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Nonproteolytic Cleavage of Aspartyl Proline Bonds in the Cellulosomal Scaffoldin Subunit from *Clostridium thermocellum*

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Abstract

Previous work from our group [Morag (Morgenstern), E., Bayer, E. A., and Lamed, R. (1991), *Appl. Biochem. Biotechnol.* **30,** 129–136] has demonstrated an anomalous electrophoretic mobility pattern for scaffoldin, the 210-kDa cellulosome-integrating subunit of *Clostridium thermocellum*. Subsequent evidence [Morag, E., Bayer, E. A., and Lamed, R. (1992), *Appl. Biochem. Biotechnol.* **33,** 205–217] indicated that the effect could be attributed to a nonproteolytic fragmentation of the subunit into a defined series of lower-molecular-weight bands. In the present work, a recombinant segment of the scaffoldin subunit was employed to determine the site(s) of bond breakage. An Asp-Pro sequence within the cohesin domain was identified to be the sensitive peptide bond. This sequence appears quite frequently in the large cellulosomal proteins, and the labile bond may be related to an as yet undescribed physiological role in the hydrolysis of cellulose by cellulosomes.

Index Entries: Cellulosome; multienzyme complex; cohesin domain; Asp-Pro bond; cellulases; *Clostridium thermocellum*.

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Introduction

Various cellulolytic bacteria produce a cohesive, high-molecular-weight, multicomponent, cellulolytic complex, called the cellulosome *(1–4)*. The definitive subunit of this complex is the scaffoldin subunit, which both serves to bind the cellulosome to its substrate and to integrate the other (hydrolytic) subunits into the cellulosome.

Scaffoldin is composed of multiple types of domains. One, the cellulose-binding domain (CBD), provides the substrate-binding function of the cellulosome. A second type of scaffoldin domain, the cohesin domain, occurs in multiple copies and participates in a novel affinity interaction with complementary dockerin domains of the enzymatic subunits, thereby incorporating them into the cellulosome complex.

Most of the early work on the cellulosome was performed using the thermophilic anaerobe *Clostridium thermocellum*. The cellulosome of *C. thermocellum* is a particularly stable complex, and, historically, the complex could be dissociated only under denaturing conditions *(1)*—i.e., the presence of a detergent and high temperatures (>70°C). Altering the pH or ionic strength led to an anomalous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) migratory pattern of the scaffoldin subunit and some of the larger enzymatic subunits *(5)*. It was postulated that these conditions led to nonproteolytic covalent bond cleavage of an unusually sensitive type of peptide bond *(6)*. In this communication, we demonstrate that the sensitive bond is an aspartyl proline bond.

Materials and Methods

Cellulosome Preparations

The cellulosome of *C. thermocellum* strain YS was prepared by affinity digestion as described previously *(7)*.

Expression and Purification of Scaffoldin Segments

A recombinant scaffoldin segment (Coh2-CBD) comprising the proximate cohesin 2 and cellulose-binding domains, separated by a Pro/Thr-rich linker sequence, was expressed in *Escherichia coli* containing an inducible T7 polymerase BL21(DE3), as described by Yaron et al. *(8)*. The expressed protein was isolated by affinity chromatography on a microcrystalline cellulose column by virtue of the resident CBD of the construct.

pH-4 Pretreatment

Coh2-CBD and cellulosome samples (0.5 mg/mL in 50 m*M* Tris-HCl buffer, pH 7.5) were dialyzed overnight against 25 m*M* sodium acetate buffer, pH 4. The samples were then mixed with 1 vol of sample buffer (3% SDS, 5% mercaptoethanol, and 10% glycerol in 62.5 m*M* Tris-HCl buffer, pH 6.8), and subjected to SDS-PAGE, performed on 10% gels. The gels were stained using Coomassie brilliant blue R250.

Following dialysis against pH-4 buffer, control samples were neutralized by adding a 0.1 vol aliquot of 0.5 *M* Tris-HCl buffer, pH 7.5, prior to SDS-PAGE.

Peptide Sequencing

SDS-PAGE-separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes by the method of Matsudaira *(9)*. The blots were stained with Coomassie brilliant blue R-250, and the desired bands were excised. The N-terminal amino acid sequence was determined by Edman degradation using an Applied Biosystems model 470A protein peptide sequencer.

Results and Discussion

In previous work, we described the fragmentation of the scaffoldin subunit of the cellulosome of *C. thermocellum*, resulting from acid treatment, carried out *prior* to SDS-PAGE *(5)*. We postulated that the fragmentation resulted from nonproteolytic covalent bond breakage when the subunit reverts to a fragile conformation *(6)*. This phenomenon was suggested to reflect the dynamics of cellulosome interaction with the cellulose substrate. It was thus of interest to determine which bond(s) might be cleaved.

The sequence of the scaffoldin subunit is subdivided into a dozen functional domains (Fig. 1a), including one CBD and nine repeated cohesin domains *(10)*. Since numerous fragments of the intact scaffoldin were produced by acid pretreatment, we designed a simpler experimental system to determine which bond(s) might be affected. For this purpose, we used a defined recombinant segment (Coh2-CBD) of this subunit which contains a single cohesin domain and a single cellulose-binding domain (Fig. 1b) *(8)*.

The recombinant Coh2-CBD segment was subjected to the identical pH-4 pretreatment, prior to SDS-PAGE. As a result of this treatment, a major polypeptide fragment was produced that migrated somewhat faster on SDS-PAGE than Coh2-CBD (Fig. 2). Densitometry tracings showed that, under these conditions, about 30% of the recombinant construct was converted to the fragment.

The lane was transferred electrophoretically onto PVDF membranes, the designated fragment was extracted and subjected to N-terminal sequence analysis. The resultant sequence is shown in Fig. 3a. On the basis of this sequence, the sensitive bond was identified as the bond between aspartic acid in position 57 and proline in position 58 (Fig. 3b). The other two Asp-Pro bonds of cohesin 2 appeared to be relatively stable under the indicated conditions, since no signs of other sequences were evident.

The sensitive Asp57-Pro58 sequence is located in an exceptionally well-defined loop stabilized by a multiplicity of hydrogen-bonding interactions *(11,12)*. The sequence of this loop is exquisitely conserved in all nine cohesin domains of the scaffoldin subunit. This fact, combined with the

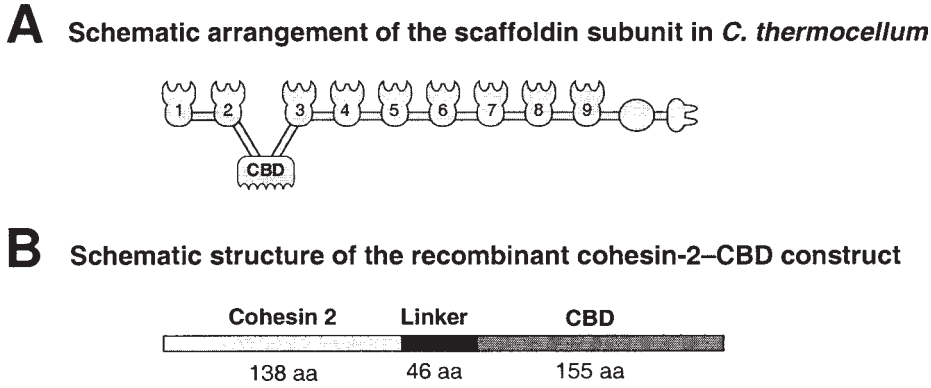

Fig. 1. Organization of the domains in the cellulosomal scaffoldin subunit from *C. thermocellum*. (a) Schematic arrangement of scaffoldin, showing its multiple domain structure. The nine cohesin domains are numbered, and the CBD is designated. (b) Schematic structure of the recombinant cohesin-2–CBD (Coh2-CBD) construct. The two domains are separated by a 46-residue linker sequence. The size of each domain is also shown.

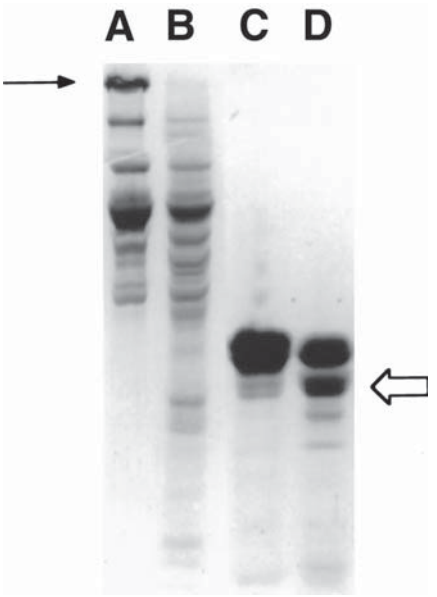

Fig. 2. Effect of pH 4 pretreatment on the integrity of the native scaffoldin and the recombinant scaffoldin fragment. Samples were first subjected to pretreatment in dilute sodium acetate buffer, pH 4. They were then mixed with sample buffer, boiled, and analyzed by SDS-PAGE. Lane A, cellulosome, no pretreatment. Lane B, cellulosome sample, pretreated by dialyzing against pH-4 buffer prior to SDS-PAGE. Note the disappearance of the scaffoldin subunit (small arrow). Lane C, recombinant scaffoldin fragment (Coh2-CBD construct), no pretreatment. Lane D, Coh2-CBD construct, pretreated as in lane B prior to SDS-PAGE. Note the major fragment (open arrow) resulting from mild acid pretreatment of the recombinant construct. The gels were stained with Coomassie brilliant blue.

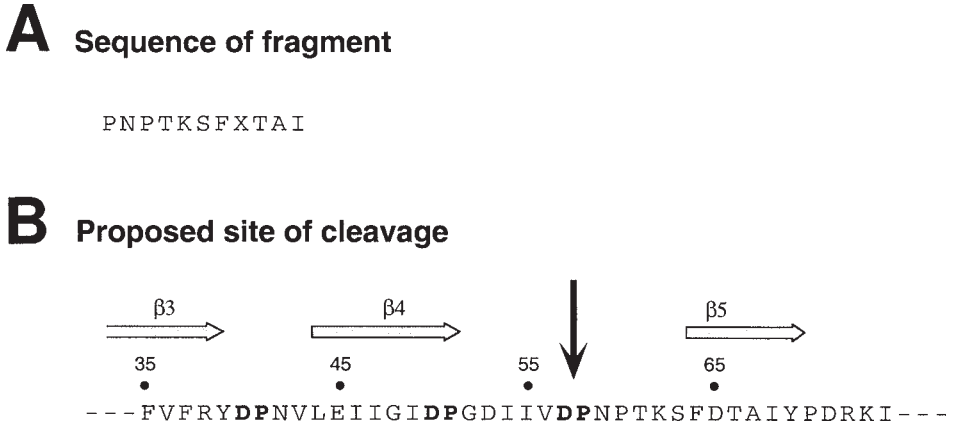

Fig. 3. Position of the sensitive Asp-Pro bond in the cohesin-2 domain. (a) N-terminal sequence (first 11 residues) of the major Coh2-CBD fragment, indicated in Fig. 2, lane D. (b) Proposed site of cleavage within the cohesin-2 domain. The domain contains three Asp-Pro bonds (residues 40–41, 50–51, and 57–58), located in the 3/4 turn, and near or in the 4/5 loop. Two of these bonds (40–41 and 57–58) are conserved in all of the cohesins of this subunit. The proposed sensitive bond (Asp57-Pro58), consistent with the amino acid sequence shown in (a), is in the 4/5 loop (designated by the arrow).

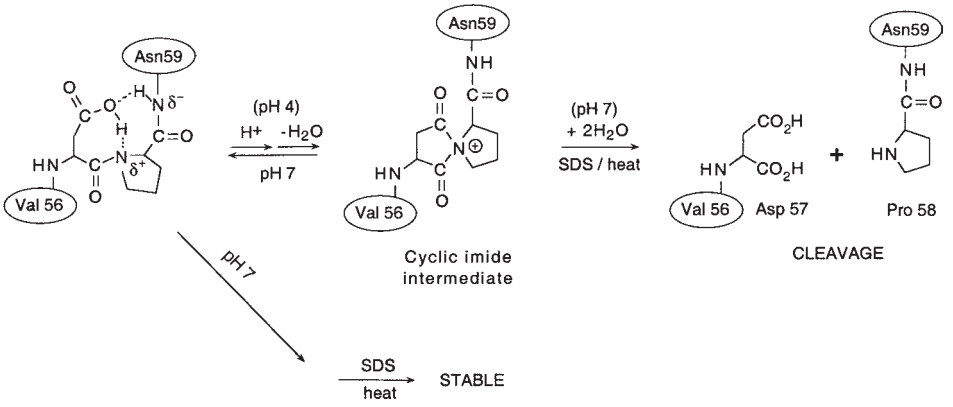

Fig. 4. Proposed mechanism for hydrolysis of the labile Asp57-Pro58 peptide bond.

observation that only about 30% of the construct was cleaved under the described conditions, explains the reported breakdown of the intact subunit into a multiplicity of defined fragments *(6)*.

The lability of aspartyl-proline peptide bonds is well documented *(13)*. Such bonds are known to be selectively hydrolyzed under mildly acidic conditions for prolonged periods of time, particularly at high temperatures. Indeed, the procedure described in this communication included a mild-acid treatment (dialysis at pH 4), followed by a second treatment with SDS (neutral pH) and heat. The acid pretreatment presumably serves to generate a stable intermediate (Fig. 4), consistent with the cyclic imide proposed earlier *(13)*.

Although the results are rather straightforward, the interpretation of the process is quite subtle: cleavage occurs only under relatively specific conditions. For this reason, the phenomenon apparently failed to be observed by other researchers in the field. For example, Bhat and Wood *(14)* did not detect cleavage of CipA after the cellulosome was subjected to mildly acidic conditions followed by SDS-PAGE. In this case, however, the samples were not heated. On the other hand, some cleavage was observed by Salamitou et al. *(15)* following treatment of the cellulosome with 80% formic acid, but the extent of degradation was much less than that observed in our previous work *(6)*.

Bond cleavage thus appears to be dependent upon two concurrent phenomena: the formation of the stable intermediate and the application of SDS and heat. Upon simple adjustment of the medium to neutral conditions, the stable intermediate presumably reverts to the original structure and the Asp-Pro bond is preserved. On the other hand, the intermediate is susceptible to heat, and upon boiling in the presence of SDS, the peptide bond is cleaved even under neutral conditions. It is interesting to note that both SDS and heat are required for the observed cleavage.

It is still unclear why the Asp57-Pro58 bond of the cohesin domain is particularly sensitive to hydrolysis by the above-described procedure. The cohesin contains three Asp-Pro domains (Fig. 3). Close examination of the three dimensional crystal structure of the cohesin domain [PDB ID code 1ANU *(11)*] reveals that the side chain of Asp50 does not face its neighboring proline ring and would thus be unlikely to form a cyclic intermediate. In contrast, the side chains of both Asp40 and Asp57 exhibit a similar configuration, each facing their adjoining proline residues, where the carboxyl oxygen forms a hydrogen bond with the peptide bond nitrogen of the residue which follows proline (Asn42 or Asn59). This arrangement brings into close proximity the carbonyl carbon of the Asp residue and the nitrogen of the proline ring, thus facilitating the formation of a cyclic imide intermediate. Interestingly, the distance between the carbonyl and the proline nitrogen is 3.9 and 3.6 Å for Asp50-Pro51 and Asp57-Pro58, respectively. Thus, based on the crystal structure, Asp57-Pro58 sequence is most likely to form the imide intermediate.

The Asp-Pro sequence is rather common, both to other multidomain cellulosomal components from *C. thermocellum (6)* [notably, the catalytic subunits, such as CelJ *(16)* and CbhA *(17)*] and to the scaffoldin subunits from the other known clostridial cellulosomes (18–20). In the latter scaffoldins, however, the sequence is usually located in interdomain linker regions and not in the cohesin domains. It should also be noted that the peptide bond immediately downstream to aspartic acid or asparagine residues are, in general, notoriously sensitive to a variety of chemical treatments *(13,21–23)*.

Native and recombinant cellulosomal subunits are currently being produced for potential biotechnological application *(3,22,24,25)*. These subunits are relatively large polypeptides, composed of a multiplicity of

functional domains, connected by a series of linker sequences. In order to maintain the continuity of the intact polypeptides, future analytical and purification procedures should take into account the tendency of sensitive peptide bonds to undergo hydrolysis, even under relatively mild conditions.

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