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Thermoinactivation of cellobiohydrolase I from *Trichoderma reesei* QM 9414

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Abstract

Irreversible thermoinactivation of cellobiohydrolase I from *Trichoderma reesei* has been analyzed at 70°C and pH 4.8. The time course of thermal inactivation and the dependence of the inactivation rates on protein concentration suggested that aggregation followed by precipitation was the main process leading to irreversible thermoinactivation. The enzyme activity was very resistant to 4 M urea which stabilized the enzyme against thermal inactivation. Deamidation of Asn/Gln residues and hydrolysis of peptide bonds were responsible for the loss of enzyme activity at long times of exposure at 70°C.

Keywords: Cellulase; Cellobiohydrolase I; Thermal inactivation; *Trichoderma reesei*

1. Introduction

The filamentous fungus *Trichoderma reesei* produces a variety of cellulolytic enzymes including endoglucanases, exoglucanases (cellobiohydrolases), and β-glucosidase [1]. The cellulolytic system of *Trichoderma reesei* contains two cellobiohydrolases CBH I and CBH II, which cleave the disaccharide cellobiose from the nonreducing end of the cellulose chain [2,3]. Both are strongly adsorbed onto insoluble cellulose substrates and interact synergically together with endoglucanases to hydrolyze microcrystalline cellulose [4]. During the past years much work has been done to obtain highly pure and

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active cellulase preparations for use in biological reactors to accomplish the optimal hydrolysis of cellulose materials [5].

Enzymes are remarkably good catalysts in terms of their catalytic activity, selectivity, and ability to function under mild conditions. However, in order to be suitable for technical applications, catalysts should be stable under operational conditions for weeks or months. Most enzymes do not satisfy this requirement. From a practical point of view, thermal inactivation is by far the most important mode of enzyme inactivation in biocatalytic reactors where it is necessary to work at elevated temperatures because the rates of enzymatic reactions generally accelerate upon heating, which also reduces bacterial contamination and in most cases increases the solubility of substrates. Thus, a knowledge of the mechanisms of thermoinactivation of a particular enzyme should be of great practical interest.

Unfolding is a general step, usually reversible in enzyme thermoinactivation [6]. The subsequent steps giving rise to irreversible thermoinactivated molecules are specific for individual enzymes and can be conformational or covalent in nature [7]. According to Klibanov and Mozhaev [8], there can be irreversible conformational unfolding of enzymes. Unfolded molecules can intramolecularly refold into new structures that are kinetically or thermodynamically stable and are catalytically inactive. With respect to covalent changes that can contribute to irreversible thermoinactivation of enzymes, deamidation of Asn/Gln residues, hydrolysis of peptide bonds, interchange of disulfide bonds, and modification of amino acids have been described [9]. Whereas the mechanisms involved in the reversible steps are well understood, more work has to be done to throw light on the nature of the irreversible denaturation which accounts for the deterioration of the enzymes in bio-reactors.

The aim of the present work was the analysis of the mechanisms that contribute to the irreversible thermal inactivation of cellobiohydrolase I from *Trichoderma reesei* QM 9414.

2. Materials and methods

Materials.—*p*-Nitrophenyl β-cellobioside (pNPC) was from Sigma (USA). DEAE-Sepharose CL-6B was from Pharmacia (Sweden). Ultrogel AcA-44 and the ampholines used in isoelectric focusing were from LKB (Sweden). Electrophoresis reagents were from Serva (USA). Urea (molecular biology grade) was from USB (USA). All other reagents were of analytical grade from Merck (Germany).

Cellobiohydrolase I source.—Cellobiohydrolase was purified from cultures of *Trichoderma reesei* QM 9414. The fungus was grown on wheat straw as previously described [10] Cellulolytic enzymes were precipitated from culture supernatants with $(NH_4)_2SO_4$ (20–60% saturation). The precipitate was resuspended in 5 mM sodium phosphate buffer, pH 7.0, and loaded onto a DEAE-Sepharose CL-6B column (2.6 × 42 cm) equilibrated in the same buffer. Elution was carried out with this buffer (250 mL), a linear gradient of 0–500 mM NaCl in the same buffer (250 mL), and buffer containing 500 mM NaCl (250 mL). The fractions with cellobiohydrolase activity were pooled, dialyzed against water, lyophilized, and applied on an Ultrogel AcA-44 column (1.6 × 95

cm) equilibrated in 100 mM NH_4OAc buffer, pH 5.0. The fractions with cellobiohydrolase activity were pooled, freeze-dried, resuspended in 10 mM NH_4OAc buffer (pH 4.5), and loaded onto a Sepharose CL-6B column (2.5 × 20 cm) equilibrated in the same buffer. Elution was completed with the initial buffer (100 mL), a linear gradient of 10–500 mM NH_4OAc (300 mL), and 500 mM NH_4OAc (pH 4.5, 100 mL). The homogeneity of the sample eluted from the last chromatography step was demonstrated by SDS-polyacrylamide gel electrophoresis and by analytical isoelectrofocusing.

Enzyme assay.—The enzymatic activity of cellobiohydrolase was determined using pNPC as substrate as previously described [11]: 1.8 ml of a 1 mg/mL solution of pNPC in 100 mM NH_4OAc buffer (pH 4.8) and 0.2 mL of enzyme solution were incubated at 30°C for 30 min. The released *p*-nitrophenol was estimated spectrophotometrically at 410 nm by mixing the incubation solution (1 mL) and aq 2% Na_2CO_3 (1 mL) (molar extinction coefficient, 18.5 mL/μmol/cm).

Kinetics of irreversible thermoinactivation of cellobiohydrolase.—Solutions of the enzyme (0.2 mL) in 100 mM NH_4OAc buffer (pH 4.8) in the presence or absence of 4 M urea were incubated at 70°C in a thermostatically controlled bath. Aliquots (0.2 mL) were periodically withdrawn and kept at 20°C during 2 h, in order to allow refolding of reversibly inactivated enzymes. Afterwards, cellobiohydrolase activity was determined, as described for the enzyme assay, in triplicate experiments. Data of the remaining activity versus thermoinactivation time were fitted to the corresponding equations by using the BMDP program [12].

Soluble protein after thermoinactivation.—Samples of cellobiohydrolase I were thermoinactivated at 70°C and pH 4.8 during different times. Soluble protein in thermoinactivation assays was determinated by using spectrophotometric measurements, at 280 nm, after centrifugation and filtration through Millipore filters (0.45 μm pore diameter).

Thermal stability of cellobiohydrolase.—The stability of cellobiohydrolase I towards heating was monitored by incubating the enzyme dissolved in the appropiate buffer at different temperatures during 30 minutes. Then, after cooling, the enzymatic activity was assayed as described above.

Absorption spectra.—Absorption spectra were recorded in a single beam Beckman DU-70 spectrophotometer using 1-cm optical path cells at 2 nm/s scanning speed. Net absorbance at 280 nm was obtained by correcting spectra for dispersion with the use of a computer program inherent to the apparatus.

Circular dichroism spectra.—Circular dichroism (CD) spectra were obtained in a Jobin Yvon Mark III dichrograph fitted with a 250-W Xenon lamp; the spectra were recorded at 0.5 nm/s scanning speed. Samples were analyzed in 0.1-cm optical path cells in the far ultraviolet (below 250 nm) region. The reported CD values were the average of at least three independent measurements. These values were expressed as mean residue weight ellipticities, in units of degree cm^2 $dmol^{-1}$ on the basis of 104.9 as the average molecular weight per residue for cellobiohydrolase I.

Before the spectra were recorded all samples were previously centrifuged and filtered through Millipore filters (0.45 μm pore diameter). Protein concentration was determined after each CD spectrum by spectrophotometric methods and it was in the range of 1 mg/mL.

Amino acid composition.—Samples of native and thermoinactivated cellobiohydrolase I were reduced and carboxymethylated. Afterwards they were hydrolyzed in sealed evacuated tubes with 5.7 M HCl for 24 h at 110°C. Amino acid analysis was performed with a Beckman amino acid analyzer.

Determination of free thiol groups.—The number of thiol groups in thermoinactivated samples of cellobiohydrolase I was determined by using 5,5′-dithiobis(2-nitrobenzoic acid) following the procedure described by Ellman [13].

SDS-Polyacrylamide gel electrophoresis.—Polyacrylamide gel electrophoresis in the presence of 3% SDS was carried out according to Laemmli [14] at a constant current of 55 mA in 12.5% polyacrylamide slab gels (1.5 mm thick). Visualization was by Coomassie Blue staining.

Isoelectric focusing.—Analytical isoelectric focusing was carried out in 4.5% polyacrylamide slab gels (1.5 mm thick) in the pH range 2.5–6.0 according to the method described by Rodríguez de Córdoba et al. [15]. Samples were focused for 15 h at 15 mA. Visualization was by Coomassie Blue staining.

3. Results

At 70°C and pH 4.8, the pH for the maximum activity, cellobiohydrolase I undergoes a rapid irreversible thermoinactivation accompained by a visible heavy precipitation. Residual activity was measured in the supernatant solutions of the thermoinactivated samples. The activity and the amount of soluble protein decreased in parallel during the experiment.

The time course of thermal inactivation shown in Fig. 1 was non-first order, indicating the polymolecular nature of the inactivation process that was further confirmed by the dependence of the thermoinactivation on the initial protein concentration

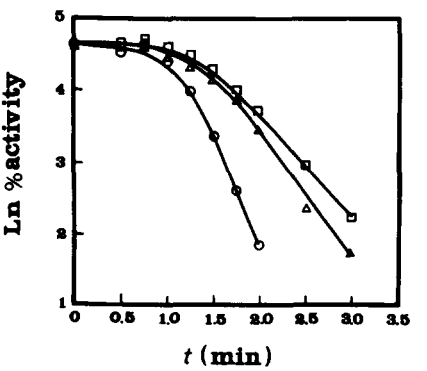

Fig. 1. Time course of irreversible thermoinactivation of cellobiohydrolase I at 70°C and pH 4.8. Samples of the enzyme (0.2 mL) at different initial protein concentration were incubated at 70°C and pH 4.8 at the indicated times. After 2 h at 20°C, the % residual activity of the samples was determined as described in the Materials and Methods section. Initial protein concentrations were 2 (—○—), 1 (— △ —), and 0.5 mg/mL (— □ —).

in the range of 0.5–2 mg/mL. As can by seen in Fig. 1, there is a lag period from the start of thermoinactivation to 1.5 min where activity was unchanged, and whose length was dependent on cellobiohydrolase concentration. From 1.5 min to the end of the experiment, activity decreased with time. This kind of behaviour has been described by Sadana [16] who proposed a lag period before deactivation. After this lag period, the inactivation can be explained by several models. In the present case the data fitted well to a first-order inactivation pattern:

$$\text{Ln } A = -kt$$

where A is the enzyme activity at each time and k is the rate of inactivation. Table 1 depicts the inactivation rates as a function of the initial protein concentration. It can be seen that a decrease in protein concentration reduces the rate and extent of thermally induced aggregation.

Table 1
Thermoinactivation rates of cellobiohydrolase I

Protein	2 mg/mL	1 mg/mL	0.5 mg/mL
k (s^{-1})$\times 10^3$	43.39 ± 2.84	28.29 ± 1.61	22.93 ± 2.11

When thermoinactivation of cellobiohydrolase I was carried out in the presence of 4 M urea, which avoids aggregate formation by preventing non-specific interactions between protein molecules, the process obeyed first-order kinetics and was independent of the initial protein concentration (Fig. 2). The rate of inactivation calculated from the slope was $7.2 \pm 0.27 \times 10^{-3}$ s^{-1} an order of magnitude lower than the corresponding rates (Table 1) in the absence of urea; $t_{1/2}$ was ca. 84 min in the presence of urea and 0.8–1.8 min in its absence, indicating the protective effect of the chaotropic agent against irreversible thermoinactivation.

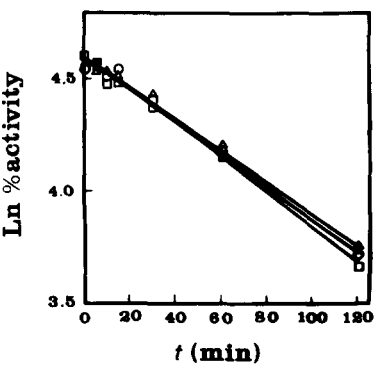

Fig. 2. Time-course of irreversible thermoinactivation of cellobiohydrolase I in the presence of 4 M urea. Samples of the enzyme (0.2 mL) at different initial protein concentration were preincubated at 70°C in 0.1 M NH_4OAc buffer (pH 4.8) containing 4 M urea at the indicated times. After 2 h at 20°C, the % residual activity of the samples was determined as described in the Materials and Methods section. Protein concentrations were 5 (—△—), 1 (—□—), and 0.25 mg/mL (—○—).

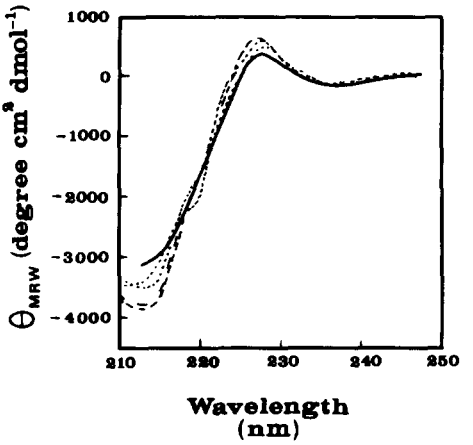

Fig. 3. CD spectra of cellobiohydrolase I. Samples of the enzyme (1 mg/mL) were dissolved in 0.1 M NH_4OAc buffer (pH 4.8) and preincubated at 70°C during different times. Aliquots were periodically removed and after 2 h at 20°C, the samples were filtered and their CD spectra recorded as indicated in the Materials and Methods section. Samples containing 4 M urea (———). Samples without urea: control (· · · · · ·), 30 s at 70°C (---), 1 min at 70°C (— — —), and 2 min at 70°C (— —).

The effect of 4 M urea on the structure of cellobiohydrolase I has been analyzed by circular dichroism. The CD spectra of the protein in the far UV region indicate no significant change by exposure of the enzyme to 4 M urea even at 70°C (Fig. 3). Moreover, enzyme activity was unchanged in the presence of urea until a concentration of 4 M urea in the incubation assays, in standard conditions (data not shown).

The high resistance of this protein to urea could be ascribed to a reduced flexibility of its structure, which should be correlated to a rigid structure constrained by 12 disulfide bridges [17].

In order to ascertain the importance of disulfide bridges in the structure of cellobiohy-

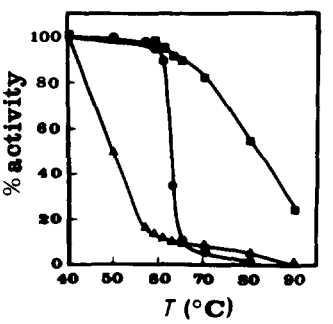

Fig. 4. Thermal stability of cellobiohydrolase I. Samples of the enzyme were preincubated for 30 min at different temperatures and their % residual activities tested after 2 h at 20°C. Enzyme in 0.1 M NH_4OAc buffer (pH 4.8) (—●—), enzyme in the same buffer containing 4 M urea (—■—), and enzyme in the same buffer containing 4 M urea and 2 mM DTE (—▲—).

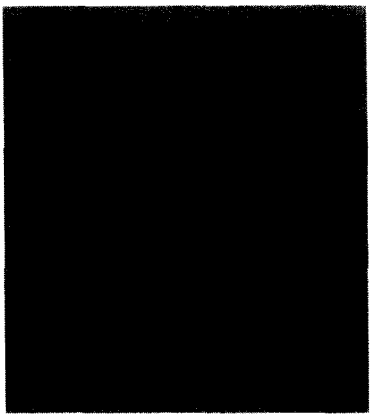

Fig. 5. Isoelectric focusing on polyacrylamide gel of cellobiohydrolase I heated at 70°C for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), and 4 h (lane 6). The isoelectric focusing was run in the pH range 2.5–6.0. Lane 1 shows native cellobiohydrolase I and corresponds to a homogeneous preparation of the enzyme as shown by SDS-PAGE. The two bands on lane 1 correspond to isocomponents of the enzyme which are immunologically identical [25].

drolase I, the thermostability of the enzyme was analyzed at 70°C in 100 mM ammonium acetate buffer (pH 4.8) containing 4 M urea and 2 mM dithioerythritol (DTE). The results in Fig. 4 show that the addition of the reducing agent results in a significant weakening of cellobiohydrolase I.

The present results point to aggregation as the main process of thermoinactivation of cellobiohydrolase I. Nevertheless, thermoinactivation in the presence of 4 M urea (Fig. 2) shows that processes apart from aggregation contribute to irreversible inactivation of the enzyme at times longer than when aggregation occurs.

In order to identify deleterious covalent processes that could participate in irreversible inactivation, samples of thermally inactivated cellobiohydrolase I were analyzed for deamidation of Asn/Gln residues, hydrolysis of peptide bonds, interchange of disulfide bonds, and amino acid modification according to Zale and Klibanov [9].

Heat-induced deamidation of Asn and Gln residues seems to be a general phenomenon in irreversible inactivation of enzymes [9]. Since deamidation converts Asn into Asp and Gln into Glu, the extent of deamidation can be followed by isoelectric focusing. Samples of cellobiohydrolase I were heated at 70°C, pH 4.8 during different times. It can be seen from Fig. 5 that, upon heating, the native band of the enzyme gradually disappears and is replaced by bands with decreasing pI's after 4 h of heat treatment.

To determine the possible contribution of peptide bond hydrolysis to thermal irreversible inactivation of cellobiohydrolase I, samples of the native enzyme and of the enzyme heated at 70°C for several periods of time, at pH 4.8, were analyzed by SDS/PAGE. After 2 h of heating, new bands corresponding to proteins of low M_r appeared, possibly indicating the involvement of peptide bond hydrolysis in the irreversible thermoinactivation of the enzyme (Fig. 6).

Reactions involving sulfur-containing amino acid residues are often responsible for thermoinactivation of enzymes. Cystine residues in proteins undergo destruction via two

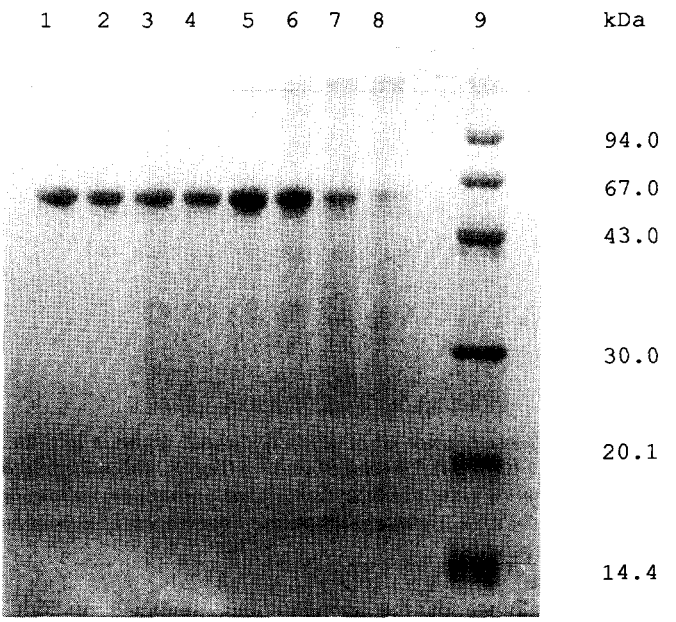

Fig. 6. SDS-PAGE of cellobiohydrolase I heated at 70° for 0 min (lane 1), 1 h (lane 2), 4 h (lane 3), 10 h (lane 4), 24 h (lane 5), 48 h (lane 6), 72 h (lane 7), and 96 h (lane 8). Molecular weight markers are shown in lane 9.

different mechanisms: β-elimination and disulfide interchange catalyzed by thiols formed during the β-elimination or by thiols from cysteine residues in the native enzyme [9]. Although there are no free cysteine residues in native cellobiohydrolase I, we have tested the possible involvement of β-elimination and subsequent disulfide interchange in the thermoinactivation of this enzyme. Cellobiohydrolase I was thermoinactivated at 70°C, pH 4.8; samples were removed after several time periods and assayed for free thiol groups with Ellman's reagent. The absorbance at 412 nm was constant along the inactivation process, indicating the absence of free thiol groups that could contribute to irreversible thermoinactivation by the mechanisms described above.

We also evaluated the possible contribution of amino acid modification to the irreversible thermoinactivation of cellobiohydrolase I. In this way, samples of the enzyme were heated at 70°C, pH 4.8 for 2, 5, 10, 24, and 48 h. The amino acid analysis of the corresponding protein hydrolysates showed no changes in the amino acid composition of cellobiohydrolase I during the inactivation process in agreement with the absence of β-elimination. Amino acid analysis employed by us cannot detect deamidation of Asn or Gln residues.

4. Discussion

The present data indicate that irreversible thermoinactivation of cellobiohydrolase I from *Trichoderma reesei* at 70°C and pH 4.8, the pH for the maximal activity, is caused by a rapid aggregation.

The time course of thermal inactivation showed a biphasic behaviour with a first stage where the enzyme was fully active and whose duration was dependent on protein concentration. There followed a second stage where the decay in activity was fitted to first-order kinetics with a rate of inactivation that was dependent on the initial protein concentration in the thermoinactivation assays. Taking into account that the residual activity corresponded to the soluble protein and that activity and soluble protein decreased in parallel during inactivation, the first stage could be ascribed to a "nucleation time" where aggregates are being formed.

Although a polymolecular process such as aggregation generally obeys non-first-order kinetics [9,18,19], the results presented here fitted first-order kinetics, probably because the aggregates of cellobiohydrolase I, formed during the first stage, are fully active and their precipitation is responsible for the enzyme inactivation.

It is known that thermoinactivation of proteins is accompanied by aggregation. Aggregation is not the cause of irreversible thermoinactivation but a subsequent reaction to irreversible unfolding when high concentrations of proteins are present [20]. Thus, when urea was added to the reaction mixture, the half-life was increased indicating that non-specific interactions have an effect on the thermoinactivation process and lead to thermoaggregation of cellobiohydrolase I in the absence of urea.

The present data suggest that cellobiohydrolase I is rather resistant to the chaotropic agent urea; this stability probably arises from the presence of disulfide bridges in its structure. The results in Fig. 4 support this hypothesis, showing that in the presence of 1,4-dithioerythritol the enzyme was more easily inactivated. Moreover, the enzyme was fully active when assayed in 4 M urea in standard conditions, and its secondary structure did not change in these conditions. A similar effect has been described by Woodward et et al. [21] for cellobiohydrolase I in the presence of guanidine hydrochloride, by Domínguez el al. [22] for endoglucanase I in the presence of 8 M urea, and by Chaffotte et al. [23] for endoglucanase D in the presence of 8 M urea.

Samples of thermally inactivated cellobiohydrolase I were analyzed for peptide chain integrity, amino acid destruction, and disulfide bond interchange in order to identify covalent processes that could contribute to inactivation. Results presented here show the deamidation of Asn/Gln residues at intermediate times of thermal treatment and hydrolysis of peptide bonds at longer times of inactivation.

Finally, destruction of cystine residues via β-elimination [24], interchange of disulfide bonds, or amino acid modification could not be observed under our assay conditions, excluding these mechanisms as responsible for the irreversible thermoinactivation of cellobiohydrolase I.

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