which occurs in the BglH gene and the GlcA gene.

of BglH. The pKSAH contained a hybrid gene encoding a hybrid β-1,3-glucanase, GlcAH. A mature form of this chimera enzyme was composed of 836 amino acid residues, in which Ala-1 to Asp-628 was derived from GlcA and Ile-629 to Glu-836 was derived from BglH. As a result, the enzyme was mainly GlcA with 192 amino acid residues identical to the C-terminal region (Glu-648 to Glu-839) of BglH.

The recombinant β-1,3-glucanases were purified from the periplasmic fractions (spheroplast juice prepared by the lysozyme treatment) of *E. coli* JM101 transformants in the same manner (ion-exchange chromatography and hydrophobic chromatography) [6]. SDS-PAGE showed that molecular masses of purified BglH and GlcAH were approximately 91 kDa and those of GlcA and BglHΔC were approximately 72 kDa.

The specific activities of BglH, BglHΔC, GlcA and GlcAH were 192, 134, 309, and 127 U/mg, respectively. These activities were equivalent to specific molar activities of 18, 9.7, 22 and 9.1 U/nmol. The specific molar activity of each chimera enzyme for laminarin was reduced from the original enzyme. These results indicated that activity levels of the enzymes were not determined simply by the presence of the C-terminal region.

The temperature-activity and pH-activity profiles of BglHΔC were almost the same as those of BglH (Fig. 2). BglHΔC was most active at pH 4–5 and 70°C, like BglH. The activity profiles of GlcAH were similar to those of GlcA. GlcAH did not acquire high activity at elevated pH or temperature despite the presence of the C-terminal region. These results indicate that the high activity of the enzyme BglH at elevated temperature and pH was due to the region from N-terminal to central of BglH. There was 15.4% difference in amino acid sequence beyween BglHΔC and GlcA. We consider that the difference is important for the high activity of the enzyme at elevated temperature and pH. It was concluded that the C-terminal region did not participate in the activity at high temperature and pH.

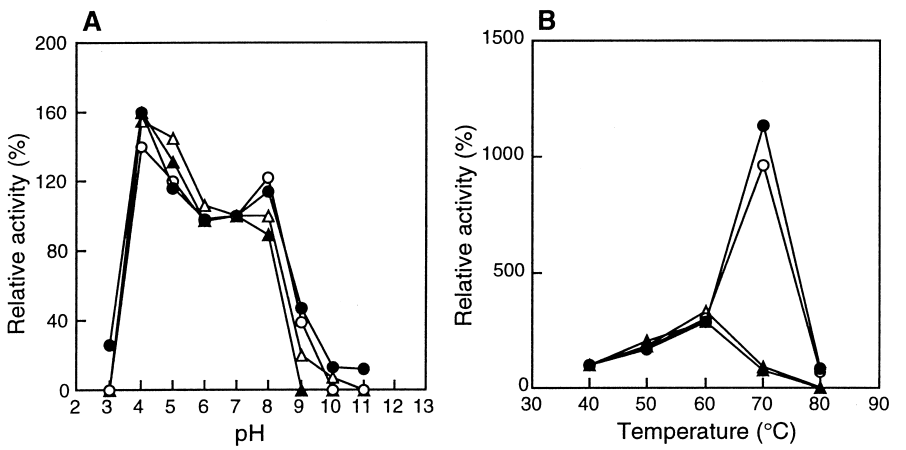

Fig. 2. Effects of temperature and pH on activity. A: Activity-temperature curve. The activity was measured at various temperatures under the standard assay conditions. B: Activity-pH curve. The activity was measured at various pH under the standard assay conditions. Symbols: ○, BglH; △, GlcA; ●, BglHΔC; ▲, GlcAH.

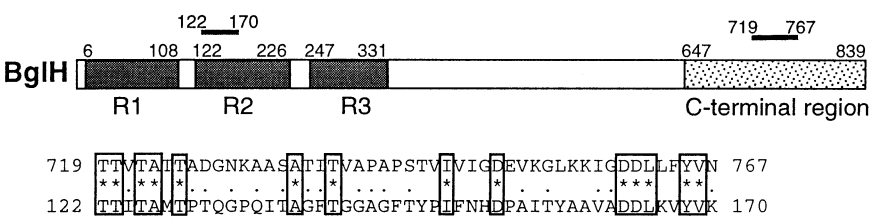

Fig. 3. Homology of the C-terminal region of BglH. The shaded boxes show N-terminal-repeated sequences R1–3 proposed for binding domains to colloidal pachyman. The dotted box indicates the C-terminal region of BglH. The amino acid sequences of R2 (aa 122–170) and the C-terminal region (aa 719–767) are shown in the figure. Among these amino acids, identical amino acids are shown with asterisks and similar ones are shown with dots.

Table 1
Binding of β-1,3-glucanases to insoluble polysaccharides

Polysaccharide	Enzyme binding to the saccharides[a] (%)			
	BglH	BglHΔC	GlcAH	GlcA
Pachyman	30 (2.1)	25 (1.0)	37 (1.1)	27 (1.5)
Colloidal pachyman	80 (0.5)	78 (2.5)	87 (2.3	83 (1.4)
Curdlan	79 (2.0	54 (1.8)	77 (0.6)	60 (3.4)
Lichenan	80 (0.5)	79 (0.8)	79 (0.4)	79 (0.1)
Fungal cell wall	47 (3.0)	15 (2.8)	32 (0.4)	19 (1.7)
Chitin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a]Data are means of 3–5 independent experiments.
Figures in parentheses are the standard deviation.

In a previous study, N-terminal repeated domains of GlcA were shown to bind β-1,3-glucans, colloidal pachyman (alkali-soluble β-1,3-glucan prepared from pachyman) and laminarin [10]. BglH and GlcA have the three-repeated N-terminal domains [4]. A part of the C-terminal region (Thr-719 to Asn-767) of BglH showed a low homology to a portion of the second N-terminal domain (Thr-122 to Val-226) (Fig. 3). In addition, the region Gly-649 to Ala-739 is somewhat homologous to C-terminal sequence of κ-carrageenase derived from *Alteromonas carrageenovora* (results not shown), although any biological meaning of the sequence has not been shown in the κ-carrageenase [11]. These homology analyses suggested that the C-terminal region of BglH might possess some binding activity for polysaccharides.

To examine the possibility that the C-terminal region might function as a polysaccharide-binding domain, binding activity of each enzyme was evaluated using several polysaccharide preparations (Table 1). All the enzymes bound effectively to colloidal pachyman and lichenan (β-1,3-1,4-glucan). The enzymes bound less abundantly to pachyman and fungal cell wall than to colloidal pachyman, curdlan (alkali-soluble β-1,3-glucan of *Agrobacterium* sp.) or lichenan. BglHΔC bound less abundantly to pachyman and curdlan than BglH. The binding rate of BglH to pachyman decreased to 25% by deletion of the C-terminal region. The binding rate of GlcA to pachyman was similar to that of BglHΔC. Furthermore, BglH and GlcAH adsorbed highly to fungal cell wall or curdlan, compared with BglHΔC and GlcA, respectively. No enzyme bound to chitin, which is a minor component of the fungal cell wall. These results indicate that the enzymes not containing the C-terminal region were partially impaired in affinity for pachyman, fungal cell wall and curdlan. However, they retained a high binding ability to colloidal pachyman and lichenan. These facts suggest that the binding activity of the enzymes depended not only on a local linkage between glycoside residues but also on the overall conformation of the glucan preparations, and that a significant portion of the binding ability was also contributed by the N-terminal region of BglH and GlcA.

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