

R82-33²

TECHNICAL LIBRARY
U.S. ARMY NATICK R & D LABORATORIES
NATICK, MA 01760

From: SOLUTION BEHAVIOR OF SURFACTANTS, Vol. 2
Edited by Mittal and Fendler
(Plenum Publishing Corporation, 1982)

PROTECTION OF TRICHODERMA REESEI CELLULASE FROM INACTIVATION
DUE TO SHAKING

Elwyn T. Reese

Food Sciences Laboratory
U.S. Army Natick Research & Development Command
Natick, Massachusetts 01760

Cellulases can be added to the list of enzymes that are inactivated by shaking, agitation, or shearing. Of the cellulases, the most susceptible component is cellobiohydrolase (CBH). A survey of compounds that protect CBH against shaking reveals that some non-ionic surfactants are most potent, being active in the range of one molecule per molecule of enzyme. Under shaking conditions (50°, 350 rpm) where the CBH half-life is 1.5 hours, the presence of Zonyl-FSN increased the half-life to 180 hours. The addition of surfactant to enzyme already inactivated by shaking does not lead to recovery of activity. The presence of surfactant does not improve heat stability of the enzyme under unshaken conditions; nor does it affect the initial rate of hydrolysis. Long time (3 day) hydrolyses of crystalline cellulose under shaking conditions are markedly improved by the presence of surfactant, the improvement resulting from protection of the CBH against inactivation.

INTRODUCTION

In preceding papers^{12,16}, we have shown that cellobiohydrolase (CBH) is the least stable component of the Trichoderma reesei cellulase complex. Destabilization has been effected by a wide range of chemical agents (biocides, etc.), by heat, and by shaking. The inactivation by shaking may be due in part to a shear effect,¹⁷ similar to that reported earlier for other enzymes by Charm^{5,6,7} and Tirrell^{23,24,25}. The most severe damage by shearing is observed when a protein is forced through pores of small diameter, such as

blood capillaries,⁵ or hollow fibers and membranes used in ultrafiltration. Conditions in shake flasks are much milder, but examples of inactivation of fungal endo- β 1,4 glucanases (C_x) and of exo- β 1,4 glucanase (CBH) have been reported.^{3,17,20} In this paper, we report on factors affecting inactivation by shaking, and demonstrate how this type of inactivation may be minimized. A practical process for the enzymatic hydrolysis of cellulose to glucose requires that enzymes maintain their activity for the longest possible time under the conditions of usage. Stability of enzymes is a relative term, and the stability of the *T. reesei* enzymes ranks high. But it must be recognized that saccharification takes place at elevated temperature (50°), over an extended period of time (1-3 days), and in the presence of shaking.

METHODS

Most of this work was done with crude enzyme solutions of *T. reesei*, such as would be used in a practical hydrolysis reaction. Stability of cellobiohydrolase (CBH, E.C. 3.2.1.91) varies from strain to strain,¹⁶ and for this work, we have selected a strain (Rutgers C30; 13) whose CBH is readily inactivated by shaking, so that we may more easily evaluate the factors responsible. A single lyophilized preparation was used in most of the experiments. It contains Avicelase 2.0 IU/mg; protein (Folin) 90%; and residual salts of the medium. The CBH of *T. reesei* has a pI of 4.0 and a molecular weight of about 44,000 (unreported data).

The effect of shaking was determined by incubating the enzymes in 0.025 M citrate buffer pH 5.0 (1 mg E/ml Buffer); 20 ml per 125 ml flask on a rotary shaker (1" circle, 200 rpm) at 50°C. The effect of substrate (i.e., cellulose) on the inactivation was also determined. Samples taken at different times were routinely checked for pH, Avicelase activity, and protein.^{11,16} When preservatives were required, tetracycline (0.01 mg/ml) was used in experiments at 50°C, - and sodium azide (0.12 mg/ml) at 30°.

The Avicelase assay measures the action of enzymes on crystalline cellulose (Avicel pH 10.2), and is thus a function of both CBH and endo- β 1,4 glucanase (C_x) activity. To 1 ml enzyme (properly diluted in 0.025 citrate pH 5.0) is added an equal volume of an Avicel suspension (2.5% in same buffer). The mixture is incubated at 50°C for 1 hour, centrifuged, and 1 ml of supernatant used for a reducing sugar determination by the dinitrosalicylic acid method.^{11,16} A unit of activity yields 1 μ mol reducing sugar (as glucose) per minute under these conditions.

The endo- β 1,4 glucanase (C_x ; E.C. 3.2.1.4) is determined in a similar way.^{11,16} To 0.5 ml of diluted enzyme is added an equal volume of carboxymethyl cellulose (CMC 7L2, Hercules; 2% solution in M/20 citrate pH 5.0). After 30 minutes incubation at 50°, reducing sugar is determined, - and the activity unit defined as above.

RESULTS

A. Effect of Various Factors on Inactivation of *T. reesei* Cellulases Due to Shaking

1. Time (Figure 1)

Shaking inactivates both the C_x and Avicelase of *T. reesei* C30, but the rate of inactivation of Avicelase is much greater than that of C_x . When the solutions were incubated unshaken at 30°, no inactivation could be detected (5 days). The inactivation of Avicelase due to shaking does not go to completion, but levels off at about 70-75%. This is because one of the two CBH components⁴ is more susceptible to shaking than is the other.

Inactivation is accompanied by precipitation of protein. The amount of precipitate, however, is small (<20%), even when inactivation has reached a levelling off point (~75%). Absence of precipitate is a good indication that inactivation has not occurred.

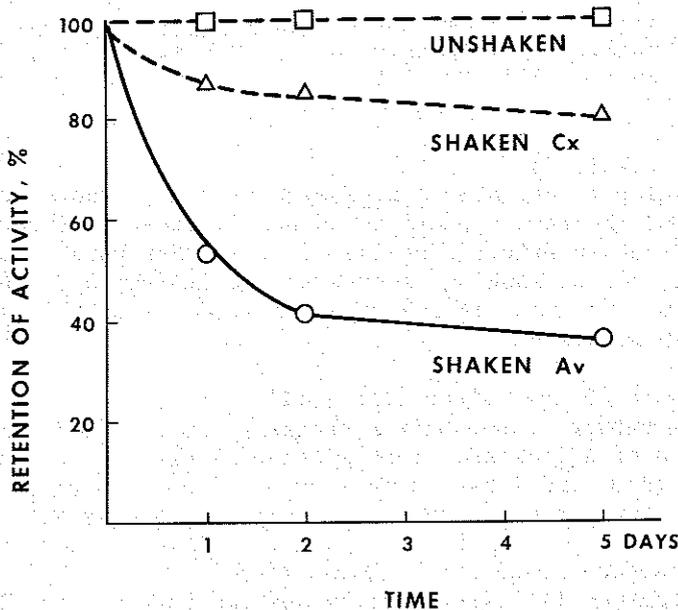


Figure 1. Effect of shaking on inactivation of *T. reesei* cellulase C30 enzymes. Conditions: 30°; pH 4.9; 300 rpm 1" circle; 0.2 mg NaN_3 /ml (preservative). (□) Unshaken Controls (both C_x and Avicelase); (o) Shaken, Avicelase; (Δ) Shaken, C_x .

2. Temperature (Table I)

Shaking inactivates both Avicelase and C_x about ten times more rapidly at 52° than at 30°. This temperature effect is quite

different from that reported by Charm^{5,6} who found that temperature had no effect on destruction of heparin and fibrinogen by shearing (4° vs 37°).

Table I. Effect of Temperature on Inactivation Due to Shaking.

Temp.	$t_{1/2}^*$	
	Avicelase	C_x
30°	57 hr	> 600 hr
52°	5.3 hr	53. hr

* $t_{1/2}$ = half-life of enzyme (*T. reesei* C30) at pH 5.0. (The shaking at 30° was at 300 rpm; that at 52° 200 rpm)

3. pH (Figure 2)

Shaking inactivates Avicelase over the pH range tested (e.g., 2.8-6.5), and the rate appears to be nearly constant (when correction is made for the pH effect itself). On the other hand, the inactivation of C_x due to shaking is greatest at low pH, and minimal at about pH 6.0. The rate of inactivation of C_x is much less than that of Avicelase at all pH's.¹²

4. Nature of the buffer (Table II)

The nature of the buffer exerts an influence on the rate of inactivation of Avicelase. Inactivation is greatest in citrate, less in acetate, and least in phosphate. Ionic strength has little or no effect over the concentration range 0.025-0.10 M.

5. Presence of substrate (Table III)

Avicelase is inactivated by shaking both in the presence and in the absence of substrate (Avicel). After digestion much less enzyme is found in solution in shaken flasks than in the unshaken, - and this correlates well with the amount of protein remaining. But a quantitative measure is difficult to obtain, since there is no way of evaluating the amount of active enzyme adsorbed to the Avicel in each case. We have found, however, that with the enzyme of Rutgers C30 in shaken flasks, no further digestion of cellulose takes place after 24 hours incubation,¹⁶ and this correlates well with the low value for soluble enzyme. The rate of CBH

inactivation due to shaking is essentially the same whether substrate is present or absent.

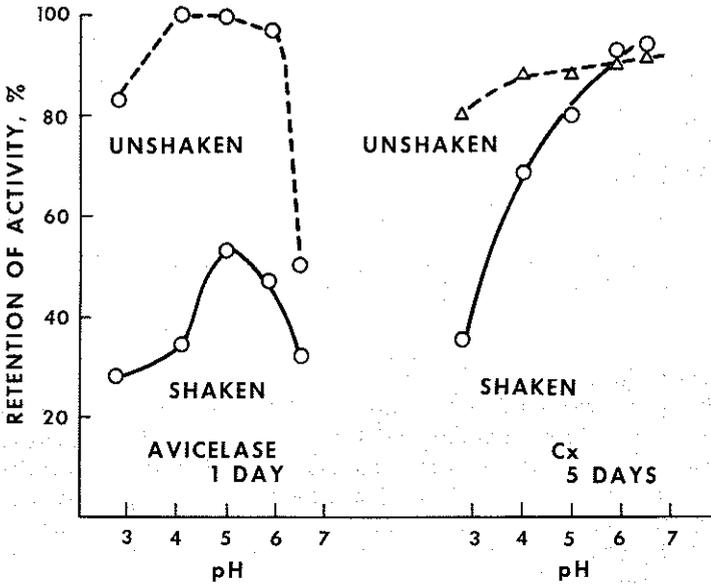


Figure 2. Effect of pH on inactivation of cellulase by shaking. Cellulase of *T. reesei* (C30) 1 mg/ml in 0.025 M citrate containing 0.2 mg/ml NaN_3 . Temperature 30° ; Shaken at 300 rpm. Avicelase data at 1 day of incubation; C_x at 5 days.

Table II. Effect of the Nature of the Buffer on Inactivation by Shaking.

Buffer 0.025 M pH 5.0	Avicelase Inactivation (k_d)*
Citrate	0.077 hr ⁻¹
Acetate	0.050
Phosphate	0.017

* $k_d = \frac{0.693}{t_{1/2}}$; Temp 50°

Table III. Effect of Shaking on the Inactivation of Avicelase in the Presence of Substrate.

<u>T. reesei</u> strain	Avicelase retention, %	
	Shaken	Unshaken
Rutger's C30	1.	53.
QM 9414	21.	41.

*Conditions: 10% Avicel pH 10.2 in enzyme (2 mg/ml) in 0.025 M citrate pH 5.0; 70 Hrs @ 50°; 200 rpm.

6. Other factors

The selection of a T. reesei C30 preparation for this work was based on the high susceptibility to shaking inactivation. More recent work on a number of samples indicates that strain MCG77 appears to be most stable, but compounds present in the reaction mixture may affect the inactivation rate. There are quite large differences in the k_d values of different preparations from the same strain (Table IV).

Table IV. Inactivation Constants of Avicelase of Strains of T. reesei.

Cellulase of <u>T. reesei</u>	Number of Samples	k_d * range
QM 6a	1	0.114 hr ⁻¹
QM9414	3	0.02-0.085
QM MCG77	4	0.029-0.049
Rutgers C30	5	0.031-0.139

*Conditions: Citrate buffer pH 5.0; 50°; shaken at 200 rpm

The vigor of shaking is an important factor in the rate of enzyme inactivation. At low rpm in shake flasks or in stirred

vessels, and at low flow rates in fine tubes, no inactivation is detected. The rate of inactivation increases dramatically above a shear stress of 15 dynes/cm².¹⁷ In stirring tests in a Bioflow fermentor, for example, the k_d increased from 0.001 hr⁻¹ at 200 rpm, to 0.128 hr⁻¹ at 500 rpm.

B. Inactivation of Enzymes (other than CBH) Due to Shaking

The inactivation due to shaking is not limited to enzymes of *T. reesei*. The C_x of 11 other species are similarly affected (Table 5). The relative susceptibility of the various C_x 's to heat alone (i.e., unshaken controls), and to shaking shows that *T. reesei* C_x 's are among the most stable, both to temperature (50°) and to shaking. The C_x 's of *Pestalotiopsis* and of *Irpex* are the least stable of those studied. (The Avicelase values of preparations, other than from *T. reesei*, were too low to evaluate.)

Table V. Susceptibility of the C_x 's of Various Organisms to Inactivation by Shaking.

Source of C_x	Inactivation, %	
	Shaken	Unshaken
<i>Aspergillus niger</i> (Wallerstein)	61.	16.
<i>Chrysosporium lignorum</i> QM 9145	69.	20.
<i>Irpex cinnamoni</i> (Driselase)	89.	43.
<i>Pestalotiopsis westerdijkii</i> QM 351	86.	52.
<i>Polyporus cinnabarinus</i> QM 8846	32.	14.
<i>Poria</i> sp. (SEAB)	63.	28.
<i>Sclerotium rolfsii</i> QM 7739	40.	0
<i>Sporotrichum dimorphosporum</i> QM 806	52.	23.
<i>Sporotrichum pruinosum</i> QM 826	76.	8.
<i>Streptomyces</i> sp. QM B814	60.	42.
<i>Trichoderma reesei</i> strains	0-35	0
<i>Trichoderma viride</i> (Onozuka P500)	70.	27.

Conditions: 50°, pH 5.0, 0.025 M citrate, enzyme at 1 mg/ml 69 hours @ 200 rpm

The β -glucosidase of *A. phoenicis* QM 329 (often used to supplement *T. reesei* cellulase in hydrolysis experiments) is the most

stable enzyme we have tested.¹⁹ No inactivation took place in 5 days of shaking at 50°. The β -glucosidase of T. reesei is also very stable (Table VI). When used in combination, the Aspergillus β -glucosidase and the Trichoderma Avicelase each retained the k_d values of the preparations tested individually. It appears, at least in this case, that the stability or instability is inherent in the enzyme, and that it is not due to the presence of some other factor in the preparation. This conclusion is also supported by the fact that there is appreciable variability in the inactivation rates of different enzymes found in the same preparation. In a T. reesei QM 9414 preparation (Table VI), shaking most rapidly inactivated Avicelase, trehalase and β -1,3 glucanase while C_x , β -glucosidase, amylase and β -1,4 mannanase were much less affected.

Table VI. Effect of Shaking on Various Enzymes Present in T. reesei QM 9414 "Cellulase".

Enzymes	Inactivation*, %
Cellulase-Avicelase	66
" C_x	10
β -glucosidase	8
β -1,3, glucanase	52
β -1,4 mannanase	22
α -1,4 glucanase	16
α - α -trehalase	68

*Inactivation due to shaking @ pH 5.0, 50°, 200 rpm; 17 hrs.

C. Prevention of the Inactivation Due to Shaking

1. Screening of compounds (Table VII; Figure 3)

In the preceding report,¹⁶ several compounds were found to protect the Avicelase of T. reesei from heat inactivation under unshaken conditions. Here we are looking for compounds that protect against the shaking effect. Compounds with surfactant action are most promising, - but there appears to be no correlation between chemical structure and protective action (Table VII). The most protective compounds, - i.e., those effective at the lowest

concentrations, were the fluorinated surfactants of which all four types (anionic, cationic, amphoteric, non-ionic) were effective. The best protectants were the non-ionic Zonyl N (a perfluoroalkylethoxylate) and a compound of quite a different nature, polypropylene glycol; followed closely by the high molecular weight polyethylene glycols (PEG). The poly-glycols were much less effective in lowering surface tension than were the many surfactants found in the list (Table VII),—yet most of the effective compounds do have surfactant properties.

Table VII. Compounds which Protect Avicelase of *T. reesei* C30 Against Inactivation Due to Shaking*.

Protective Effects

@ < 0.01 mg/ml	Zonyl A, B, C, N (fluorinated surfactants, duPont); Polypropylene glycol 2700
@ 0.01-0.1 mg/ml	Polyethylene glycols of high mol. wt. (> 4000) Polyvinyl pyrrolidone (mol. wt. 37,000) Triton X-100; Zwittergent 12 Digitonin; Saponin Methocel (Methyl cellulose, DS 1.89; Hercules) Hydroxy-propyl cellulose (Klucel E, Hercules) Roccal (a quaternary ammonium compound) β -lactoglobulin; ovalbumen
@ 0.1-1.0 mg/ml	Bovine plasma albumen; gelatin Na lauryl sulfate n-octanol; n-decanol Ustilagic acid; steviol bioside
@ > 1.0 mg/ml	Tween 20; Tween 80; Span 80 Na oleate; Na deoxycholate Ethanol; n-pentanol Cholesterol; sucrose non opalmitate Glucoustilic acid Polyethylene glycol 400; 600
<u>No effect</u>	Mercaptoethanol; lactose NaCl; CaCl ₂ ; cyclohexamide SAG 100 (silicone antifoam); carbopol 934 Zwittergent 08

Adverse Effects

Toluene (5 mg/ml); Thymol (1 mg/ml)

*Conditions: Enzyme @ 1 mg/ml; 0.025 M Citrate pH 5.0; 50°; 300 rpm

As reported previously,¹⁶ some enzyme-protective compounds inhibit the activity of the enzyme on cellulose. These include Roccal, and the substrate - or product analogues, e.g., methocel, ustilagic acid and glucoustilic acid. Fortunately, PEG 6000, and the Zonyl surfactants do not inhibit the activity of the enzyme in the ranges where they are effective protectants. Since PEG 6000 was one of the first protective agents found, a number of experiments were done with it. PEG 6000 is a nice white powder, readily soluble in aqueous solutions, and shows little or no interference with the assay procedures. It has very low toxicity and is relatively cheap (< \$0.01/gram). Many non-ionic surfactants (Tweens; Tritons; etc.) contain a polyethylene glycol moiety. PEG is known to complex with phenols and polyphenols (tannins), and perhaps an affinity for phenolic amino acids may contribute to its interaction with protein.²⁶

The protective agents gave very similar curves for concentration vs. activity. In solutions containing about 1.0 mg enzyme protein, both Avicelase and C_x were protected (Figure 6) by as little as 0.02 mg PEG per ml. The protective effect is a function of the size of the PEG molecule. Preparations of low molecular weight (400-600) were not effective; preparations of 4000-4,000,000 were highly effective. The protective effect of PEG 6000 extends over the pH range (Figure 3). pH: Inactivation curves for shaken flasks containing PEG, coincided closely with those for unshaken flasks lacking PEG. As a result, enzyme inactivation can be minimized either by reducing the rate of shaking, - or by adding a protectant. The amphoteric surfactants, Zwittergents (sulfobetaines, Cal. Bio. Co.), are available with varied lengths of the hydrocarbon side chain. When tested for their effect on shaking, it was found that protection increased from a very low level for the octyl derivatives to high values for the dodecyl (Figure 4A). There was no further enhancement with chain lengths of 14 or 16. Dose-response curves for two Zwittergents are shown in Figure 4B.

The soluble cellulose derivatives, methocel and hydroxypropyl cellulose (Klucel E), were protective at low concentrations. Both are highly substituted celluloses resistant to the action of cellulases. They differ, however, in that methocel inhibits cellulase activity at very low concentrations (0.004 mg/ml), whereas Klucel does not inhibit even at much higher concentrations (1 mg/ml). The latter, therefore, has much greater potential than the former, as a protective agent.

Proteins differ in their protective effect on Avicelase. Those which are themselves susceptible to denaturation (i.e., precipitation) by shaking (e.g., β -lactoglobulin, ovalbumen) are more protective of Avicelase than those which are not readily denatured (e.g., bovine plasma albumen, gelatin).

Not all added compounds protected proteins against inactivation by shaking. Some had the reverse effect. Toluene has been reported to increase the rate of denaturation of haemoglobin.¹ In our tests, toluene and Thymol similarly increased the rate of

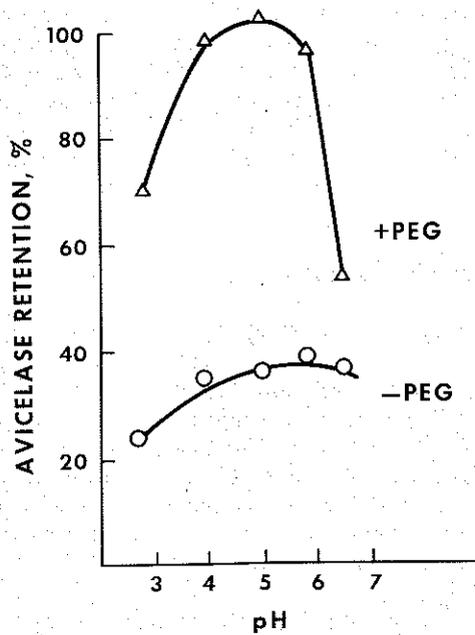


Figure 3. Effect of pH on the protective effect of PEG 6000 against the shaking inactivation of Avicelase. Conditions: as in Figure 2; time 5 days; PEG at 0.2 mg/ml.

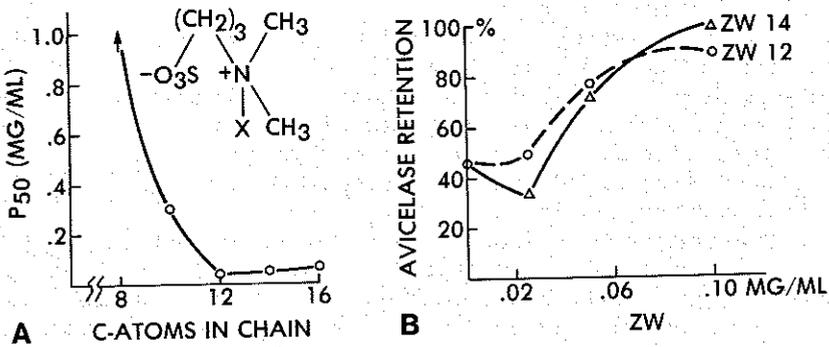


Figure 4. Protection of Avicelase by amphoteric surfactants. A - Effect of chain length on the concentration of Zwittergent required to reduce inactivation due to shaking by 50% (P50). Formula of Zwittergents, X = hydrocarbon side chain. B - Effect of Zwittergent concentration on retention of Avicelase activity after shaking at 350 rpm (50°, pH 5.0) for two hours Zwittergent 14 (14C chain); Zwittergent 12 (12C chain)

inactivation of Avicelase (Table VII). This destructive effect of Thymol could be overcome by the addition of a protectant, as little as 0.02 mg/ml of Zonyl N (or of PEG 6000) being sufficient to neutralize the effect of 0.75 mg/ml of Thymol (i.e., ca. 1 molecule Z·N/200 mol Thymol). On the other hand, the protective effect of Zonyl N could be enhanced by the addition of surfactants (Zwittergent 08; aerosol OT) at concentrations of these which by themselves have no protective effect.

2. Effect of surfactants on half-life (t) of enzymes under shaking conditions

The above data (Table VII) indicate the effectiveness of a compound in terms of the amount required. Equally important is the evaluation in terms of prolonging enzyme activity; i.e., of increasing the half-life of the enzyme. T. reesei Avicelase has a t of 1.5 hours (50°; pH 5.0; 350 rpm). Addition of polypropylene glycol (0.02 mg/ml) or of Zonyl N (0.02 mg/ml) increased the t by over 100 times. A similar increase was observed at 30°. Other surfactants gave comparable increases, but at the somewhat higher concentrations shown in Table VII.

3. Effect of surfactants on the enzymatic hydrolysis of Avicel

As indicated above, shaking inactivated cellulase in the presence of substrate as well as in its absence. In this experiment (Figure 5), Avicel hydrolysis effectively came to a standstill within 8 hours in the absence of surfactant, and the amount of free enzyme decreased on further incubation. In the presence of surfactant, (Zonyl N), digestion continued over the entire 4-day period, and the amount of free Avicelase increased greatly. The extent of hydrolysis was markedly increased as a result of the protective action of surfactant.

The dose response curves in the presence and in the absence of substrate (Figure 6) for protection of Avicelase, by PEG 6000 are very similar. Curves for the endo- β -1,4 glucanase (C_x) showed the same relationship. This suggests that PEG is not being removed from solution by adsorption on cellulose. We have tested several surfactants, and found no detectable adsorption on Avicel under the reaction conditions.

Some surfactants showed their protective effect only over a very narrow range of concentration. The quaternary ammonium compound, Roccal, effectively doubled the extent of hydrolysis (Figure 7) of Avicel when used at 0.1 mg/ml in shaken flasks, - but hydrolysis was much less at higher concentrations. This results from the fact that Roccal, in this concentration range, is a strong inhibitor of Avicelase activity (Figure 7B). At 0.1 mg/ml, the protective effect against shaking is obviously greater than the inhibitory effect on activity; at higher concentrations of Roccal, the inhibitory effect on activity; at higher concentrations of Roccal, the inhibitory effect becomes dominant. Methocel resembles Roccal in this respect.¹⁶

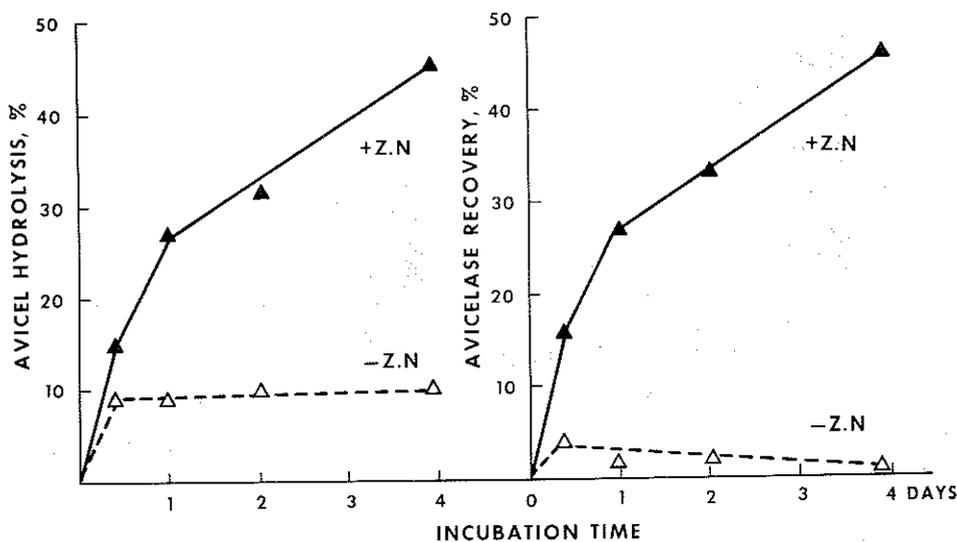


Figure 5. Effect of Zonyl N on hydrolysis of Avicel (10%) by *T. reesei* C30 enzyme under shaken conditions. Conditions: 50°; pH 5.0; 200 rpm; Zonyl N 0.02 mg/ml; 0.05 M PO₄ buffer.

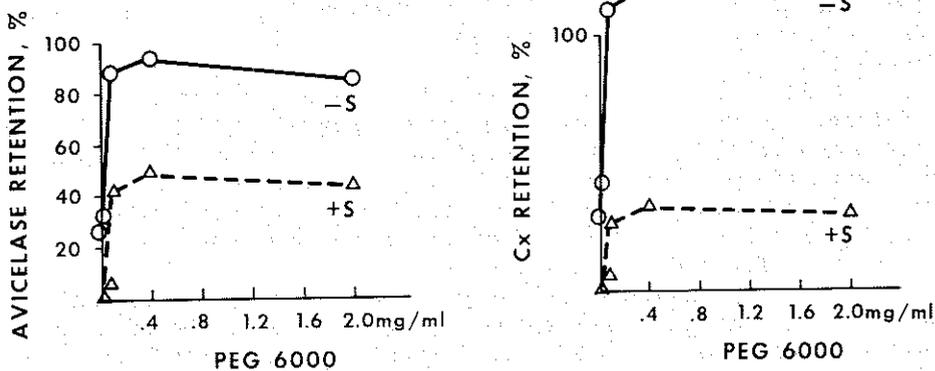


Figure 6. Effect of polyethylene glycol (PEG 600) on the inactivation of *T. reesei* enzymes due to shaking. Enzyme (1 mg/ml) in 20 ml 0.025 citrate pH 5.0; 50°; 200 rpm; 65 Hrs.; ± substrate (Avicel 10%)

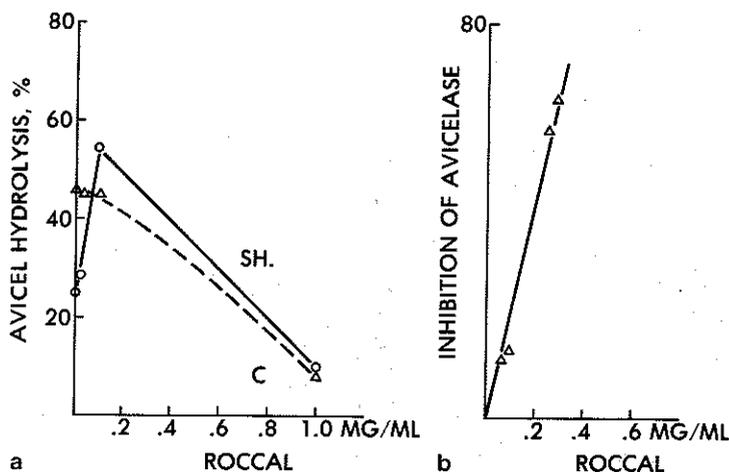


Figure 7. Effect of Roccal on Avicel hydrolysis (A) (shaken vs. unshaken); and on Avicelase activity (B).

The curves (Figure 7) show that Roccal always has an inhibitory effect when the digestion of Avicel is under unshaken conditions. All protective agents are effective because they protect against the shaking action. Under unshaken conditions, this type of inactivation is not occurring, and so these agents have no beneficial effect. Most protective compounds - unlike Roccal and methocel - do not inhibit Avicelase activity, at least over the range of concentrations in which they have proved useful (Figure 6).

While the data (above) describe results with only three surfactants, many more of the protective compounds (Table VII) have been tested in shake flasks for this effect on Avicel hydrolysis. Their effectiveness here is essentially of the same magnitude as their effectiveness when tested in the absence of substrate, - i.e., the results are similar, but the concentrations required for maximal effect differ for each surfactant (Table VII).

When Avicel digestion was carried out in unshaken flasks, the Avicelase was not inactivated, and there was no need for surfactant. The extent of digestion in unshaken flasks lacking surfactant was just slightly less than that in shaken flasks containing surfactant. In either case, the extent of hydrolysis (3 days) was about 4 times that in shaken flasks lacking surfactant. These results reflect the fact that the cellobiohydrolase component

of cellulase, which is so sensitive to shaking, is the component required for digestion of crystalline cellulose (Avicel). Similar results have been obtained using as substrates cotton (highly crystalline), and Solka Floc (partly crystalline). As highly amorphous cellulose is degraded most rapidly⁹ by the endo- β -1,4-glucanase (C_x), and since C_x is much more stable to shaking, the digestion of amorphous materials is much less affected by shaking than is the digestion of crystalline cellulose.

DISCUSSION

With the seven fungal carbohydrates reported here and the thirteen glycolytic enzymes of erythrocytes of Asakura² the number of enzymes known to be inactivated by shaking is rapidly increasing. To these must be added numerous examples of protein denaturation.^{1,5,14,18} Indeed it is likely that all proteins may be affected by shaking, although not to the same extent.¹⁰ Some proteins, e.g., haemoglobin,¹⁴ and interferon¹⁸ exist in a number of forms which differ in the rate at which they are denatured on shaking. Other proteins, like the enzymes described in this report, vary in susceptibility as a result of the conditions under which they were produced.

Factors which enhance denaturation due to shaking are: (a) the inherent nature of the protein, (b) the conditions of shaking, e.g., time, temperature, and (c) the nature of the buffer and of other components of the system. For the Avicelase of *T. reesei* under the optimum conditions for cellulose hydrolysis (50° pH 4.8-5.0), the half-life can be reduced to less than 2 hours by increasing the rate of shaking to 350 rpm. Under the conditions for growth of the fungus (30° pH 3.5-4.0), the half-life is much longer (24-48 hours), but even this rate of inactivation may be enough to reduce enzyme yields. The use of surfactants for increasing enzyme production¹⁵ may, in part, be due to this protective effect against shaking inactivation.

The inactivation by shaking is irreversible. It is accompanied by precipitation of about 18% of the protein. HPLC chromatograms of the clear supernatant reveals the disappearance of the first (of two) CBH peaks (F. Bissett, pers. comm.).⁴ Foaming does not occur under the experimental conditions. Inactivation is temperature dependent for Avicelase, but independent of temperature for β -lactoglobulin (unreported results).

Protection of a protein against the adverse effects of shaking has not been adequately investigated. Addition of other proteins, especially bovine serum albumen, has been successfully used to protect against heat inactivation, and its value in shaking tests demonstrated for haemoglobin,¹ cellulase (C_x) of *Pellicularia*² and other fungi,³ and - in the present work - cellobiohydrolase. Some organic solvents, e.g.; ethanol, butanol, acetone, have been found to protect haemoglobin,² but at relatively high concentration (1-10⁺ mg/ml). The most effective protectants reported here,

active at as low a concentration as 0.002 mg/ml, are surfactants. Tween 80 (at 1 mg/ml) has previously been shown¹⁸ to protect interferon against denaturation by shaking. For our most active agents, one molecule (or less) per molecule of enzyme appears to be sufficient to obtain complete protection. In most cases, these concentrations are below the critical micellar concentration (CMC) of the surfactant so it appears that we need not be concerned with the special properties of micelles.

Our explanation of the shaking effects on protein has been continually changing. Shearing seemed to be a good possibility in light of the experiments of Charm,^{5,6,7} Tirrell^{23,24,25} and others, and - when we forced our enzymes through a fine tubing - we did indeed observe deactivation.¹⁷ There is a possibility that shearing effects may be enhanced by the presence of other components of the medium such as pH, or the presence of metal ions. Thus, Tirrell implicated metals in urease inactivation.²⁴ In the present case, it seems that the shearing effect - while definitely detectable - may be only a minor factor in the total inactivation; i.e., that the passage through fine tubing represents shearing, but that the action in shaken flasks may involve additional factors. Thomas et al.^{21,22} have recently questioned the extent of inactivation that results from shear. According to this explanation, shearing would result in a conformational change in the protein, with the new conformation subsequently attempting to revert to the native form. Surfactants react with protein in both conformations. Their protective effects may be a result of (a) reducing the amount of unfolding, and (b) aiding in refolding to the native form.

A second possibility is that proteases are involved in the inactivation.⁸ Proteases have been found in *T. reesei* preparations. Under shaken conditions at 50°, the action is very slow, - but as the vigor of shaking increases, inactivation is progressively more rapid. According to this hypothesis, shaking changes the conformation of the cellobiohydrolase in such a way that it becomes more susceptible to proteolysis. The greatly increased rate of inactivation as the temperature is raised (from 30° to 50°) is compatible with this hypothesis. In this scheme, the protective effect of surfactants results from their interaction (hydrophobic bonding) with the enzyme in the native or altered conformation, the complex being resistant to protease action. If this explanation is valid, then inhibition of the protease should prevent inactivation. To test this, three known protease inhibitors were added to the enzyme solutions:

- (1) pepstatin @ 0.25-25 µg/ml vs. acid proteases
- (2) iodoacetamide @ 200. µg/ml vs. SH-proteases
- (3) phenylmethane sulfonyl fluoride @ 500. µg/ml vs. serine proteases

None of these reduced the inactivation due to shaking.

A third hypothesis is that inactivation by shaking is a surface related phenomenon. As we increased the volume of enzyme solution per flask - thus reducing the surface-volume ratio, -

inactivation decreased. Asakura and Macritchie (both pers. comm.) favor this explanation. A rough calculation indicates that, under our conditions, a monomolecular layer of enzyme at the solution surface would represent about 0.01% of the total enzyme. Shaking tends to displace the surface layer, the faster the shaking the greater the exposure of fresh enzyme to surface conditions. According to Macritchie¹⁰ "the steps in the inactivation are firstly an adsorption of the enzyme, followed by unfolding at the interface, and finally a two-dimensional precipitaton . . . of the protein." Inactivation may occur during the unfolding, - but it is more likely that it is associated with the subsequent re-folding. We have observed that another enzyme, β -glucosidase, retains much of its activity (66%) when adsorbed on Kaolin (where we assume it is unfolded). But when the enzyme-Kaolin mixture is dried from acetone, and acetone dried, it retains all of its activity. The acetone effect here is determined by the conformation of the enzyme that is exposed to it.

According to this concept, the protective effect of surfactants results from competition with the protein for the available air-liquid interface. There is an energy barrier to adsorption of a compound at an interface.¹⁰ Only 1 molecule of protein in 150 striking the interface is adsorbed. For a surfactant, the barrier is much less, 1 of 3 molecules is adsorbed. The surfactant is, therefore, more likely to occupy the surface layer, and by so doing to prevent adsorption and subsequent unfolding of the protein. This simplified explanation does not preclude surfactant-protein interaction. Such complexes would orient themselves at the gas-liquid interface with the most hydrophobic part (e.g. surfactant) towards the gas phase, - and again reduce the unfolding and re-folding of protein that lead to inactivation.

The nature of denaturation by shaking appears to be quite different from that of denaturation by heat. While we can effectively prevent shaking inactivation by the addition of surfactants, under no conditions have we been able to increase heat stability by this means.

REFERENCES

1. T. Asakura, K. Adachi and E. Schwartz, *J. Biol. Chem.*, 253, 6423 (1977).
2. T. Asakura, P. Herridge, P. K. Ghory and K. Adachi, *J. Biol. Chem.*, 252, 1829 (1977).
3. S. N. Basu and P. M. Pal, *Science*, 178, 312 (1956).
4. F. H. Bissett, *J. Chromatog.*, 178, 515- (1979).
5. S. E. Charm and B. L. Wong, *Biotechn. Bioeng.*, 12, 1103- (1970).
6. S. E. Charm and B. L. Wong, *Science*, 170, pp. 466- (1970).
7. S. E. Charm and B. L. Wong, *Biotechn. & Bioeng.*, 12, 451- (1978).

8. J. Feder, D. Kochavi, R. G. Anderson and B. S. Wilde, *Biotechn. Bioeng.*, 20, 1865 (1978).
9. R. G. Kelsey and F. Shafizadeh, *Biotechn. Bioeng.*, 22, 1025- (1979).
10. F. Macritchie, *Adv. Protein Chem.*, 32, 283- (1978).
11. M. Mandels, R. Andreotti and C. Roche, *Biotechn. Bioeng. Symp.*, 5, 21- (1976).
12. M. Mandels, S. Dorval and J. Medeiros, *Proc. Second Ann. Symposium Fuels from Biomass, R.P.I.*, pp. 627-669, Ed. W. W. Schuster (1978).
13. B. J. Montenecourt and D. E. Eveleigh, *Appl. Env. Microbiol.*, 34, 777- (1977).
14. T. Ohnishi and T. Asakura, *Biochem. Biophys. Acta*, 453, 93- (1976).
15. E. T. Reese, *Biotechn. Bioeng. Symp.*, 3, "Enzyme Engineering", pp. 43-62, John Wiley and Sons, Inc., NYC (1972).
16. E. T. Reese and M. Mandels, *Biotechn. Bioeng.*, 22, 323- (1980).
17. E. T. Reese and D. Ryu, *Enz. and Microbiol. Techn.*, 2:239-40.
18. J. J. Sedmak and S. E. Grossberg, *Texas Rept. on Biology and Medicine*, 35, 198- (1977).
19. D. Sternberg, P. Vijayakumar and E. T. Reese, *Can. J. Microbiol.*, 23, 139- (1977).
20. M. Tanaka, S. Takenawa, R. Matsuno and I. Kamikubo, *J. Ferment. Techn.*, 56, 108- (1978).
21. C. R. Thomas, A. W. Nienow and P. Dunnill, *Biotechn. Bioeng.*, 21, 2263- (1979).
22. C. R. Thomas and P. Dunhill, *Biotechn. Bioeng.*, 21, 2279- (1979).
23. M. Tirrell and S. Middleman, *AIChE Symp. Ser. Rheology*, 74, 102- (1978).
24. M. Tirrell and S. Middleman, *Biophys. J.*, 23, 121- (1978).
25. M. Tirrell, *J. Bioeng.*, 2, 183- (1978).
26. N. Toyama and K. Ogawa, in "Enz. Hydrolysis of Cellulose" Symp., (SITRA), Aulanko, Finland, Edit. M. Bailey, T. M. Enari, and M. Linko, pp. 375-387 (1978).