

Design and Production in *Aspergillus niger* of a Chimeric Protein Associating a Fungal Feruloyl Esterase and a Clostridial Dockerin Domain

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A chimeric enzyme associating feruloyl esterase A (FAEA) from *Aspergillus niger* and dockerin from *Clostridium thermocellum* was produced in *A. niger*. A completely truncated form was produced when the dockerin domain was located downstream of the FAEA (FAEA-Doc), whereas no chimeric protein was produced when the bacterial dockerin domain was located upstream of the FAEA (Doc-FAEA). Northern blot analysis showed similar transcript levels for the two constructs, indicating a posttranscriptional bottleneck for Doc-FAEA production. The sequence encoding the first 514 amino acids from *A. niger* glucoamylase and a dibasic proteolytic processing site (*kex-2*) were fused upstream of the Doc-FAEA sequence. By using this fusion strategy, the esterase activity found in the extracellular medium was 20-fold-higher than that of the wild-type reference strain, and the production yield was estimated to be about 100 mg of chimeric protein/liter. Intracellular and extracellular production was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, dockerin-cohesin interaction assays, and Western blotting. Labeled cohesins detected an intact extracellular Doc-FAEA of about 43 kDa and a cleaved-off dockerin domain of about 8 kDa. In addition, an intracellular 120-kDa protein was recognized by using labeled cohesins and antibodies raised against FAEA. This protein corresponded to the unprocessed Doc-FAEA form fused to glucoamylase. In conclusion, these results indicated that translational fusion to glucoamylase improved the secretion efficiency of a chimeric Doc-FAEA protein and allowed production of the first functional fungal enzyme joined to a bacterial dockerin.

Plant cell walls are composed of various polysaccharides and lignin, forming a rigid and complex matrix recalcitrant to microbial degradation. This structure is strengthened by cross-linkages such as diferulic acid bridges between adjacent hemicellulose chains (28) or between lignin and hemicellulose (24), increasing its resistance to microbial invasion. Aerobic and anaerobic microorganisms have developed two main efficient mechanisms of plant cell wall degradation. In the first system, microorganisms secrete free extracellular enzymes in contact with a substrate. The products of degradation constitute both nutrients for growth and regulators of the production of lignocellulolytic enzymes (9, 42, 43). Among these microorganisms, filamentous fungi such as *Trichoderma* spp. and *Aspergillus* spp. are especially good secretors of lignocellulolytic enzymes (1). Moreover, *Aspergillus niger* produces enzymes named feruloyl esterases (EC 3.1.1.73), a subclass of the carboxylic ester hydrolases, that are able to hydrolyze diferulate cross-links in plant cell walls, facilitating the access of main-chain-degrading enzymes to the polysaccharide backbone (8, 46). Furthermore, feruloyl esterases release cross-linked aromatic acids, such as

ferulic acid, which is an attractive industrial compound by virtue of its antioxidant, photoprotectant properties (18) and its potential biotransformation to vanillin as a food flavor precursor (25).

A second system of degradation has evolved among anaerobic microorganisms that are subjected to more-drastic energetic constraints. Anaerobic fungi and bacteria produce high-molecular-mass complexes called cellulosomes, where different types of enzymes are bound to a scaffolding protein (for reviews, see references 38 and 39). Among bacteria, the cellulosomes from *Clostridium* spp. are the most studied complexes. In addition to a catalytic domain, all cellulosomal enzymes include a dockerin domain, usually in the C terminus, that allows their incorporation along the scaffoldin. The scaffoldin contains a cellulose-binding domain (11) and multiple copies of cohesin domains that interact with the dockerin on the cellulosomal enzymes. This binding is mediated by a high-affinity interaction ($\geq 10^9 \text{ M}^{-1}$) among complementary dockerin-cohesin domains (13, 29). The highly conserved dockerin domain is characterized by two Ca^{2+} -binding sites with sequence similarity to the EF-hand motif (27). The multienzymatic complex facilitates a stronger synergistic effect among catalytic subunits than the free-enzyme system (5, 15, 22). Moreover, the fixation to the substrate mediated by the scaffoldin-borne carbohydrate binding module prevents large-scale diffusion of enzymes away from the cell, leading to a lower requirement of proteins for hydrolysis. In previous studies,

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TABLE 1. Expression vectors and strategy of constructions used in this work

Construct	Vector used	Plasmid	Secretion system	Reference or source
FAEA	pAN52.3	pF	ss (<i>faeA</i>)	34
FAEA-Doc	pAN52.3	pFD	ss (<i>faeA</i>)	This study
Doc-FAEA	pAN52.4	pDF	ss (<i>glaA</i>)	This study
Doc-FAEA	pAN56.1	pGDF	<i>glaA</i> carrier	This study

domain of cellulase Cel48S from *C. thermocellum*. Both fusions were placed under the control of the *gpdA* promoter and the *trpC* terminator and contained the glucoamylase preprosequence of *A. niger* to target the secretion (Table 1, pFD and pDF). *A. niger* D15#26 protoplasts were cotransformed with a mixture of plasmid pAB4.1 and expression vectors. Transformants were selected for their abilities to grow on a minimal medium plate without uridine. For each construct, approximately 100 uridine prototrophic transformants were obtained per μg of expression vector. A fungal PCR colony was effected to control the integration of the expression cassette into the *A. niger* genome. Forty transformants were inoculated into a glucose-containing minimal medium, repressing endogenous *faeA* gene expression, and their extracellular medium was tested for esterase activity. Esterase activity was detected for transformants containing the FAEA-Doc expression cassette, whereas no activity was measured for any of the clones transformed by plasmid pDF or for the untransformed host. For FAEA-Doc transformants, esterase activity against MFA as the substrate ranged from 0.1 to 0.4 nkat/ml. Esterase activity was detected on day 3 and increased strongly until day 10 to reach a maximum of 0.4 nkat/ml for the clone exhibiting the highest activity (Fig. 1). This activity was estimated to be fivefold higher than that obtained with the wild-type strain BRFM281 under optimal conditions of production using maize bran as an inducer (25).

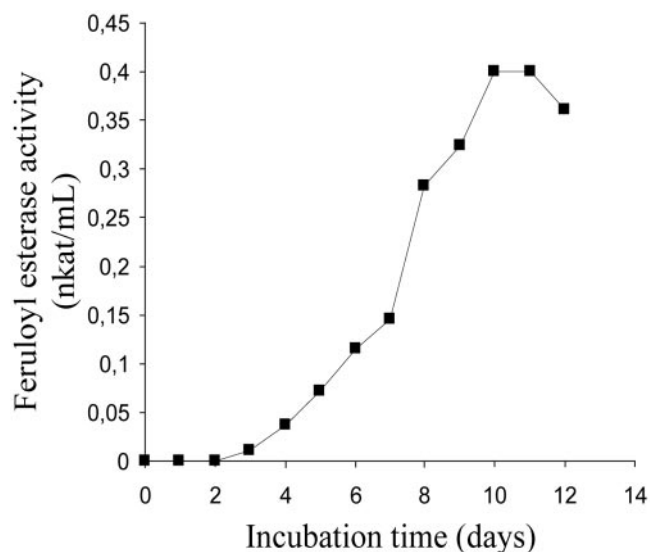


FIG. 1. Time course activity of the feruloyl esterase produced by the FAEA-Doc transformant. Activity was measured in the extracellular medium of the fungus by using MFA as the substrate.

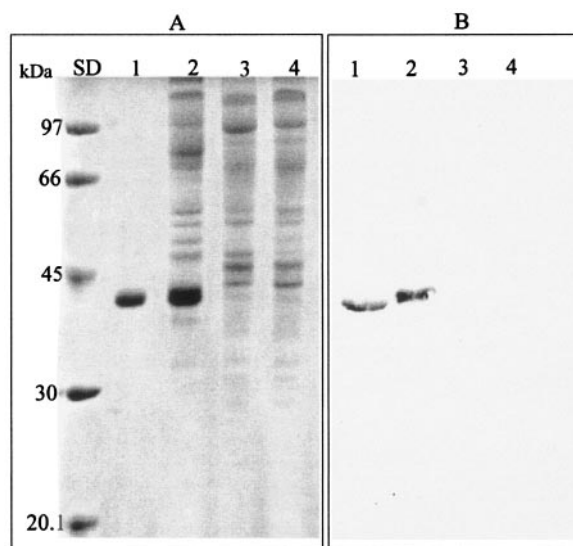


FIG. 2. SDS-PAGE and Western blot analysis of extracellular proteins produced by the FAEA-Doc and Doc-FAEA transformants. The purified FAEA (lanes 1) and total proteins from the FAEA-Doc transformant (lanes 2), the Doc-FAEA transformant (lanes 3), and a non-transformed strain (lanes 4) were loaded onto an SDS-PAGE (11% polyacrylamide) gel. The gel was stained with Coomassie blue (A), or Western blotting using antibodies raised against *A. niger* FAEA protein was performed (B). Lane SD, molecular size standards.

Biochemical characterization of the chimeric proteins. Total proteins in the supernatant were analyzed by SDS-PAGE, and a major band with a molecular mass close to 38 kDa was revealed for FAEA-Doc transformants (Fig. 2A). For Doc-FAEA transformants, no predominant band was observed, indicating that the lack of activity was due to a lack of Doc-FAEA protein production rather than to an inactivated chimeric protein. Immunodetection of the FAEA-Doc and Doc-FAEA proteins was performed using antibodies raised against FAEA (Fig. 2B). Western blot analysis showed a unique band corresponding to the 38-kDa protein, demonstrating that this protein was the chimeric FAEA-Doc fusion. The difference in molecular mass between purified FAEA (36 kDa) and the FAEA-Doc fusion (38 kDa) did not match with the expected size, since the molecular mass of the chimeric protein was estimated at around 43 kDa. Moreover, far-Western blotting using biotinylated cohesins from *C. thermocellum* as the probe confirmed the absence of the dockerin module in the supernatant produced by the FAEA-Doc (and Doc-FAEA) transformant, since no interaction signals were detected (data not shown). To exclude intracellular retention of the Doc-FAEA protein, we verified that the corresponding recombinant proteins had not accumulated in the mycelium cells of *A. niger*.

Gene expression analysis. In order to analyze gene expression in both FAEA-Doc and Doc-FAEA transformants at the transcriptional level, Northern blot analysis was performed for 5-day-old cultures (Fig. 3). A transformant containing the *faeA* gene without dockerin was used for *faeA* gene transcription control (lane 1). An 18S gene probe was used as an internal control to monitor the amount of RNA loaded in each lane. By using FAEA and dockerin probes, similar levels of expression

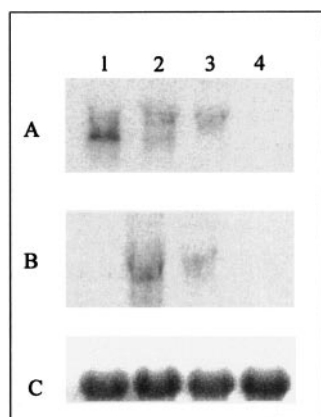


FIG. 3. Northern blot analysis of total RNAs isolated from biomass aliquots of *A. niger* transformants. Total RNAs (10 μ g) from *A. niger* transformed with pF (lane 1), pFD (lane 2), or pDF (lane 3) and from nontransformed strain D15 (lane 4) were electrobotted and hybridized with *faeA* and dockerin cDNA probes from *A. niger* (A) and *C. thermocellum* (B), respectively. The PCR-amplified 18S DNA was used as a probe for the loading control (C).

were detected for the FAEA-Doc and Doc-FAEA transformants (Fig. 3A and B, lanes 2 and 3). Moreover, for the two fusions, *faeA*-dockerin mRNAs of similar sizes were detected. Consequently, the absence of chimeric DF protein production was clearly not related to a transcriptional bottleneck.

Study of the chimeric Doc-FAEA protein production using a glucoamylase carrier strategy. The results described above indicate that the localization of the dockerin module downstream of the FAEA leads to production of a truncated chimeric protein without dockerin. Moreover, when the bacterial dockerin-encoding sequence was expressed as a 5' fusion to the fungal *faeA* gene, no protein was produced. Based on these results, we used a second strategy based on the fusion of the Doc-FAEA chimera to a carrier protein. With this strategy, the sequence encoding the first 514 amino acids of the well-secreted glucoamylase from *A. niger* (GLA1-514) is believed to improve the secretion efficiency of the Doc-FAEA protein by facilitating translocation and subsequent folding in the endoplasmic reticulum. In this genetic construction (pGDF [Table 1]), this sequence was fused upstream of the *doc-faeA* sequences, and a dibasic proteolytic processing site (*kex-2*) was introduced between the corresponding sequences encoding the GLA1-514 and Doc-FAEA proteins. Time course analysis of the feruloyl esterase activity produced by the best Doc-FAEA transformant showed a maximal activity of about 1.7 nkat/ml at day 8 (Fig. 4). Then esterase activity decreased, probably due to proteolytic degradation. This activity is approximately 20-fold-higher than the activity measured for the wild-type strain BRFM281 using maize bran as an inducer.

Biochemical characterization of Doc-FAEA. (i) SDS-PAGE analysis of extracellular and intracellular proteins. To monitor the production of the chimeric Doc-FAEA protein in the intracellular and extracellular fractions of the fungus, total proteins were analyzed by SDS-PAGE from days 3 to 10 (Fig. 5). From day 3, two predominant bands around 80 and 43 kDa were observed in the extracellular medium, in contrast to that of the wild-type strain D15, where no major band was present.

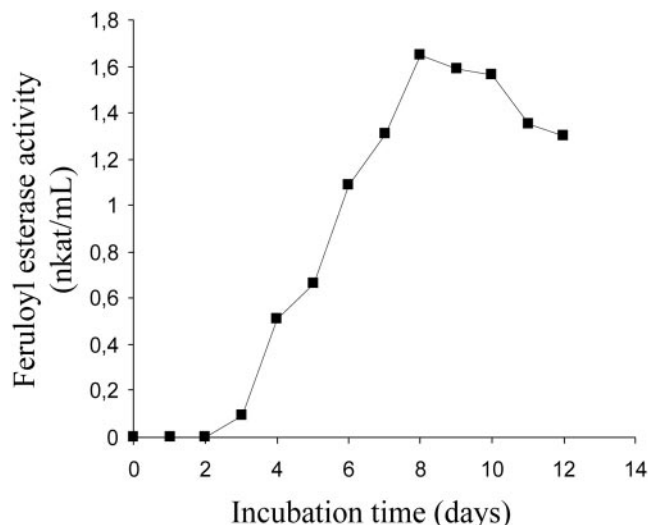


FIG. 4. Time course activity of the feruloyl esterase produced by the Doc-FAEA transformant (pGDF construct). Activity was measured in the extracellular medium of the fungus by using MFA as the substrate.

The intensity of bands increased with incubation time, in accordance with the time course activity observations. These molecular masses were in complete agreement with those estimated for GLA1-514 and an intact chimeric Doc-FAEA protein, respectively, indicating correct processing of the GluA carrier. With regard to total intracellular proteins, no clear protein accumulation was observed for the Doc-FAEA transformant.

(ii) Detection of the chimeric Doc-FAEA protein by cohesin-dockerin interaction and Western blot analysis. In order to confirm the production of an intact and functional chimeric Doc-FAEA protein, dockerin-cohesin interaction tests were carried out by using extracellular and intracellular fractions from day 3 to 10 (Fig. 6). Proteins from the untransformed strain D15 and engineered Cel9E (12) from *C. cellulolyticum* with a *C. thermocellum* dockerin appended served as negative and positive controls for dockerin-cohesin interactions, respectively. From day 6, two proteins with molecular masses around 43 and 7 kDa were detected in the extracellular medium. According to these molecular masses, we supposed that the labeled cohesins interacted with a whole Doc-FAEA protein (7 + 36 kDa) but also with a single dockerin module (7 kDa) cleaved from the Doc-FAEA protein. In the intracellular fraction, a very high molecular mass was detected from day 5, 1 day earlier than detection of secreted Doc-FAEA protein. This result suggests that Doc-FAEA is present in the secretion pathway, as an intermediary protein fused to the carrier glucoamylase, with a global molecular mass close to 120 kDa. This protein is processed in the late Golgi apparatus, where the fungal Kex-2-like protein is expected to be active (40). Consequently, this high-molecular-mass protein could be the immature Doc-FAEA form fused to glucoamylase.

To complete and validate previous results, total extracellular and intracellular proteins were loaded onto an SDS-PAGE gel and immunodetected by using antibodies raised against FAEA protein (Fig. 7). In the extracellular medium (Fig. 7, lane 2),

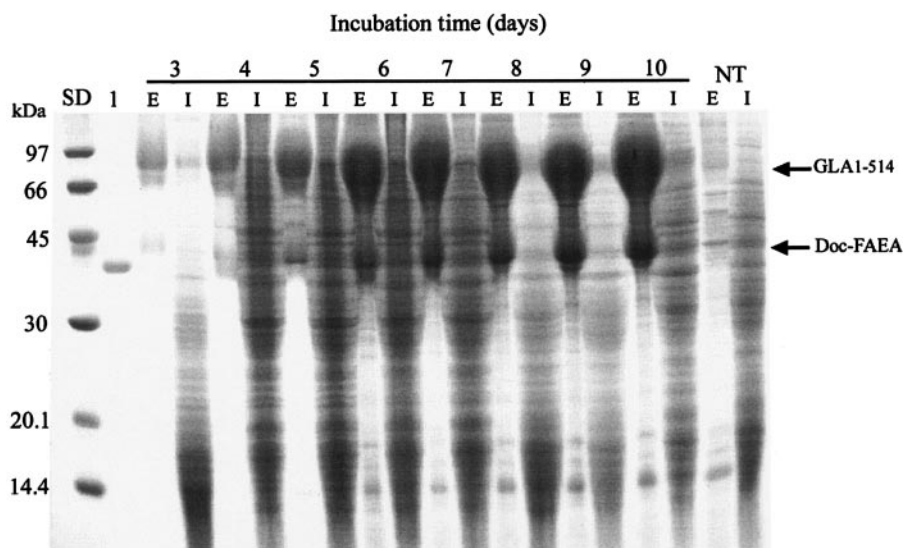


FIG. 5. Production of chimeric Doc-FAEA protein in the extracellular and intracellular fractions from *A. niger* transformants. Lanes: SD, molecular size standards; 1, purified FAEA; NT, nontransformed strain D15 at day 8; E and I, extracellular and intracellular fractions, respectively.

protein bands of approximately 43 and 36 kDa were detected. The first, large band corresponds to the whole Doc-FAEA protein, whereas the small band, with a molecular mass close to that of the purified FAEA, is a cleaved form of Doc-FAEA (Fig. 7, lane 1). In the intracellular fraction, both 36- and 43-kDa proteins could be observed. In addition, a protein with a molecular mass of around 120 kDa, corresponding to the noncleaved GLA1-514-Doc-FAEA form, was also detected.

DISCUSSION

The bacterial cellulosome is a very effective system for increasing the synergistic effect between enzymes. These positive

effects were determined on crystalline cellulose by using hybrid cellulases containing dockerin domains of different clostridial species incorporated into chimeric scaffoldin protein (12, 14). In the present work, our aim was to produce a fungal enzyme different from bacterial cellulase, to be grafted into a cellulosome complex. This work will be included in future studies comparing the synergistic effects of free and grafted fungal and nonfungal enzymes for plant cell wall degradation. Therefore, we developed a strategy to secrete a chimeric fungal enzyme fused to a bacterial dockerin module in *A. niger*. This fungus is a well-known host for protein overproduction, producing complex enzymes that require posttranslational modifications, such as glycosylation. Thus, the production of chimeric proteins

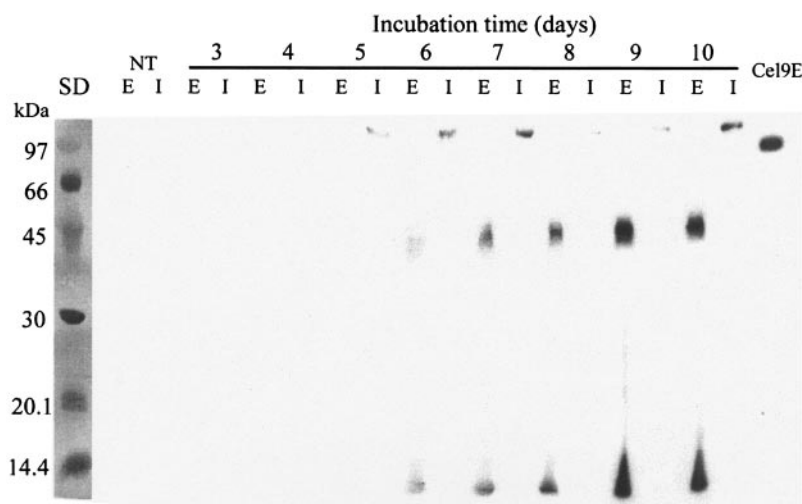


FIG. 6. Cohesin-dockerin interaction assay. Extracellular (E) and intracellular (I) media from days 3 to 10 were loaded onto an SDS-PAGE (11% acrylamide) gel, transferred, and probed with a cohesin-containing protein (C2CBD) labeled by biotinylation. Proteins from the nontransformed strain D15 and an engineered cellulase from *C. cellulolyticum* with a *C. thermocellum* dockerin appended (Cel9E) served as negative and positive controls for dockerin-cohesin interactions, respectively. Detection was performed using a streptavidin-peroxidase conjugate. SD, molecular size standards stained with Coomassie blue.

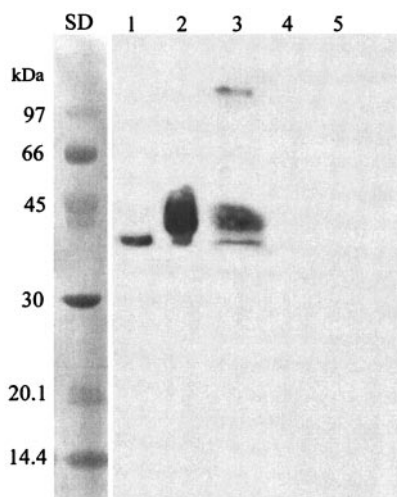


FIG. 7. Western blot analysis of extracellular and intracellular proteins from the Doc-FAEA transformant. Lane SD, molecular size standards; lane 1, purified FAEA; lanes 2 and 4, extracellular medium at day 9 from the Doc-FAEA transformant and the wild-type strain, respectively; lanes 3 and 5, intracellular medium at day 9 from the Doc-FAEA transformant and the wild-type strain, respectively.

consisting of a complex eukaryotic enzyme fused to a bacterial dockerin domain provided an extension to the production of *E. coli* hybrid cellulases.

In the first approach, the dockerin module from *C. thermocellum* was fused to a well-secreted protein, *A. niger* FAEA. Both N-terminal and C-terminal fusions of the bacterial module were attempted to allow a correct folding of both partners. Feruloyl esterase activity in the extracellular medium from FAEA-Doc transformants was fivefold higher than that of the reference strain. This improvement factor is less than that obtained for homologous production of FAEA in *A. niger* (34). Apparently, as seen for other fusion proteins, the production yield was negatively affected by the presence of the bacterial domain (21, 31, 35, 36). In addition, we observed that FAEA-Doc protein was produced as a truncated form. The cleavage site is located at the beginning of the dockerin N-terminal end, since a very weak molecular mass difference between FAEA-Doc and native FAEA was observed. The C-terminal fusion of the bacterial domain generates an unstable structure, leading to a proteolytic or physical cleavage between the two partners, and as a consequence, the corresponding construct was not considered in the subsequent experiment.

The Doc-FAEA construct produced no chimeric protein, in agreement with the lack of feruloyl esterase activity in the extracellular medium. The detection of mRNA encoding the chimeric Doc-FAEA protein indicates that the bottleneck for Doc-FAEA production lies at a posttranscriptional level. Thus, the location of the bacterial domain next to the fungal protein is an essential parameter to consider for this overproduction. In earlier studies, methods were developed to alleviate limitations for protein production in fungal hosts. An efficient strategy is based on the use of translational fusions in which the targeted protein is fused to an endogenous secreted carrier protein at the N-terminal end of the heterologous protein (17, 35). The N-terminal carrier protein is believed to improve the

secretion efficiency of the heterologous protein by facilitating translocation and folding in the endoplasmic reticulum. These results could explain differences between FAEA-Doc and Doc-FAEA protein production. Indeed, for the FAEA-Doc construction, the N-terminal FAEA played the role of carrier protein, in contrast to Doc-FAEA, where the FAEA was located C-terminal to the dockerin. In addition, Ward and co-workers (44) demonstrated that an autocatalytic release between the carrier and the heterologous protein was possible, which could explain the fact that only the truncated form of FAEA-Doc was detected.

We designed a new construct in which the Doc-FAEA sequence was located downstream of a partial sequence from *A. niger* glucoamylase (GLA1-514) and the *kex-2* site (4, 6). Thus, the recombinant protein will be recognized and processed in vivo at this consensus dibasic proteolytic processing site (Kex2 site) in the trans-Golgi network by the kexin family of proteases (30, 40). By use of this strategy, the negative effect generated by the bacterial dockerin was reduced and an increased esterase activity was detected in the extracellular medium, demonstrating the efficiency of the fusion to the carrier protein. Moreover, the esterase activity was even 4- and 20-fold higher, respectively, than those obtained for the FAEA-Doc transformant and the wild-type strain under optimal induction conditions, confirming that glucoamylase is the carrier protein of choice for such a construct. Glucoamylase was used in previous work to successfully produce recombinant proteins such as porcine pancreatic phospholipase A2, human interleukin-6, or hen egg white lysozyme (6, 17, 20, 21, 35). Based on the specific activity of purified Doc-FAEA, we estimated a production yield of 100 mg per liter. The cohesin-dockerin interaction assay revealed two extracellular bands of about 43 and 8 kDa interacting with the labeled scaffolding protein. The recombinant extracellular Doc-FAEA protein was partially cleaved, producing two forms: an intact Doc-FAEA protein containing a dockerin domain and a truncated form where the dockerin was separated from the enzymatic partner. This cleaved dockerin possessed two conserved duplicated sequences, each containing a calcium binding motif, allowing the dockerin domain to bind to cohesins (27). In addition to a unique extracellular band of 43 kDa interacting with cohesins, antibodies raised against FAEA recognized a second, minor band of about 36 kDa. This form corresponded to the FAEA without dockerin from partially cleaved Doc-FAEA, confirming previous results of cohesin-dockerin assays. Therefore, the cleavage occurred near the linker region between dockerin and FAEA, leading to two functional domains with the majority of the Doc-FAEA in the intact form, as shown by Western blot analysis. In order to improve the stability and the final recovery of intact Doc-FAEA, this linker region might be modified, i.e., by adding glycosylation sites [N-X-(S/T)]. In the cellular extract, a very high molecular weight protein was detected by the cohesin-dockerin interaction assay and Western blotting. This form was suggested to be a nonprocessed GluA-Kex2-Doc-FAEA protein. Immunodetection also revealed two other proteins, corresponding to a major, intact Doc-FAEA and a minor, cleaved Doc-FAEA. The intracellular Doc-FAEA protein (around 43 kDa) could be observed in the terminal step of the secretion pathway, because the Kex2 site was already cleaved in the trans-Golgi apparatus. However, interaction of this Doc-

FAEA protein form was not detected with labeled cohesins, indicating that this protein was not completely processed until the extracellular medium. No experiments fusing the FAEA-Doc construct to a carrier protein were carried out, because the aim of the study was achieved, but future work to investigate if the C-terminal dockerin end would be protected by the glucoamylase protein is envisaged.

The aim of this study was to produce in *A. niger* a chimeric protein fusing a fungal enzyme and a bacterial dockerin domain. In this work, a strategy was developed to produce the first fungal enzyme able to be incorporated in vitro into a bacterial cellulosome from *C. thermocellum*. Future work will be performed to modify the linker region between the dockerin and the fungal enzyme. Moreover, other complementary enzymes, such as metalloenzymes which cannot be produced in bacterial hosts, will be fused to dockerin from different species. All these chimeric enzymes might be assembled onto chimeric cellulosomes, and the resultant synergy of degradation could be estimated.

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