Structural Insights into the β-Xylosidase from *Trichoderma reesei* Obtained by Synchrotron Small-Angle X-ray Scattering and Circular Dichroism Spectroscopy†

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ABSTRACT: The enzyme β-xylosidase from *Trichoderma reesei*, a member of glycosil hydrolase family 3 (GH3), is a glycoside hydrolase which acts at the glycosidic linkages of 1,4-β-xylooligosaccharides and that also exhibits α-L-arabinofuranosidase activity on 4-nitrophenyl α-L-arabinofuranoside. In this work, we show that the enzyme forms monomers in solution and derive the low-resolution molecular envelope of the β-xylosidase from small-angle X-ray scattering (SAXS) data using the ab initio simulated annealing algorithm. The radius of gyration and the maximum dimension of the β-xylosidase are 30.3 ± 0.2 and 90 ± 5 Å, respectively. In contrast to the fold of the only two structurally characterized members of GH3, the barley β-D-glucan exohydrolase and β-hexosaminidase from *Vibrio cholerae*, which have respectively two or one distinct domains, the shape of the β-xylosidase indicates the presence of three distinct structural modules. Domain recognition algorithms were used to show that the C-terminal part of the amino acid sequence of the protein forms the third domain. Circular dichroism spectroscopy and secondary structure prediction programs demonstrate that this additional domain adopts a predominantly β conformation.

Trichoderma reesei β-xylosidase is a glycoside hydrolase that acts on unbranched xylans, glucuronoxylans, and β-1,4-xylooligosaccharides with different degrees of polymerization. The only resulting product of all these reactions is D-xylose, which characterizes *T. reesei* β-xylosidase as an exoglycosidase (*1*). Xylan is a major structural polysaccharide in plant cells and is the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on earth (*2*). Xylan together with cellulose (1,4-β-glucan) and lignin (a complex polyphenolic compound) makes up the major polymeric constituents of plant cell walls (*3*). Recently, there has been significant industrial interest in xylan and its hydrolytic enzyme complex as a supplement in animal feed as well as for the manufacture of bread, food, and drinks. Further industrial applications include the production of textiles, ethanol, and xylitol and the bleaching of cellulose pulp (*4*).

Xylan degradation is a multistep process involving a number of enzymatic activities (*5*). Xylanases (1,4-β-D-xylan xylanohydrolases; E.C. 3.2.1.8) hydrolyze the internal β-1,4-xylosidic linkages of the xylan backbone. Xylosidases (1,4-β-D-xylan xylohydrolases; E.C. 3.2.1.37) in turn catalyze the hydrolysis of 1,4-β-D-xylans, removing successive D-xylose residues from the nonreducing termini, whereas arabinofuranosidases (β-L-arabinofuranoside arabinofuranohydrolase; E.C. 3.2.1.55) hydrolyze terminal nonreducing β-L-arabinofuranoside residues in β-L-arabinosides.

β-Xylosidases are produced by bacteria, yeast, marine algae, protozoans, snails, crustaceans, insects, and plants, but the principal commercial source is filamentous fungi. β-Xylosidases from various organisms have been grouped into glycoside hydrolase (GH)[1] families 3, 39, 43, 52, and 54 in the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/) (*6*). β-Xylosidase from *T. reesei* belongs to GH3. There are more than 500 known members of this family, most of which are classified as β-D-glucosidases, β-D-xylosidases, or *N*-acetyl-β-D-glucosaminidases.

Barley β-D-glucan exohydrolase is one of the best characterized members of GH3, both in functional and structural terms, and its crystal structure has been determined to 2.2 Å resolution (*7*). It folds into two distinct domains. The first domain consists of an $(\alpha/\beta)_8$ TIM-barrel domain, whereas the second consists of an αβα sandwich, which contains a β sheet of five parallel β strands and one antiparallel β strand, with three α helices on either side of the sheet. β-Hexosaminidase from *Vibrio cholerae* is the only other member of GH3 whose crystal structure is known (PDB IDs 1Y65 and 1TR9). This enzyme folds into a single $(\alpha/\beta)_8$ TIM-

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[1] Abbreviations: GH, glycoside hydrolase; GH3, glycosil hydrolase family 3; SAXS, small-angle X-ray scattering; 3D, three dimensional; 2D, two dimensional; CD, circular dichroism; CBM, carbohydrate binding modules; PSD, X-ray position sensitive detector; R_g, radius of gyration; NSD, normalized spatial discrepancy.

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barrel domain. Although members of each glycoside hydrolase family have similar three-dimensional (3D) conformations, comparison of the small-angle X-ray scattering (SAXS) data of the *T. reesei* β-xylosidase with the theoretical scattering curves for the β-D-glucan exohydrolase and β-hexosaminidase shows poor agreement, indicating gross differences between the structures. A search for possible domains of the protein was performed using the Superfamily HMM library and genome assignments server (*8*) and the primary structure of *T. reesei* β-xylosidase. In this way two known domains, comprising the first 643 residues of *T. reesei* β-xylosidase, could be identified, but the last 146 residues form an additional, third domain of unknown fold. Circular dichroism (CD) spectroscopy and secondary structure prediction algorithms indicate that the third domain has a predominantly β conformation. Docking of the β-D-glucan exohydrolase structure into the molecular envelope of the *T. reesei* β-xylosidase demonstrates that all three domains together form a heart-shaped structure.

MATERIALS AND METHODS

Protein Purification, Sample Preparation, and SAXS Data Collection. The β-xylosidase was purified from a culture of *T. reesei* after 48−60 h of cultivation as previously described (*26*, *27*). The resulting purified enzyme was dialyzed against water and lyophilized. This lyophilized enzyme was dissolved in 90 mM Tris buffer, pH 7, at a concentration of 7.3 mg/mL. The sample was centrifuged for 3 min at 20000*g*.

The small-angle X-ray scattering experiments were carried out at the LNLS, Campinas, Brazil, using a synchrotron SAXS beamline (*30*). The wavelength of the incoming monochromatic X-ray beam was $\lambda = 1.608$ Å. A 1D X-ray position sensitive detector (PSD) was utilized to record the scattering intensity as a function of the modulus of the scattering vector *q*. The parasitic scattering from air and beamline windows were subtracted from the total measured intensities. Desmearing of the experimental results was performed since the entrance window of the 1D PSD was 8 mm high.

The sample-to-detector distance (702 mm) was adjusted in order to record the scattering intensity for *q* values ranging from 0.01 to 0.25 Å^{-1}. The samples were encapsulated inside a cell with two thin parallel mica windows. Data analysis was performed using the GNOM program package (*9*, *10*). Model simulations were done using the program GASBOR (*11*, *12*). At least 10 independent reconstructions starting from random approximations yielded reproducible results.

The superposition of crystallographic structures and free atom models was done using the program SUPCOMB (*21*). To keep the original low-resolution limit of the SAXS data, molecular envelopes were computed from coordinate files output by GASBOR (*11*, *12*).

Circular Dichroism. Circular dichroism spectra were acquired in-house on a J715 spectropolarimeter (Jasco Corp.). The protein, at a concentration of 0.1 mg/mL, was placed in a 10 mm path-length cuvette. Spectra were obtained in 10 mM Tris buffer, pH 7, and spectra for the buffer were subtracted from those for the protein. Secondary structure content was estimated using the programs CDSSTR (*16*, *17*) and CONTINLL (*15*). Data were submitted online to the DichroWeb website (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) (*28*) via the online submission procedure. Data over the range 190−240 nm were used. The NRMSD (*29*) of fit parameter was found to be 0.013 with CDSSTR and 0.038 with CONTIN, in both approaches using the reference database number 7.

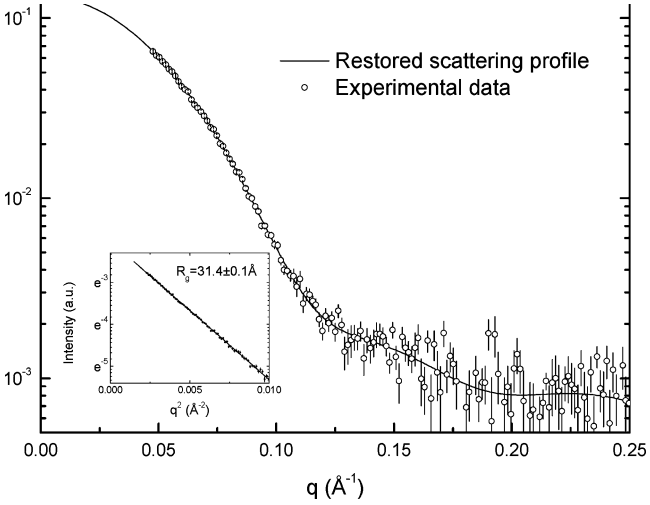

FIGURE 1: X-ray small-angle scattering curve of the β-xylosidase in 90 mM Tris buffer at pH 7 superimposed with the theoretical scattering curve from the restored low-resolution model. The radius of gyration obtained from the Guinier plot is $R_g = 31.4 \pm 0.1$ Å, which is just slightly larger than $R_g = 30.3 \pm 0.2$ Å obtained from the integral analyses of the scattering curve using the method implemented in GNOM.

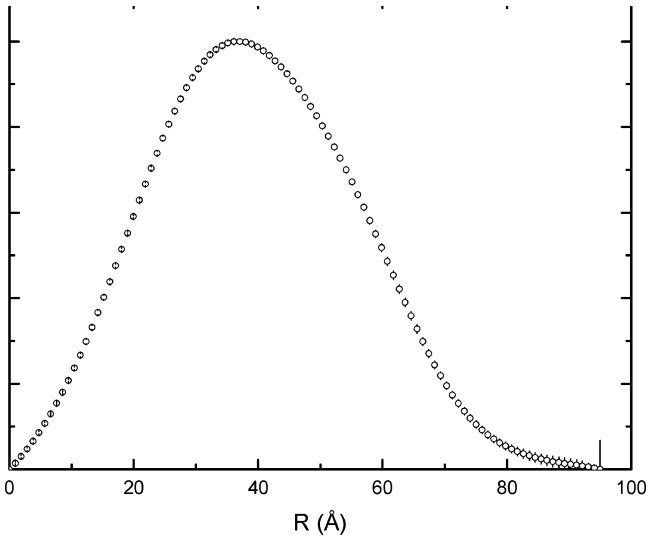

FIGURE 2: Distance distribution functions, *p*(*r*), of *T. reesei* β-xylosidase computed by indirect Fourier transform with the program GNOM (*9*, *10*).

RESULTS AND DISCUSSION

Shape of T. reesei β-Xylosidase. To obtain information about the tertiary structure of *T. reesei* β-xylosidase and its molecular shape, we submitted the protein to SAXS analysis. The X-ray scattering curve of β-xylosidase obtained as described in Materials and Methods is shown in Figure 1. The radius of gyration (R_g) evaluated with the Guinier approximation is equal to 31.4 ± 0.1 Å. The Guinier plot was linear in the qR_g range up to 3.14 (here $q = 4\pi \sin\theta/\lambda$, θ being half the scattering angle). The distance distribution functions, *p*(*r*), evaluated by the indirect Fourier transform with the program GNOM (*9*, *10*), estimated the radius of

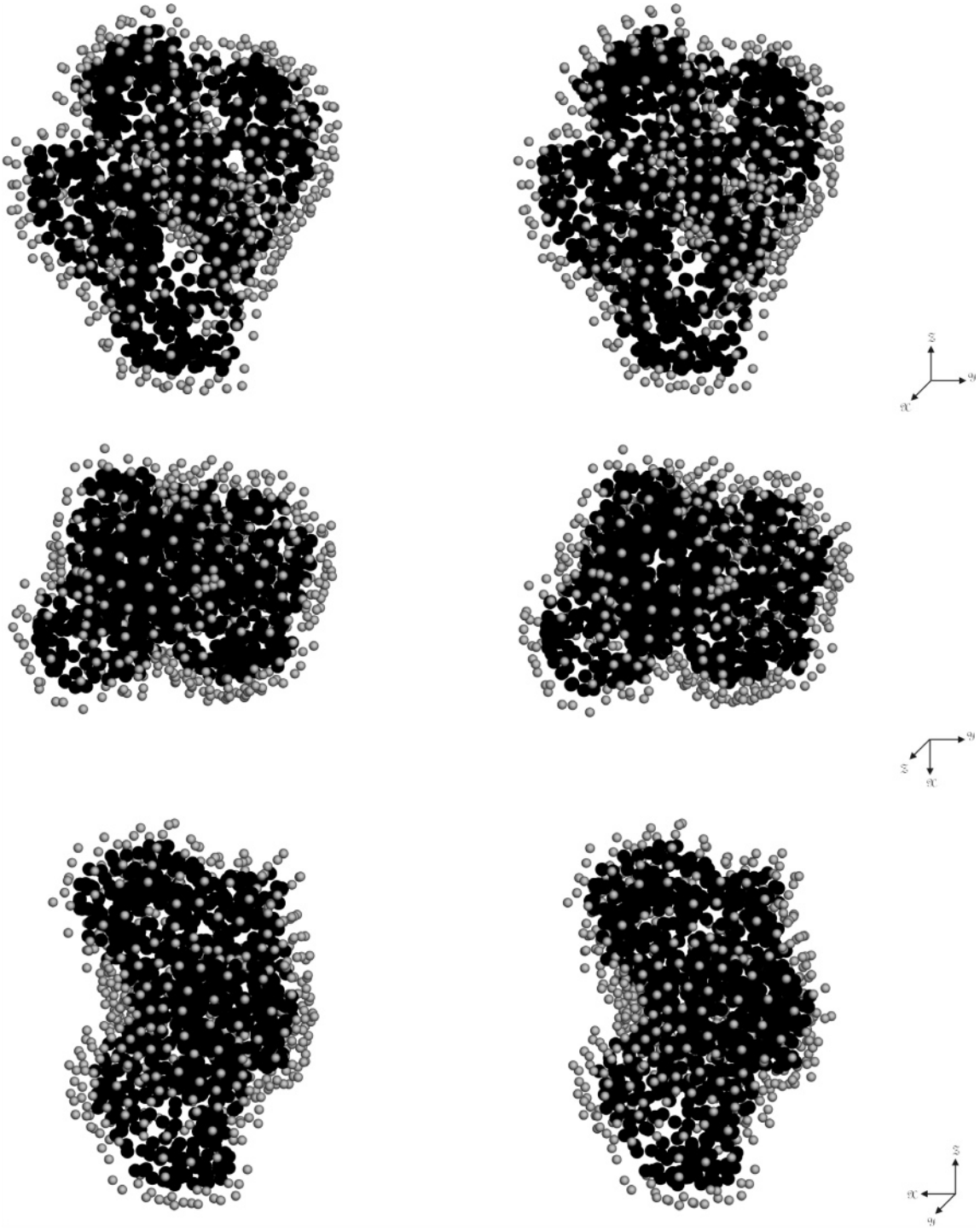

FIGURE 3: The shape of β-xylosidase in solution was restored from synchrotron X-ray scattering data to 20 Å resolution using an ab initio simulated annealing algorithm implemented in the program GNOM (*9*, *10*). Only the best model [normalized spatial discrepancy (NSD) = 1.27, χ against experimental data = 1.42] is shown. Three different stereoviews of the protein shape are given. The middle and bottom rows are rotated counterclockwise by 90° around the *Y* and *Z* axes, respectively.

gyration and the maximum dimension of the molecule to be 30.3 ± 0.2 and 90 ± 5 Å, respectively (Figure 2). The shape of the protein was determined from the X-ray scattering data with the program GASBOR (*11*, *12*), using a real-space algorithm. The results of 10 independent simulated annealing computations were compared with each other, and the best model is given in Figure 3. The molecular envelope of the protein adopts the form of a heart with a longitudinal dimension of about 90 Å and a transverse dimension of approximately 55 Å.

Secondary Structure of T. reesei β-Xylosidase. In a search directed to obtaining secondary structure information for *T. reesei* β-xylosidase, we initiated a CD spectroscopy study. The CD spectrum of the protein in solution obtained as described in Materials and Methods is shown in Figure 4a. The secondary structure content was estimated using the programs K2D (*13*), SELCON3 (*14*), CONTINLL (*15*), and CDSSTR (*16*, *17*). The best fit between experimental data and the reconstructed data was found using CDSSTR (Figure 4b). However, it has been reported that CDSSTR tends to

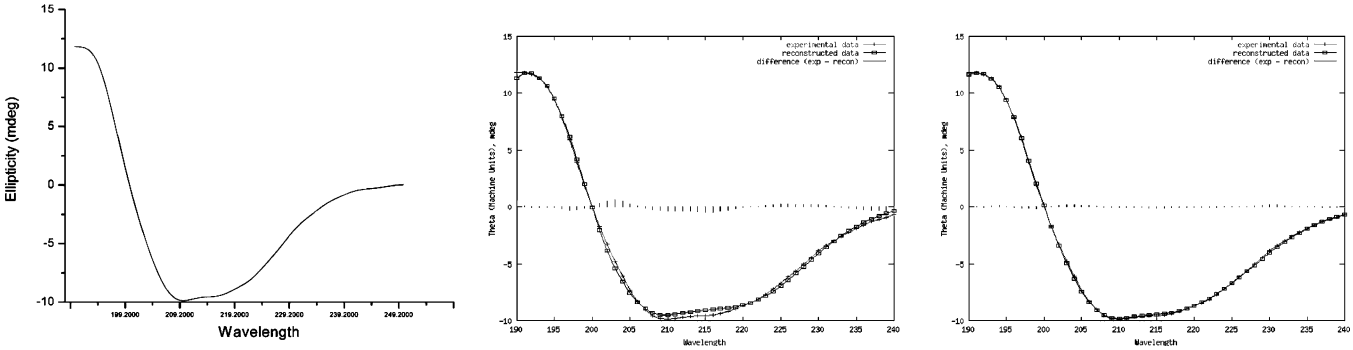

FIGURE 4: (a) Circular dichroism spectra of *T. reesei* β-xylosidase. (b) Fit to experimental data obtained with the program CONTINLL (*15*). (c) Fit obtained with the program CDSSTR (*16*, *17*).

Table 1: Secondary Structural Content of the *T. reesei* β-Xylosidase Evaluated from CD Spectra Using CONTINLL and CDSSTR

program	helix (%)	β (%)	turn (%)	random (%)	NMRSD
CDSSTR	23.0	27.0	20.0	30.0	0.013
CONTINLL	24.1	25.5	21.7	28.6	0.038

give the lowest NRMSD values, even though this does not necessarily mean that these results are the most correct (*18*). For this reason the fit to experimental data obtained with CONTINLL is also given in Figure 4c.

The spectra are strongly characteristic of an α/β protein, with spectral bands attributable to electron transitions in the amide groups of the protein backbone. The carbonyl oxygen lone pair rotational transition nπ* gives rise to the minimum at ∼222 nm, indicative of a right-handed α helix (*19*). The local minimum at ∼210 nm is an average between the minimum at ∼208 nm consequential from ππ* transition caused by light polarized parallel to the helical axis and the minimum at ∼215 nm from the nπ* transition in β strands. This is indicative of an α/β structure for the protein. The secondary structure content estimated with both programs is given in Table 1.

The Putative Fold of β-Xylosidase from T. reesei. Barley β-D-glucan exohydrolase and β-hexosaminidase from *V. cholerae* are the members of GH3 with known three-dimensional structures (Figure 5a,c). It is tempting, therefore, to use these structures as a template for a structural model of *T. reesei* β-xylosidase. Following this reasoning we calculated theoretical scattering curves for the β-D-glucan exohydrolase and β-hexosaminidase using the program CRYSOL (*20*). To our surprise, comparison of these theoretical curves with the experimental SAXS data of the *T. reesei* β-xylosidase gave $\chi = 6.9$ and $\chi = 13.0$, respectively (Figure 5b,d), which clearly indicated gross difference between the structures. Superposition of the crystallographic structure of β-D-glucan exohydrolase with the free atom model of β-xylosidase, performed by the program SUPCOMB (*21*) (Figure 5c), shows that the β-xylosidase is significantly larger than the β-D-glucan exohydrolase, indicating the presence of an additional domain in the β-xylosidase structure that could fill the region inside the circle in Figure 5c. To determine the amino acid sequence extension and organization of possible domains, we conducted an analysis of the β-xylosidase primary structure using the server http://supfam.org/SUPERFAMILY. As a result, the N-terminal part of the protein (residues 61−413) was predicted to fold as a TIM-barrel domain followed by an α/β domain (residues 414−643), in agreement with the classification of this enzyme as a member of GH3. The last 146 residues remained unclassified.

The 2D sequence alignment of *T. reesei* β-xylosidase and barley β-D-glucan exohydrolase, another member of GH3, also indicates that the C-terminal region of β-xylosidase could form the third domain (Figure 6). This is not surprising since some fungal β-xylosidases from other families have already been reported to possess three structural domains (*22*). Furthermore, the secondary structure prediction performed using JUFO: Secondary Structure Prediction for Proteins server (*23*) showed that the third domain should be for the most part composed of β strands. This notion was confirmed by CD analysis of *T. reesei* β-xylosidase. While in total 232 amino acid residues of *T. reesei* β-xylosidase are estimated to adopt the helical conformation and 129 form β structures, by contrast 225 residues form helices and only 64 form β strands in the β-D-glucan exohydrolase X-ray structure. Assuming the existence of the additional structural domain in the former structure and taking into account that the first two domains of these proteins have the same fold and similar secondary structure composition, the third domain of *T. reesei* β-xylosidase should be a β-fold domain. This fact is in accord with the general observation that GHs consist of a catalytic domain joined by flexible linker sequences to one or more carbohydrate binding modules (CBM). The function of the CBMs is largely to attach the enzyme to its substrate and, therefore, enhance the rate of catalysis by increasing the probability of enzyme/substrate interaction (*24*). This observation opens up the possibility that the two auxiliary domains of *T. reesei* β-xylosidase play a role in recognizing the substrate xylooligosaccharides. Moreover, in line with our studies, a number of xylan binding domains found in xylanases adopt β (predominantly β sandwich) folds (*25*). The amino acid sequence of the putative third domain presents significant sequence similarities with the β-xylosidases from *Aspergillus fumigatus*, *Aspergillus oryzae*, *Talaromyces emersonii*, *Aspergillus nidulans*, *Emericella nidulans*, and *Aspergillus niger*, indicating the presence of a similar structural module. Furthermore, pairwise sequence comparison of the putative third domain of β-xylosidase from *T. reesei* with the representative numbers of all known CBMs revealed limited similarity (both overall and within the binding site) with the carbohydrate binding domain of *Rhodothermus marinus* xylanase, a member of the CBM

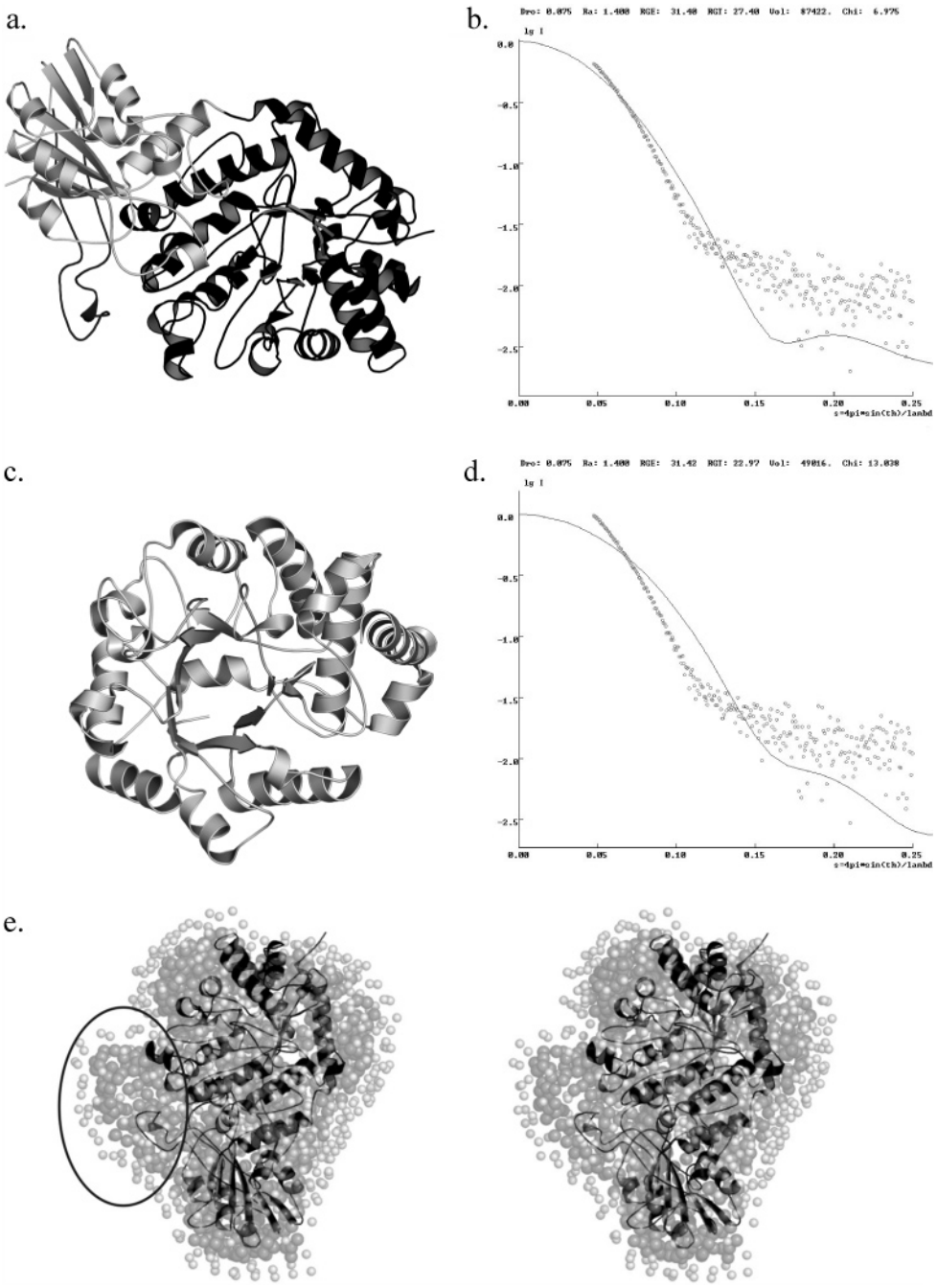

FIGURE 5: (a) Crystallographic structure of barley β-D-glucan exohydrolase, a member of GH3. (b) Comparison of the SAXS data of the *T. reesei* β-xylosidase (dots) with the theoretical scattering curve of the barley β-D-glucan exohydrolase (solid line) shows poor agreement ($\chi = 6.9$), indicating gross differences between these two structures. (c) Crystallographic structure of β-hexosaminidase from *V. cholerae.* (d) The SAXS data of the *T. reesei* β-xylosidase (dots) do not agree well with the theoretical X-ray scattering curve of the β-hexosaminidase, shown as a solid line ($\chi = 13.0$). (e) Superposition of the *T. reesei* β-xylosidase molecular envelope with the 3D structure of barley β-D-glucan exohydrolase indicates that the β-xylosidase could have an additional, third domain.

family 4. However, low identity between these two domains (~16%) does not allow to conclude unambiguously that the third domain of *T. reesei* β-xylosidase belongs to the CBM family 4 or, otherwise, is a member of a new family of CMBs.

CONCLUSIONS

The shape of β-xylosidase in solution was restored at 20 Å resolution from synchrotron X-ray scattering data. It demonstrates that the enzyme forms monomers in solution and indicates that it is composed of three distinct domains. Fold recognition algorithms and 2D sequence alignment show that the first two domains fold, respectively, as a TIM barrel and an α/β domain and, furthermore, confirm the existence of the third, C-terminal, domain. CD data and secondary structure prediction analysis provide evidence that this putative domain should have a predominantly β fold. On the basis of this evidence, we propose that the first two domains of *T. reesei* β-xylosidase have a molecular fold similar to that of barley β-D-glucan exohydrolase, another member of GH3, and, in addition, this monomeric enzyme has an extra, predominantly β-fold domain that could

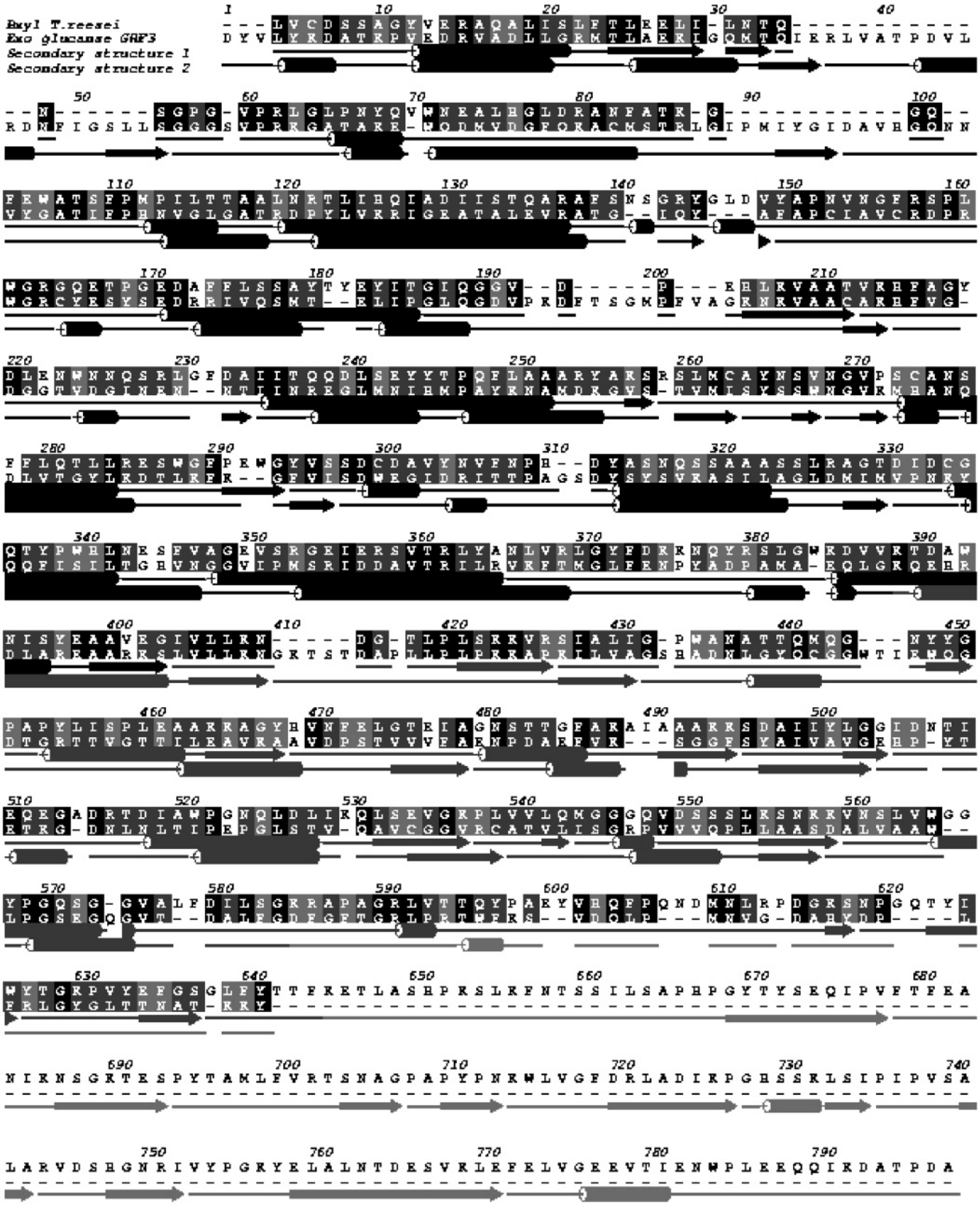

FIGURE 6: Amino acid sequence alignment of the *T. reesei* β-xylosidase (797 amino acids) and barley β-D-glucan exohydrolase (605 amino acids). The amino acid similarity between primary structures as calculated by the program ALSCRIPT is shown in three different shades of gray. The darker color represents higher sequence similarity. Secondary structure elements of the *T. reesei* β-xylosidase domains 1−3, predicted by the JUFO server (*23*), are depicted in black, gray, and light gray, respectively.

possibly function as a carbohydrate binding module involved in the recognition of the substrate. Although crystallization of the β-xylosidase from *T. reesei* has been reported (*27*), these crystals did not yield the crystallographic structure of the enzyme. Independent crystallization of the isolated domains may, therefore, be a possibility. The crystallographic

models of these domains could subsequently be positioned within the SAXS envelope in order to restore the entire three-dimensional structure of the β-xylosidase.

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