The competitive inhibition of *Trichoderma reesei* C30 cellobiohydrolase I by guanidine hydrochloride

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The *p*-nitrophenylcellobiosidase (PNPCase) activity of *Trichoderma reesei* cellobiohydrolase I (CBH I) was competitively inhibited by concentrations of guanidine hydrochloride (Gdn HCl) that did not affect the tryptophan fluorescence of this enzyme. The K_m of CBH I, 3.6 mM, was increased to 45.4 mM in the presence of 0.14 M Gdn HCl, the concentration that was required to inhibit the enzyme by 50%. A similar concentration of lithium chloride and urea had little effect on the PNPCase activity of CBH I. Maximal inhibition was pH dependent, occurring in the range of pH 4.0 to 5.0, which is in the range for maximal activity. Analysis of the inhibition data indicated that 1.2 molecules of Gdn HCl combine reversibly with 1 molecule of CBH I. Other hydrolases and proteases were also inhibited by Gdn HCl. It is suggested that the inhibition of CBH I by Gdn HCl occurs as a result of the interaction between the positively charged guanidinium group of Gdn HCl and the carboxylate group of glutamic acid 126, postulated to be in the catalytic center of this enzyme.

Cellulase; Cellobiohydrolase I; *Trichoderma reesei*; Guanidine hydrochloride; Inhibition; Hydrolase; Protease

1. INTRODUCTION

Cellobiohydrolase I, CBH I (EC 3.2.1.91), catalyzes the hydrolysis of cellulose to cellobiose and is the major component of the cellulase complex, comprising as much as 60% of the total protein produced by the fungus *Trichoderma reesei* [1]. During our studies on the denaturation of CBH I by guanidine hydrochloride (Gdn HCl), we noticed that concentrations of Gdn HCl generally regarded as insufficient to denature CBH I and other proteins inhibited its ability to hydrolyze soluble *p*-nitrophenylcellobiose (PNPC) and insoluble microcrystalline cellulose [2].

The relatively low concentration of Gdn HCl required for inhibition of CBH I suggested to us that a specific interaction between the positively charged 'arginine-like' guanidinium moiety of Gdn HCl and an essential carboxylate group in the active site of CBH I [3] could result in its inhibition. To test this, the kinetics of the inhibition of CBH I by Gdn HCl were studied. Other enzymes with carboxylate groups implicated in their catalytic activity were also tested for their ability to be inhibited by Gdn HCl. The results presented suggest a specific interaction between the guanidinium group and the active site of CBH I does occur resulting in competitive inhibition especially since lithium chloride (LiCl), a chaotropic salt that disrupts water structure, has no effect on its activity. The inhibition of enzymes by Gdn HCl may be a general phenomenon, at least for enzymes possessing carboxylate or other reactive nucleophilic groups in or near their active site.

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2. MATERIALS AND METHODS

2.1. *Enzyme source, purification and assay*

A freeze-dried culture filtrate of *Trichoderma reesei* C30 was a gift from Dr Mary Mandels, US Army Natick Laboratory, Natick, MA, USA. CBH I was purified from the culture filtrate as described in [4] and assayed using PNPC as the substrate [2,5]. The β-glucosidase activity present in practical-grade (type I) cellulase from *Aspergillus niger* (Sigma Chemical Co., St. Louis, MO) was isolated, partially purified, and assayed as described previously [6,7]. An endoglucanase-rich fraction was prepared from a crude cellulase (celluclast 1.5 L – generously provided by Novo Nordisk) by fractionation on the fast protein liquid chromatograph equipped with a Mono P HR 5/20 column (Pharmacia) equilibrated at pH 5.5 [8]. Endoglucanase did not bind to the column under these conditions. The carboxymethylcellulose (CMC) viscosity reducing activity of this enzyme was measured [9]. Purified invertase grade X from *Candida utilis* was purchased from Sigma and assayed as described in [10]. The source of glucose-6-phosphate dehydrogenase used in this study was the hexokinase assay reagent (Sigma) and its activity measured by monitoring the reduction of NADP (absorbance at 340 nm) upon the addition of 2 mM glucose-6-phosphate to the reagent. Bovine pancreatic α-chymotrypsin (type II), trypsin (type I) and *Papaya latex* papain (type III) were obtained from Sigma. These enzymes were assayed by measuring the hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE), benzoyl-L-arginine ethyl ester (BAEE) and BAEE, respectively, as described in the Worthington enzyme manual.

2.2. *Inhibition studies*

The effect of Gdn HCl and urea (both being 'Baker Analyzed'

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grade from J.T. Baker, Phillipsburg, NJ, USA) on the PNPC- and Avicel-hydrolyzing activity of CBH I was determined by assaying the enzyme in the presence of different concentrations of these reagents under different conditions as given in the legends to the figures. The effect of 0.14 M lithium chloride (reagent grade, EM Science, Cherry Hill, NJ) on the activity of CBH I and the enzymes noted above was also determined at the pH for their optimum activity.

2.3. *Hydrolysis of Avicel*

Avicel PH 105 (microcrystalline cellulose) was obtained from FMC Corporation, Philadelphia, PA, USA. Approximately 50 μg of CBH I was incubated with 1% (w/v) Avicel at pH 5.0 at 50°C in a total volume of 5.0 ml in the presence and absence of 0.25 M Gdn HCl. The reaction mixture was also supplemented with *Aspergillus niger* β-glucosidase [2] purified as described in [6].

2.4. *Analytical procedures*

Protein was measured using the Coomassie blue reagent (Bio-Rad Laboratories, Richmond, CA, USA) according to the method of Bradford [11]. Glucose was determined using the hexokinase assay reagent [12]. Absorbance measurements were made with a Perkin-Elmer Lambda Array Spectrophotometer interfaced with an IBM personal computer.

3. RESULTS

3.1. *Kinetics of CBH I-catalyzed hydrolysis of PNPC in the presence of Gdn HCl*

The PNPC-hydrolyzing activity of CBH I was decreased 90 or 10% by 0.5 M Gdn HCl or urea, respectively (Fig. 1). The fact that the tryptophan fluorescence of CBH I is largely unaffected by 0.5 M Gdn HCl [2], and that much higher concentrations of Gdn HCl or urea are required to physically disrupt protein structures suggested that Gdn HCl was specifically inhibiting CBH I. Evidence to support this conclusion comes from the finding that there was no significant inhibition of the PNPC-hydrolyzing activity of CBH I by other salts (LiCl, NaCl, KCl, $ZnCl_2$) and urea at 0.14 M, which is the concentration of Gdn HCl required to inhibit the activity of CBH I by 50%. It should be noticed that the assay for PNP (produced by the action of CBH I on PNPC) was unaffected by the presence of 2.5 M Gdn HCl.

The Lineweaver-Burk plot (Fig. 2) indicated the inhibition of CBH I by 0.14 M Gdn HCl to be purely competitive: K_m was 3.6 and 45.5 mM in the absence and presence of inhibitor, respectively. From these data K_i was calculated to be 12 mM, indicating that Gdn HCl has a lower affinity for CBH I than does PNPC. Gdn HCl reversibly inhibits CBH I since dialysis to remove the inhibitor fully reactivates PNPCase activity [2]. Although the activity and Gdn HCl inhibition profile of CBH I as a function of pH are not coincidental (Fig. 3), it would appear that the catalytic groups are involved in binding to the inhibitor since maximum inhibition occurs between pH 4.0–5.0, which is similar to the pH range for maximum activity (pH 3.5–4.5). Based upon these kinetics, a reversible combination of CBH I and Gdn HCl to form an inactive complex can be assumed to occur. Analysis of the activity of CBH I as a function of Gdn HCl concentration by the method of Osborne and Chase [13] indicated that 1.2 Gdn HCl molecules combine reversibly with each CBH I molecule to cause inhibition.

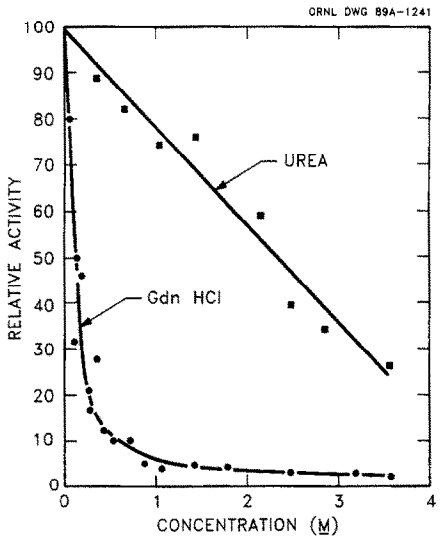

Fig. 1. The effect of Gdn HCl or urea on CBH I. CBH I (50 μl ≡ 35 μg protein) was incubated with 500 μl PNPC in 50 mM sodium acetate buffer (pH 5.0) at 23°C in the presence of Gdn HCl (●) or urea (■) at the concentrations indicated. After 45 min, aliquot (0.1 ml) of the reaction mixture was assayed for its PNP concentration. Control activity (100%) ≡ 2 nmol PNP min^{-1}.

3.2. *Hydrolysis of Avicel in the absence and presence of 0.25 M Gdn HCl*

The adsorption of CBH I to Avicel is unaffected to any great extent by the presence of 0.25 M Gdn HCl [2]. However, the hydrolysis of Avicel is inhibited by 0.25 M Gdn HCl (Fig. 4), and the extent of the inhibition (~70%) is the same as that observed during the hydrolysis of PNPC by this concentration of Gdn HCl. Since it is now established that the CBH I molecule is

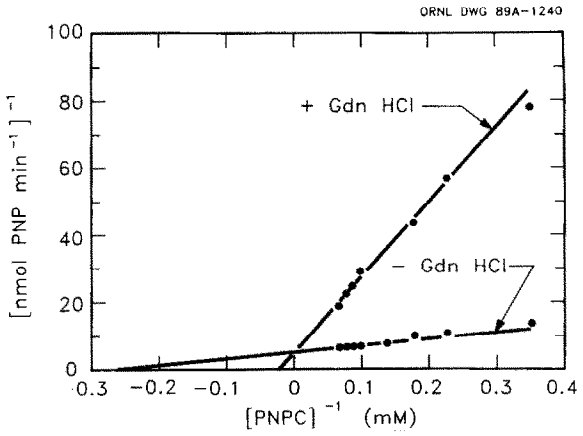

Fig. 2. Lineweaver-Burk plot for PNPC hydrolysis by CBH I in the presence of 0.14 M Gdn HCl. Slopes were calculated using the least squares method. For assay details see legend to Fig. 1.

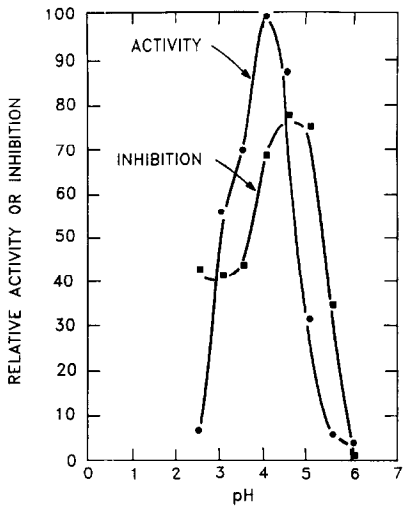

Fig. 3. The effect of pH on the relative activity and Gdn HCl-inhibition of CBH I. For details see legend to Fig. 1.

composed of two functional domains [14], namely the catalytic and cellulose-binding moieties, it can be surmised that the primary effect of 0.25 M Gdn HCl or CBH I is on the catalytic site of CBH I. In support of this, we have separated and purified the catalytic moiety of CBH I (known as 'core' CBH I) from the native enzyme [8] and found that the concentration of Gdn HCl required to inhibit 'core' CBH I by 50% is identical to that required for the inhibition of native CBH I.

3.3. *Comparison of the effect of Gdn HCl and LiCl on several enzymes*

The effect of 0.14 M Gdn HCl and 0.14 M LiCl and a combination of both of these salts on the activities of several enzymes is given in Table I. With the exception of papain and endoglucanase Gdn HCl inhibited the activity of all the enzymes tested by approximately 50% or higher. α-Chymotrypsin was inhibited 12.2 or 61% if the reaction was carried out in Tris or phosphate buffer, respectively. Interestingly, 0.14 M Gdn HCl had no effect upon endoglucanase activity.

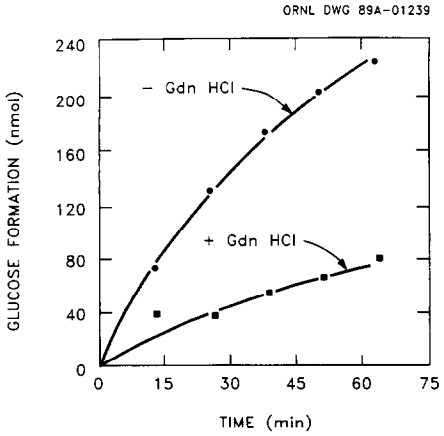

Fig. 4. The effect of 0.25 M Gdn HCl on the hydrolysis of Avicel. For details see section 2.

Table I

Inhibition of several enzyme activities by Gdn HCl and LiCl

Enzyme	Inhibition of activity (%)		
	Gdn HCl[a]	LiCl[a]	Gdn HCl/-LiCl[a]
CBH I	50	0	n.d.
Endoglucanase	0	0	0
β-Glucosidase	52.5	22.4	48
Invertase	47.4	n.d.	n.d.
Trypsin	69.2	25.5	72.3
α-Chymotrypsin[b]	12.2 (61)	0	14.3
Papain	14.8	63.8	63.5
G-6-P dehydrogenase[c]	73.8	55.6	89.1

[a] Enzyme assays were conducted in the presence of 0.14 M of each inhibitor

[b] Value in parentheses is the inhibition of α-chymotrypsin by Gdn HCl when the assay was conducted in 0.1 M sodium phosphate buffer pH 7.5. Other values obtained in Tris buffer

[c] Glucose-6-phosphate dehydrogenase

n.d., not determined

LiCl had a much less inhibitory effect on these enzymes compared to Gdn HCl, with the exception of papain and G-6-P-dehydrogenase, and there appeared to be no synergistic effect on the inhibition of activity by a combination of Gdn HCl and LiCl.

4. DISCUSSION

The competitive inhibition of *T. reesei* CBH I by concentrations of Gdn HCl insufficient to effect protein denaturation has been demonstrated. The concentration of Gdn HCl or urea required to inhibit the PNPC-hydrolyzing activity of CBH I by 50% was 0.14 and ~2.4 M, respectively. The inhibition by Gdn HCl may, therefore, result from an interference in the formation of the enzyme-substrate complex, whereas that by urea could be due to denaturation, or unfolding, of CBH I. However, it appears that CBH I is largely resistant to unfolding since its tryptophan fluorescence is only reduced 20% by incubation with 8 M urea (unpublished data). This finding is similar to that observed for the effect of 6 M Gdn HCl on the tryptophan fluorescence of CBH I [2] and suggests that CBH I possesses a tightly folded conformation. Although localized unfolding of the catalytic site by urea may be the cause of CBH I inactivation, fairly high concentrations of urea (1.0–3.5 M) have been shown to competitively inhibit the activity of several enzymes [15]. Urea, therefore, may also competitively inhibit CBH I but at much higher concentrations than required by Gdn HCl. This would be in agreement with the finding that Gdn HCl competitively inhibits xanthine oxidase, histidinase, and tyrosinase at concentrations far lower than those required for inhibition by urea [15].

Analysis of the competitive inhibition of CBH I by Gdn HCl indicates that 1.2 molecules of Gdn HCl per molecule of CBH I are involved in formation of the CBH I-Gdn HCl complex leading to inhibition. This suggests a specific effect of Gdn HCl with the active site of CBH I. Recent work [3] has implicated glutamic acid residue 126 as being essential for the catalytic activity of CBH I, and it is proposed that the positively charged guanidinium group of Gdn HCl interacts electrostatically with this residue to form the enzyme-inhibitor complex, thus causing inhibition. The finding that LiCl did not inhibit CBH I lends support to this hypothesis because, like Gdn HCl, it is a chaotropic salt that disrupts water structure, but lacks the bulky 'arginine-like' positively charged guanidinium moiety.

Carboxylic acid residues have been implicated in the catalytic mechanisms of several enzymes including glucosidases and the serine proteases trypsin and α-chymotrypsin [16]. Although endoglucanase, with a reported essential carboxylate group [3], is apparently not inhibited by Gdn HCl, the substrate for this enzyme (CMC) possesses multiple negatively charged carboxylate groups. Presumably, these would interact with the positively charged guanidium moiety and, in effect, would compete with the active site carboxylate group, resulting in little or no inhibition of the enzyme. This apparent lack of inhibition would, paradoxically, lend support to the proposed mechanism of the inhibition of enzymes possessing essential carboxylate groups by Gdn HCl.

β-Glucosidase, invertase, and trypsin are inhibited by Gdn HCl and, to a lesser extent, by LiCl, indicating the importance of the guanidinium moiety in the inhibition mechanism. In the case of trypsin, however, which cleaves proteins only at the carboxyl side of basic amino acids such as arginine, it should be noted that guanidinium compounds (e.g., phenylguanidine) have been shown to alkylate the primary active site nucleophile, serine 183 [17]. The reason for the difference between the level of inhibition of α-chymotrypsin by Gdn HCl when the reaction was carried out in Tris or phosphate buffer is unknown and warrants further investigation. Papain, a sulfhydryl protease, is inhibited to a greater extent by LiCl compared to Gdn HCl which is the converse of that seen with the other enzymes tested. The inhibition of G-6-P dehydrogenase by Gdn HCl and LiCl does not indicate any specificity of this enzyme for either salt.

For CBH I, a much lower concentration of Gdn HCl, compared with urea, is required for the inhibition of CBH I. Clearly, the inactivation of CBH I by Gdn HCl is due to a reversible inhibition of activity and not to a deleterious unfolding of the enzyme.

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