Mechanisms of thermoinactivation of endoglucanase I from *Trichoderma reesei* QM 9414

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The mechanism of irreversible thermoinactivation of endoglucanase I from *Trichoderma reesei* has been determined at 70 °C at the pH of maximum enzyme activity. The time-course of thermoinactivation did not follow first-order kinetics and kinetic constants of the process were dependent on enzyme concentration, suggesting that aggregation was the main process leading to irreversible inactivation. The enzyme was extremely resistant to urea, which in fact seemed to stabilize it against temperature. Disulphide exchange, deamidation and hydrolysis of peptide bonds were also responsible for the loss of enzyme activity at 70 °C.

INTRODUCTION

Endoglucanases (1,4- β -D-glucano-glucohydrolases, EC 3.2.1.4) are one group of isoenzymes of the cellulase complex. They hydrolyse internal β -1,4 glycosidic bonds of the cellulase polymer and show more affinity for the amorphous zones of the cellulose where weaker hydrogen bonds between cellulose fibres exist (Mandels, 1982; Enari, 1983).

During the past few years, many workers have tried to obtain highly pure and active cellulase preparations from many micro-organisms to utilize them in industrial biochemical reactors to obtain optimal hydrolysis of cellulose materials (Pokorny *et al.*, 1990). One of the main problems involved in their industrial use is their thermal inactivation. Therefore elucidation of the molecular events taking place in proteins on heating should be of great practical interest (Klibanov, 1983).

Thermoinactivation of enzymes has been described by Lumry & Eyring (1954) by the following simple mechanism:

where N is the native catalytically active enzyme, U is the partially unfolded enzyme and I is the irreversibly inactivated enzyme. 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 transformation to I which accounts for the enzyme deterioration in biocatalytic reactors.

The present paper reports the molecular mechanisms of irreversible thermal inactivation of endoglucanase I from *Trichoderma reesei* QM 9414 at 70 °C and pH 4.8, which was the pH at which maximal activity was produced.

MATERIALS AND METHODS

Materials

CM-cellulose (medium viscosity, degree of substitution 0.8) was from Fluka. DEAE-Sepharose CL-6B was from Pharmacia. Ultrogel AcA-44 as well as the ampholines used in isoelectric focusing were from LKB. Electrophoresis reagents were from Serva. Urea (molecular biology grade) was from USB. All other reagents were of analytical grade from Merck.

Enzyme source

Endoglucanase was purified from cultures of *Trichoderma reesei* QM 9414. The fungus was grown on wheat straw as previously described (Acebal *et al.*, 1986). Cellulolytic enzymes were precipitated from culture supernatants with $(\text{NH}_4)_2\text{SO}_4$

(20–60 % saturation). The precipitate was resuspended in 5 mM-sodium phosphate buffer, pH 7.0, and loaded on to a DEAE-Sepharose CL-6B column (2.6 cm $\times$ 42 cm) equilibrated in the same buffer. Elution was carried out with 250 ml of this buffer, 250 ml of a linear gradient of 0–500 mM-NaCl in the same buffer and 250 ml of buffer containing 500 mM-NaCl. Four peaks with endoglucanase activity were obtained and the last one was pooled, dialysed against water, freeze-dried and applied to an Ultrogel AcA-44 column (1.6 cm $\times$ 95 cm) equilibrated in 100 mM-ammonium acetate buffer (pH 5.0). Fractions containing endoglucanase activity were pooled, freeze-dried, resuspended in 10 mM-ammonium acetate buffer (pH 4.5) and loaded on to a DEAE-Sepharose CL-6B column (2.5 cm $\times$ 20 cm) equilibrated in the same buffer. Elution was completed with 100 ml of the initial buffer, 300 ml of a linear gradient of 10–500 mM-ammonium acetate and 100 ml of 500 mM-ammonium acetate (pH 4.5).

Homogeneity of the sample eluted from the last chromatography step was demonstrated by SDS/PAGE and by analytical isoelectrofocusing. The identity of this endoglucanase with endoglucanase I described by Penttilä *et al.* (1986) was confirmed by isoelectrofocusing, peptide mapping and sequencing of CNBr peptides (results not shown).

Enzyme assay

The enzyme activity of endoglucanase was determined using CM-cellulose as substrate as previously described (Acebal *et al.*, 1986).

Kinetics of irreversible thermoinactivation of endoglucanase I

Solutions of the enzyme in 100 mM-ammonium acetate buffer, pH 4.8, in the presence or absence of 8 M-urea and 200 μM -CuCl₂ were incubated at 70 °C in a thermostatically controlled water-bath. Aliquots were periodically withdrawn and incubated at 20 °C over 2 h. Afterwards, endoglucanase activity was determined in triplicate assays. The data for the remaining activity versus thermoinactivation time were fitted to the corresponding equations by using the BMDP program (Dixon, 1981).

Re-activation of thermoinactivated endoglucanase

A solution of endoglucanase I, at a protein concentration of 0.5 mg/ml in 100 mM-ammonium acetate buffer, pH 4.8, in the presence or absence of 8 M-urea and 200 μM -CuCl₂ was incubated at 70 °C for 2 h in a thermostatically controlled water-bath. Then urea and cupric salt were removed by desalting on a Sephadex G-

25 column (Pharmacia, PD-10) equilibrated in the same buffer. The desalted sample was then incubated at 20 °C; aliquots were periodically removed and their activities measured.

Thermal stability of endoglucanase I

The stability of endoglucanase I with temperature was monitored by incubating the enzyme dissolved in the appropriate buffer at different temperatures over 30 min. Then, after cooling, the enzyme 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-length 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.

C.d. spectra

C.d. 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 analysed in 0.1 cm optical-path-length cells in the far-u.v. (below 250 nm) region. The reported c.d. values were the average of at least three independent measurements. These values were expressed as mean residue weight ellipticities, in units of degree $\cdot$ cm² $\cdot$ dmol⁻¹ on the basis of 104.3 as the average M_r per residue for endoglucanase I.

Before the spectra were recorded, all samples were previously centrifuged and filtered through Millipore filters (0.45 μ m pore diameter). Protein concentration was determined after each c.d. spectrum by amino acid analysis and it was in the range of 0.3 mg/ml.

Amino acid composition

Samples of thermoinactivated endoglucanase I were hydrolysed in sealed evacuated tubes with 5.7 M-HCl for 24 h at 110 °C. Amino acid analysis was performed with a Durrum D-500 amino acid analyser.

Tryptophan was determined colorimetrically as described by Barman & Koshland (1967).

Determination of free thiol groups

The number of thiol groups in thermoinactivated samples of endoglucanase I was determined by using 5,5'-dithiobis(2-nitrobenzoic acid) by the procedure of Ellman (1959).

SDS/PAGE

PAGE in the presence of 3% (w/v) SDS was carried out as described by Laemmli (1970) at a constant current of 55 mA in 12.5% polyacrylamide slab gels (1.5 mm thick).

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 as described by Rodríguez de Córdoba *et al.* (1984). Samples were focused for 15 h at 15 mA.

RESULTS AND DISCUSSION

The aim of the present work was the determination of the mechanism of irreversible thermoinactivation of endoglucanase I. Most of the experiments were carried out at 70 °C, at which temperature the enzyme is inactivated at a suitable rate. Although the contribution of different mechanisms to thermoinactivation of an enzyme is strongly dependent on pH, we have carried out all the assays at pH 4.8 because that is the pH producing maximum activity of endoglucanase I.

The time-course of thermal inactivation of endoglucanase I at 70 °C and pH 4.8 is shown in Fig. 1. The semilogarithmic plot of residual activity versus time of inactivation obeys second-order kinetics. Taking into account the different kinetic models proposed by Ray & Koshland (1961) for enzyme inactivation, the kinetics of thermoinactivation of endoglucanase I could be assigned to the mechanism shown in Scheme 1.

Fig. 1. Time course of irreversible thermoinactivation of endoglucanase I

Samples of the enzyme were incubated at 70 °C as described in the Materials and methods section. Protein concentration was 0.5 mg/ml (●) and 0.25 mg/ml (▲).

Scheme 1. Ray & Koshland's (1961) model for enzyme inactivation

E, catalytically active enzyme; E₁, partially inactivated enzyme which conserves a fractional activity F_1 ; E₂ and E₃, totally inactive forms of the enzyme; k_1 and k_2 , rate constants of inactivation.

Scheme 2. Possible mechanism of thermoinactivation of endoglucanase I

This is also described by eqn. (1) but k_2 is replaced by k_2 , a kinetic rate constant that includes those processes that cause thermoinactivation of the enzyme different from those that transform E into E₁.

Table 1. Kinetic constants for thermoinactivation of endoglucanase I

The values for the constants and their standard deviations were obtained by statistical analysis of the parameter data according to eqn. (1).

Protein concentration (mg/ml)	k_1 (min ⁻¹)	$10^3 \times k_g$ (min ⁻¹)	F_1
0.5	0.172 ± 0.03	3.25 ± 0.5	0.434 ± 0.03
0.25	0.107 ± 0.02	5.00 ± 1.2	0.436 ± 0.05

Fig. 2. Time course of irreversible thermoinactivation of endoglucanase I in the presence of 8 M-urea

A sample of the enzyme at a protein concentration of 0.5 mg/ml was incubated at 70 °C as described in the Materials and methods section.

The observed activity as a function of time is given by the following equation:

$$A_R = (1 - F_1)\exp[-(k_1 + k_g)t] + F_1\exp(-k_2t) \tag{1}$$

where A_R is the residual enzyme activity at time t .

However, when one deals with thermoinactivation of enzymes, the previous model, proposed by Ray & Koshland (1961) for chemical inactivation of enzymes, can be modified (Scheme 2).

Experimental data depicted in Fig. 1 fit well to eqn. (1), indicating the validity of the proposed model at least over a range of protein concentration from 0.25 to 0.5 mg/ml. Table 1 summarizes k_1 , k_g and F_1 values as a function of the enzyme concentration in the assay. It can be observed that F_1 , the fractional activity of partially inactivated enzyme, was independent of enzyme concentration, suggesting that E_1 is a unique molecular species.

Taking into account that the time-course of thermal inactivation of endoglucanase I does not obey first-order kinetics and that kinetic parameters were found to be dependent on protein concentration (Table 1), it can be suggested that the enzyme is inactivated through a polymolecular process (e.g. formation of aggregates, E_1 , less active than the native enzyme) (Baldwin, 1975; Zale and Klibanov, 1983).

In order to test this hypothesis, thermoinactivation was carried out in the presence of 8 M-urea which disrupts non-covalent forces, thus preventing the formation of aggregates. The time-course of thermal inactivation of endoglucanase I at 70 °C in 100 mM-ammonium acetate buffer, pH 4.8, containing 8 M-urea is depicted in Fig. 2. Controls of activity were carried out under standard conditions in both the absence and presence of 8 M-

Fig. 3. Absorption spectra of thermoinactivated endoglucanase I

A sample of the enzyme (0.5 mg/ml) in 100 mM-ammonium acetate buffer, pH 4.8 (a) or in the same buffer containing 8 M-urea (b) was incubated at 70 °C. Aliquots were periodically removed and incubated at 20 °C for 2 h. The samples were then centrifuged and filtered and their spectra recorded. —, Control spectrum; —, after 5 min at 70 °C; ---, after 30 min at 70 °C; ----, after 60 min at 70 °C; ····, after 2 h at 70 °C; -·-·-, after 4 h at 70 °C. Insets: variation of the net absorbance at 280 nm with the thermo-inactivation time.

urea. The two controls exhibited identical activity. As can be seen in Fig. 2, thermoinactivation of endoglucanase I in the presence of 8 M-urea follows first-order kinetics as represented by the single scheme:

where, as in Schemes 1 and 2, E is the catalytically active enzyme, I is the irreversibly inactivated enzyme and k is the rate constant of the process. From the slope in Fig. 2, the observed rate constant has a value of $6.9 \times 10^{-3} \text{ min}^{-1}$, which is of the same order of magnitude as k_g . These results suggest that urea prevents

Fig. 4. C.d. spectra of endoglucanase I after different thermoinactivation times

A sample of the enzyme dissolved in 100 mM-ammonium acetate buffer, pH 4.8 (a) or in the same buffer containing 8 M-urea and 200 μ M-CuCl₂ (b), at a protein concentration of 0.3 mg/ml, was incubated at 70 °C. Aliquots were periodically removed and incubated at 20 °C for 2 h. The samples were then filtered and their spectra recorded as indicated in the Materials and methods section. To simplify the diagram, only some of the spectra are shown: a, control; b, after 5 min at 70 °C; c, after 30 min; d, after 120 min; e, after 240 min.

aggregate formation by eliminating the pathway to E₁ (see Scheme 2); therefore all the processes included under k must be the same as included under k_g . The higher value of the former with respect to the latter can be explained by urea affecting the enzyme structure, making it more susceptible to those processes with the increasing of the kinetic constant.

In order to demonstrate the aggregate formation, absorption spectra of the enzyme were recorded after heat-treatment for different lengths of time in both the presence and absence of 8 M-urea (Fig. 3). It should be noted that in the absence of urea the amount of solubilized enzyme (which accounts for the net absorbance at 280 nm) rapidly decreases due to precipitation concomitant with the formation of large aggregates (Fig. 3a,

Fig. 5. Thermal stability of endoglucanase I

Samples of the enzyme were incubated for 30 min at different temperatures and their activities then tested. ●, Enzyme dissolved in 100 mM-ammonium acetate buffer, pH 4.8; ○, enzyme dissolved in the same buffer containing 8 M-urea and 2 mM-dithioerythritol; ▲, enzyme dissolved in the buffer containing 8 M-urea and 200 μ M-CuCl₂; △, enzyme dissolved in the buffer containing 8 M-urea.

inset). However, in the presence of 8 M-urea the amount of solubilized enzyme remains constant (Fig. 3b, inset).

Moreover, when Figs. 1 and 2 are compared, it is evident that $t_{1/2}$ of inactivation is considerably higher in the presence of 8 M-urea (5 min versus 100 min), indicating a protective effect of urea against irreversible thermoinactivation of the enzyme. This rather striking behaviour has also been described for other enzymes (Little, 1978). As pointed out by Little (1978) for phospholipase C, it is possible that endoglucanase I contains a core of stable structure, possibly a particularly hydrophobic region which is further stabilized by high urea concentrations and which serves as a nucleation centre for refolding of polypeptide chain after denaturation. Moreover, Woodward *et al.* (1990a,b) have reported that another chaotropic agent, guanidinium chloride, causes no change in the structure of the major cellulase of *Trichoderma reesei*, i.e. cellobiohydrolase I. This great conformational stability was attributed to the large number of disulphide bridges (12) in this enzyme. Taking into account that endoglucanase I contains 11 disulphide bridges, this could also explain the stability of this enzyme against urea.

Far-u.v. c.d. spectra of endoglucanase I after different thermoinactivation times are shown in Fig. 4. The addition of 8 M-urea causes drastic changes in the c.d. spectra, suggesting extensive denaturation even without preheating of the enzyme. The absence of any residual periodic structure could indicate that the postulated nucleation centre lacks periodic structure, or its contribution is small. Alternatively non-periodic structure may be involved (Little, 1978).

In order to investigate whether disulphide bridges sustain this centre of minimal structure, the thermostability of endoglucanase I was tested in 100 mM-ammonium acetate buffer, pH 4.8, containing 8 M-urea and 200 μ M-CuCl₂ as protective agent for disulphide bonds at the indicated temperatures. Thermostability was also examined under the same conditions but in the presence of 2 mM-dithioerythritol, as a reducing agent, instead of CuCl₂. The results in Fig. 5 show that the addition of Cu²⁺ results in significant stabilization of the enzyme. In contrast, the addition of a reducing agent of disulphide bonds results in a significant weakening of endoglucanase I.

The time-course of thermal inactivation of endoglucanase I at

Fig. 6. Time course of irreversible therminoactivation of endoglucanase I in the presence of 8 M-urea and 200 μM - CuCl_2

A sample of the enzyme at a protein concentration of 0.5 mg/ml was incubated at 70 °C as described in the Materials and methods section.

Fig. 7. Time course of re-activation of therminoactivated endoglucanase I

Samples of the enzyme were therminoactivated over 2 h at 70 °C and re-activated by incubating at 20 °C for different periods of time as described in the Materials and methods section. O, Enzyme in 100 mM-ammonium acetate buffer, pH 4.8; ●, enzyme in the same buffer containing 8 M-urea and 200 μM - CuCl_2 .

70 °C in 100 mM-ammonium acetate, pH 4.8, containing 8 M-urea and 200 μM - CuCl_2 is shown in Fig. 6. The semilogarithmic plot of residual activity versus time of inactivation does not obey first-order kinetics. According to Ray & Koshland (1961), the kinetics of therminoactivation under these conditions could be expressed by the following mechanism:

where E^* is a new form of the enzyme with a different structure to E which retains full activity; k_a and k_b are the corresponding rate constants of inactivation. The observed activity as a function of time is given by the following equation:

$$A_R = [k_b/(k_b - k_a)]\exp(-k_a t) - [k_a/(k_b - k_a)]\exp(k_b t) \quad (2)$$

The experimental data depicted in Fig. 6 fit well to eqn. (2),

Fig. 8. Free thiol groups (mol/mol of protein) produced during therminoactivation of endoglucanase I

A solution of the enzyme, at a protein concentration of 0.5 mg/ml in 100 mM-ammonium acetate buffer, pH 4.8, was incubated at 70 °C. Samples (50 μl) were periodically withdrawn and 950 μl of 100 mM-Tris/HCl buffer (pH 7.5)/1 mM-EDTA was added. Then 50 μl of 10 mM-5,5'-dithiobis(2-nitrobenzoic acid) in the same buffer was added, and the samples were kept for 15 min at 25 °C. Absorbance at 412 nm was then measured and correlated with the amount of free thiol by reference to a previously constructed calibration curve.

being $k_a = 2.8 \times 10^{-4} \pm 0.16 \times 10^{-4} \text{ min}^{-1}$ and $k_b = 2.6 \times 10^{-4} \pm 0.13 \times 10^{-4} \text{ min}^{-1}$.

The c.d. spectrum of endoglucanase I in the presence of 8 M-urea and 200 μM - CuCl_2 depicted in Fig. 4 is similar to that obtained in the presence of 8 M-urea (results not shown), suggesting, in this case also, extensive denaturation with the absence of any periodic structure. These findings strongly suggest the relevance of disulphide bonds in stabilization of a centre of minimal structure which, on the other hand, allows the enzyme to retain full activity.

Unfolding is a general step, usually reversible, in enzyme therminoactivation. The subsequent steps giving rise to irreversibly therminoactivated enzyme molecules are specific for individual enzymes and can be conformational or covalent (Ahern & Klibanov, 1985). The results presented here show aggregation to be responsible for irreversible thermal inactivation of endoglucanase I. However, heat-treatment of the enzyme in the presence of a denaturing agent (8 M-urea), which prevents aggregate formation, and in the presence of a protective agent of disulphide bonds (CuCl_2), did not allow full restoration of activity after removal of the denaturing agent and cooling of the enzyme solution (Fig. 7). Thus some kind of covalent mechanism must also account for irreversible thermal inactivation of the enzyme.

Reactions involving sulphur-containing amino acid residues are often responsible for therminoactivation of enzymes owing to the thermally induced interchange of disulphide bonds which results in enzyme molecules with incorrect disulphide bond pairings (Klibanov, 1983; Zale & Klibanov, 1986). In order to test if this kind of covalent mechanism occurs in thermal inactivation of endoglucanase I, the enzyme was therminoactivated at 70 °C and pH 4.8; samples were removed after several time-periods and assayed for free thiol groups with Ellman's reagent. Results in Fig. 8 show an irregular variation of the number of free thiol groups which does not allow determination of a kinetic constant for this process. However, it clearly indicates the existence of a continuous reduction-oxidation process among cystines in the enzyme, with incorrect

Fig. 9. Isoelectric focusing on polyacrylamide gel of endoglucanase I heated at 70 °C for 15 min (lane 1), 30 min (lane 2), 60 min (lane 3), 2 h (lane 4), 4 h (lane 5), 6 h (lane 6), 10 h (lane 7) and 24 h (lane 8).

The isoelectric focusing was run in the pH range 2.5–6.0.

disulphide pairs being formed, thus helping unfolding of the molecule.

However, the results in Fig. 7 show that enzyme activity could not be totally recovered after cooling, when thermoinactivation was carried out in the presence of 8 M-urea and 200 μ M-CuCl₂. Although 80 % of initial activity was recovered, several processes other than aggregation or disulphide interchange account for the remaining 20 %. According to Zale & Klivanov (1983), these processes could be deamidation of asparagine/glutamine residues, hydrolysis of peptide bonds at aspartate residues, destruction of tryptophan or proline residues or destruction of cysteine via β -elimination.

In order to determine the possible involvement of deamidation, samples of the enzyme that had undergone thermoinactivation at 70 °C for several different periods of time were analysed by isoelectric focusing (Fig. 9). On heating, the band of native enzyme gradually disappears and is replaced by several bands with lower isoelectric points. Each of the new bands could be ascribed to enzyme that had undergone different degrees of deamidation. However, the time-course of ammonia production as a result of deamidation could not be assayed because of the limit of sensitivity of the glutamate dehydrogenase method (Kun & Kearney, 1974). Samples of native endoglucanase and endoglucanase thermoinactivated over several time-periods were reduced and carboxymethylated and subjected to acid hydrolysis and amino acid analysis. As no changes in amino acid composition during thermoinactivation was observed, destruction of proline residues or removal of cysteine residues by β -elimination

can be discarded as possible mechanisms responsible for thermoinactivation of endoglucanase I. The tryptophan content, analysed colorimetrically, also remained constant during thermal inactivation of the enzyme.

To determine whether peptide bond hydrolysis contributes to thermal irreversible inactivation of endoglucanase I, samples of the native enzyme and of the enzyme heated at 70 °C for different periods of time at pH 4.8 were analysed by SDS/PAGE. After 2 h of heating at 70 °C, new bands corresponding to proteins of low M_r appeared, possibly indicating the involvement of peptide bond hydrolysis in the irreversible thermoinactivation of endoglucanase I (results not shown). According to Inglis (1983), hydrolysis of peptide bonds at acidic pH occurs preferentially at the carboxy group of aspartate residues. As endoglucanase I contains 22 aspartate residues, it is reasonable to assume that hydrolysis of peptide bonds may be one of the mechanisms that contribute to irreversible thermoinactivation of endoglucanase I.

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