

Elution of Cellulase from Cellulose

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The following compounds at high concentrations are good eluents of the cellulase of Trichoderma reesei from crystalline cellulose (Avicel): urea (6M), guanidine-HCl (4M), DMSO (3M), DMF (3M), n-propanol (4M). The enzyme is sufficiently stable in these to permit recovery of activity under the elution conditions (pH 5.0 and 30°). The only compounds that elute at low concentrations are alkaline, but these tend to inactivate the enzyme. However, by careful adjustment to pH 10.0 with Ca(OH)₂ (0.01 - 0.02 N), low temperature (0°), and short time (10 min.), a recovery of activity of as much as 60 percent was possible.

INTRODUCTION

The commercial development of an enzymatic process for converting cellulose to alcohol is impeded primarily by the cost of the enzyme required.¹ This is not because of a lack of a good enzyme-producing system. Indeed in Trichoderma reesei we have one of the most productive of all sources of microbial enzymes². The problem is in the insolubility of the substrate, and the fact that the amount of enzyme required to hydrolyze cellulose is greater by a factor of 10-100, than that needed for a soluble system (e.g., starch). It is imperative, then, that something be done to utilize the enzyme to the maximum extent. Wilke^{3,4} and others have long recognized this necessity for re-use of the cellulase. In some commercial processes, immobilization of the enzyme has made this possible. But immobilized enzymes can function only when the substrate is soluble. Other methods will be required for the cellulase system.

The problem of enzyme recovery is related to the conditions under which the hydrolysis process is carried out. Conditions which favor enzyme inactivation - high temperature, long time, agitation - reduce the possibility of good recovery. The extent of digestion² - an engineering decision - and the enzyme:substrate ratio determine how much of the enzyme will be free in solution, and how much will exist in the adsorbed state at time of harvest. Under normal use conditions, over 90 percent of the enzyme is initially adsorbed. Much of this is subsequently released as digestion proceeds, especially under unshaken conditions⁵. The amount released, however, is a function of the enzyme:substrate ratio. Where this is high - as in Wilke's experiments⁴, very appreciable amounts of free enzyme (as much as 50% of

original) have been found in the digests. It is likely, however, that the conditions for commercial exploitation of the cellulose saccharification process will permit only low enzyme to substrate ratios, and partial hydrolysis of substrate. Under such conditions most of the cellulase will remain in the adsorbed state. (β -Glucosidase, however, will be in solution.) This report deals with the ^{recovery} elution of cellulase from the adsorbed state.

Elution of adsorbed protein is a broad problem, of which cellulase desorption from cellulose is a special case. Here the adsorbent is the substrate of the enzyme, and there is a preferential adsorption of cellulase, leaving most of the other enzymes in solution, a type of affinity binding. A specific attraction of a substrate occurs in addition to ionic bonding, hydrogen bonding, hydrophobic forces, and permeation effects.

Attempts to elute cellulase (I. reesei) from cellulose were made by King⁶ in his efforts to concentrate and purify this enzyme. The crude solution was passed through a shallow layer of Avicel (microcrystalline cellulose). Endo- β 1,4 glucanase (Cx), β -glucosidase, and protease passed through with the buffer, but cellobiohydrolase (CBH) was strongly adsorbed. The latter could be removed by reducing the ionic strength; i.e., with water. Elution was impractical, however, in that large volumes of water were required.

One of the general principles governing adsorption of proteins on uncharged but polar solids is that binding is inversely proportional to temperature. Thus elution should be at the highest temperature consistent with enzyme stability. Several investigators^{7,8} have had some measure of success in eluting cellulase by raising the temperature to 45-50°C (pH 4.8). Another generality is that the addition of a compound capable of affecting H-bonding (between protein and adsorbent) may increase the elution. This is the basis for removing native protein from that covalently immobilized on a solid matrix,

by use of 6 M urea⁹. Glycerol (10%) has been suggested for elution in the cellulase-cellulose system (R. Lovrien, personal communication). Fatty acids may play a role in elution in other systems where hydrophobic bonding may be involved.

METHODS

Cellulase of T. reesei consists of two types of components, an endo- β 1,4-glucanase (EC3.2.1.4; Cx) and an exo-glucanase (EC3.2.1.91; CBH; C₁), both being required for hydrolysis of crystalline cellulose such as Avicel. In the experiments reported here, Avicel is used as substrate, and the enzyme is referred to in a trivial manner as Avicelase. The endo-glucanase (Cx) is measured using carboxymethyl cellulose as substrate^{10,11}.

The Avicel (PH102, a microcrystalline cellulose) used for adsorption was prepared as a 2.5% suspension in .025 citrate buffer (pH 4.9). Three ml of this was centrifuged, and the wet precipitate used as the adsorbent. To this was added 2 ml of T. reesei cellulase preparation (2 mg/ml in buffer). The suspension was well mixed and incubated for one hour at 0° to accomplish the adsorption. About 85 percent of the Avicelase activity was adsorbed under these conditions.

The enzyme used was a lyophilized culture filtrate of T. reesei C30: protein content (Lowry¹²) 0.84 mg/mg; OD280 absorption .95/mg/ml; f. p. cellulase 0.44 U/mg; Avicelase 0.8 U/mg; and CMCCase 6.3 U/mg.

Enzyme was eluted from the enzyme-Avicel precipitate by the addition of 2 ml eluent, under varying conditions. The OD280 values (protein) and the Avicelase activities^{10,11} of the clear eluates were measured, and from these the amounts of protein and of enzyme activity recovered were calculated.

RESULTS

A. Elution of Enzymes Adsorbed on Avicel Columns

In a preliminary test, the I. reesei filtrate was added to an Avicel column (6½ cm dia x 6½ cm high) and washed with 0.05 M acetate buffer (pH 4.8; 25°). When this was followed by water, the flow stopped. The column was then extruded and the solids eluted successively with 20 ml portions of water. Recovery of protein was low (40%). Two important observations were made: (1) the first protein coming through the column was predominantly Cx, and (2) successive elutions of the extruded solids with water first gave clear, protein-free solutions; then opalescent solutions containing protein which was predominantly cellobiohydrolase (confirming results of King). The opalescent solutions could be clarified by addition of acid or salt to give a precipitate containing about 0.75 mg of protein (CBH) per mg of polysaccharide. This is ten times the maximum amount of cellulase previously reported bound to cellulose¹³, and indeed this bound protein may account for the "solubility" (opalescence) of the cellulosic fraction at low ionic strength.

*100' 82
122mg/ml
per mg of
w/ab. cellulase*

A similar Avicel column was set-up to determine the effectiveness of various eluents (Table 1). Flow did not stop in this column, probably because of the substitution of 10% ethanol (pH 2.5, citrate) for water. Total recovery of protein was good (88%). Again the first fraction to be eluted was Cx (high Cx/Avicelase ratio), and the fraction eluted by 10% acidic ethanol was CBH (low Cx/Avicelase ratio). The protein subsequently eluted by dilute alkali contained Cx (amounting to 40% of the total Cx), and, since 100% of the Cx was recovered, this indicates the resistance of this Cx to alkaline inactivation. (The time of contact with alkali was kept short by adding citrate to the tubes receiving the eluent.)

The recovery of Avicelase was 70%. Since we know (1) that in I. reesei ca 70% of the protein is CBH¹⁴, and (2) that all of the Cx has been accounted for, it is likely that most of the alkali-soluble protein is CBH which has been inactivated by the alkali.

Neither cellobiose nor methocel had any eluting power (Table 1).

Some digestion of the cellulose occurred during development of the Avicel column. The concentration of sugars was high in the early eluate (3-5 mg/ml), falling off in the ethanol fraction (3.4 → 1.3), and absent in the alkaline fractions. Glucose was the only sugar detectable in the first (Cx) fraction. (This suggests the occurrence of β -glucosidase as well as of Cx). Cellobiose was the dominant sugar in the acid ethanol fractions; glucose was a minor component. This is consistent with the identification of this fraction as cellobiohydrolase (CBH).

B. Urea and Guanidine as Eluents

1. Effects of urea on enzyme stability and activity

Evaluation of eluents involves a dilution prior to determining the enzyme activity. One must determine whether the diluted eluent has any effect either on the Avicelase activity, or on the dinitrosalicylic acid method (DNS) used to measure the reducing sugars produced so that corrections can be made if necessary. Urea has no effect on the DNS determination of reducing sugars even at 4 M (not shown); but it does inhibit Avicelase activity (Figure 1) even at 0.2 M. To correct for this, urea can be added to the control enzyme solution just prior to use in the assay. (The measure of OD280 is easily corrected by subtracting the absorption value of the urea solution used.) Guanidine reduces the DNS value strongly at 1 molar; and gives 50% inhibition of Avicelase activity at 0.1 M (0.6%).

The activity of enzymes in urea is variable. Our data show a loss of 50% activity at the following urea concentrations:

Avicelase (Cx + CBH)	<u>T. reesei</u>	- - - - -	1.0 M (6%) urea (Figure 1)
β -glucosidase	<u>T. viride</u>	- - - - -	4.5 M urea (Not shown)
β -glucosidase	<u>Aspergillus phoenicis</u>	- - -	4.0 M urea (Not shown)

Other data (Table 2) show that Avicelase is very stable at 25^o (pH 5.0), both in buffer and in 6 M urea; and stable at 50^o in buffer but not in 6 M urea. The instability at 50^o in 6 M urea is reflected in a high degree of inactivation, and in an increased susceptibility of the Avicelase to proteolysis. In any event, the Avicelase is sufficiently stable to permit the use of 6 M urea for enzyme elution studies.

2. Factors affecting elution of Avicelase from Avicel by 6 M urea

(1) Time (Figure 2). Most of the enzyme is eluted almost immediately.

(2) Temperature (Figure 2, Table 3). The rate and the extent of elution increases with temperature over the range studied.

(3) pH (Figure 3). There is very little effect of pH on elution over the range 4-7. This is true not only for urea and the other compounds shown in Figure 3, but also for many compounds listed in Table 4 (see, however, section D).

(4) Concentration of urea (Figure 4). Elution increases as the urea concentration increases (8 M urea was the highest concentration tested).

(5) Other factors. Ionic strength does not seem to be an important factor. The use of 2 M KCl gave only slightly greater elution than did the buffer itself (Figure 3).

Guanidine ($\text{NH}_2\cdot\text{C}(\text{NH})\cdot\text{NH}_2$) closely resembles urea ($\text{NH}_2\cdot\text{CO}\cdot\text{NH}_2$) in structure, and has also been widely used in studies of protein conformation. In our tests, it surpasses urea in elution of Avicelase from cellulose (Figure 4), but unfortunately it strongly inactivates the enzyme (Table 3).

C. Screening of Eluents (Table 4)

A screening of a broad spectrum of compounds as eluents was done using recovery as based on OD280 material only. The best of these (Table 4a) were retested using Avicelase activity as well as OD280. All of the most active eluents - except alkali - are effective only at high concentrations. DMSO (Figure 5) at 40 percent is a very good eluent, and activity is retained. n-Propanol is best of the alcohols tested with a peak at about 25%. At these concentrations, both are highly inhibitory in the Avicelase assay (Figure 1).

Organic acids (Figure 5) are good eluents (based on OD280) at 4 M, but recovery of activity falls off rapidly above 1 M. These elutions are at low pH (2.5), and are much less effective when adjusted to higher pH. Low temperature (0°C) improves the recovery of activity, but not of OD280 material.

Many compounds tested at lower concentrations (1%) had little promise; i.e., elution was no greater than that using dilute citrate. These include some sugars and modified sugars, surfactants, polyethylene glycols, salts and buffers (Table 4).

D. Elution by Alkali

The most effective agents in removing enzyme from Avicel; i.e., those active at lowest concentration, are alkaline. Unfortunately inactivation is also rapid at high pH. NaOH at .02 N removed most of the protein (or other 280 absorbing material, Table 4), but recovery of activity at 0° was only 27 percent. LiOH, NH_4OH and KOH behave similarly. A plot of the recovery of activity vs. pH shows

a peak at pH 10.0. $\text{Ca}(\text{OH})_2$ gives similar results (Figure 6), with a maximum recovery of enzyme activity of 60 percent at 0° (and much less at 40°). Recovery results based on activity are variable, differing with the time involved in subsequent handling before acidification. Attempts to improve recovery of activity by buffering more strongly at pH 10, or by the addition of stabilizing agents at this pH, have not been successful.

DISCUSSION

We have long been frustrated by the difficulty of eluting cellulase from cellulose. Not until our recent study on stability of cellulase did we discover that the enzyme is sufficiently stable in 6 M urea so that this agent could be used in elution experiments. Six M urea can even re-dissolve cellulase that has been denatured by shaking or by heat, and so looked like a fair possibility for the elution process. The enzyme is readily recovered from the 6 M urea by precipitation with 2 v cold acetone.

Both 6 M urea and 5.6 M (44%) DMSO are excellent for eluting cellulase from microcrystalline cellulose (Avicel) with recovery of activity. It is interesting that the enzyme retains some of its activity (Figure 1) in these concentrated solutions. But the rate of hydrolysis of the Avicel during the elution process is considerably less than during similar elution with buffer. The elution is rapid and temperature dependent, and the enzyme sufficiently stable that the beneficial effect of high temperature ($40-50^\circ$) for a brief time (15 min.) can be included in the procedure.

The use of organic solvents as eluents of protein from Avicel may be related to their uses in isolation of protein from membranes. If so, the effects may be primarily on hydrophobic interactions, and to a lesser degree on hydrogen

bonding or on ionic interaction¹⁵. Organic solvents reduce surface tension, and increase the tendency of protein molecules to unfold. The denaturing effect of alcohols (methanol < ethanol < propanol < butanol) is in agreement with their ability to elute cellulase from Avicel. It may be that a conformational change is required to reduce the affinity of cellulase for cellulose, and that, therefore, only agents which cause such change; e.g., denaturants, can be good eluents.

The stability of Cx at high pH (as seen in the column experiment), and to some degree of CBH at pH 10.0, may be sufficient to develop an elution procedure involving alkali. The low concentration of alkali is a major advantage over use of urea or DMSO. A search for a protective agent useful at alkaline pH seems warranted.

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Table 1. Elution of T. reesei Cellulase from Avicel Column

Fraction	Volume ml	Protein mg	Cx/Avicelase	% of Recovered Protein
Filtrate Buffer	150 ml	4.1	100. +	} (38.%)
Acetate (0.05 M 4.8)	175	.48	5.5	
Ethanol 10% Containing	100	.12	0.75	} (21.%)
Citrate pH 2.5	180	.66	1.25	
" " "	390	.42	1.20	
" " "	505	.34	1.40	
Cellulose, 1%	600	0	-	
Methocel 0.1%	600	0	-	
Ethanol 20.0%	600	0	-	
HCl 0.05 N	600	0	-	
PO ₄ 0.05M	48	0.22	100. +	} (41.%)
NaOH 0.05 N	160	5.2	45.	
NaOH 0.05 N	95	.32	1.8	
NaOH 0.05 N	500	.08	-	
Original (Conc.)	110	23.	3.2	

Column run at room temperature; 6.5 cm dia. x 6.5 cm high. Volumes = combination of 20 ml fractions. The above fractions represent elution under the sequence shown.

Table 2. Effect of 6 M Urea on Inactivation and Proteolysis of *T. reesei* Avicelase

Enzyme in	Inactivation at 25°	Inactivation @ 50°		
	+ Buffer	+ Buffer	+ Mutanolysin	+ Pepsin
1. Buffer	5.%	5.0%	5.%	5.%
2. 6 M Urea	0.	43.	79.	81.

Conditions: Citrate (.05M) pH 5.0; 60 minutes. Assay vs. Avicel. Mutanolysin is a protease derived from a *Streptomyces*, a gift from T. Marumo of Dainippon (Japan). Pepsin (3x cryst.) was obtained from Nutritional Biochemical Company.

Table 3. Comparison of Urea and Guanidine as Eluents of Avicelase from Cellulose

Condition	Eluted as % of Adsorbed*			
	0°	OD280 40°	Enzyme Activity* 0°	40°
Buffer	7	20.	5	34.
Urea 6 M	50	71.	39	60.
Guanidine 4 M	55	98.	18	22.

*Not corrected for inhibitory effects of Urea and Guanidine in assays.
Elution time 30 minutes; pH 4.9.

Szejtli, J. et al 1977 Acta Chim. Acad. Sci. (Hungary) 94: 383

Adsorbed CTB on agarose - α -cyclodextrin (affinity Am)

Washed off other components.

Eluted with 5 M urea

A 113-fold concentration in one step to homogeneity

*Elution could also be done with α -cyclodextrin soln
(6 mg/ml)*

Table 4. Compounds Tested for Elution of Cellulase from Avicel

Compound	Concentration		*Eluted/Adsorbed %
	M	%	
A.			
Sodium hydroxide	.02		100
Guanidine · HCl	4.0		98
Urea	6.0		90
DMSO	3.2		80
DMF	3.4		66
Formamide	8.0		68
n-Propanol	4.2	25.	66
Acetic acid	4.0		90
Propionic acid	4.0		90
B.			
Ethylene glycol monomethyl ether		30.	59
Ethylene glycol		30.	50
Ethanol		30.	50
Glycerol		30.	50
Acetonitrile		25.	51
Isopropanol		25.	54
KCNS		20.	52
C.			
Citrate pH 5.0	0.025		30 ± 4
Phosphate pH 7.0	0.05		32
Mannitol		1.	24
Glucose		1.	25
Cellobiose		1.	25
βCH ₃ glucoside		1.	26
PEG 400		25.	37
PEG 6000		1.	18
Zonyl N		1.	14
Tween 80		1.	24
Na lauryl SO ₄		5.	33
Ethanol amine		1.	26
Methanol		25.	39
Mercapto ethanol		0.5	13
EDTA, Na		5.	14
Tris pH 7.0	0.2		34
CaCl ₂		10.	22
LiCl		5.	28
NaHCO ₃		5.	36
Na ₂ CO ₃		5.	38
K ₂ HPO ₄	1.0		12
Ammonium sulfate		15.	8

*Elution 40°, 15 minutes. Based on OD280 only. All compounds were in 0.025 M Citrate pH 5.0, except for the salts and other buffers.

Figure 1. Inhibition of Cellulase (Avicelase) Activity by Compounds which are good Eluents.

□ — □ U = urea; * — * GU = guanidine · HCl; o — o = n-propanol;
Δ — Δ = DMSO.



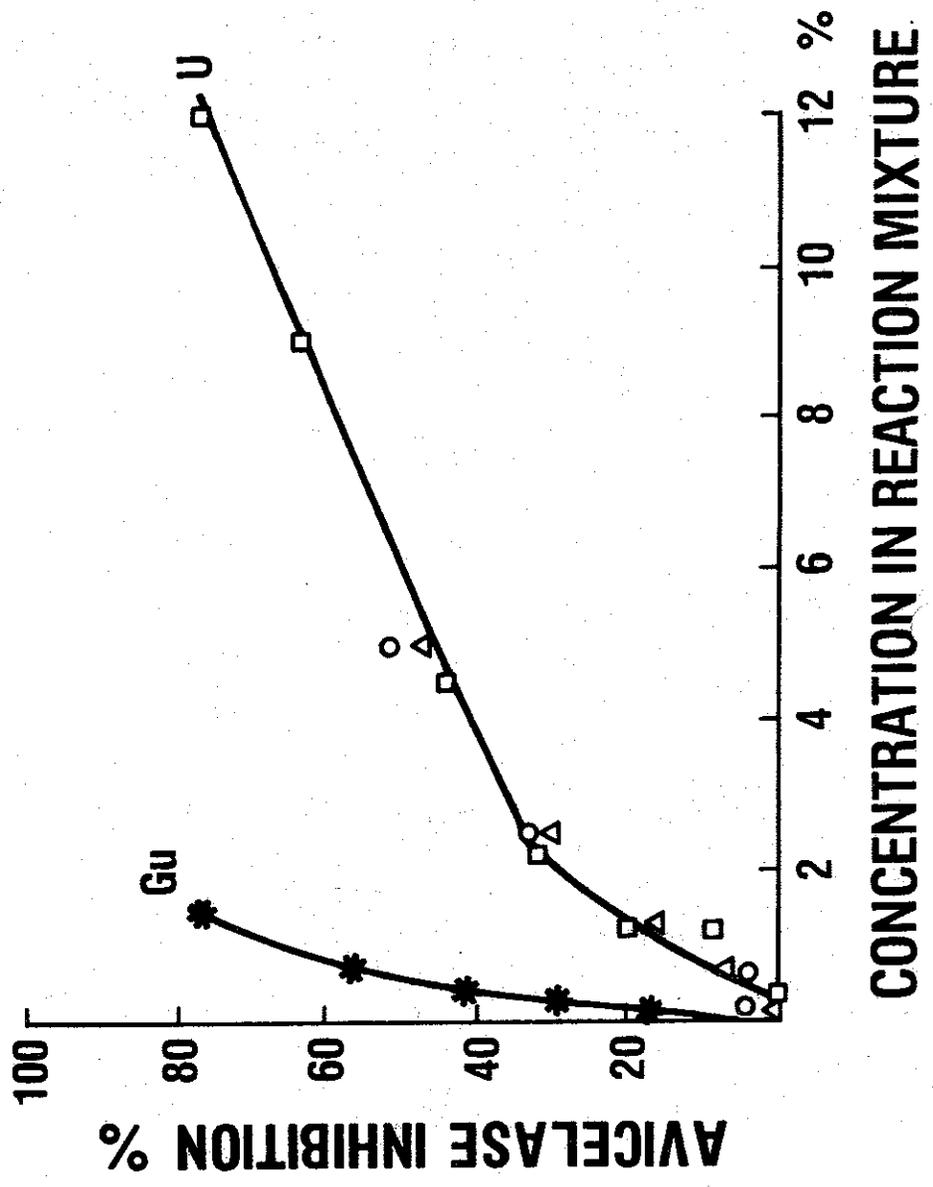


Figure 2. Effect of Time and Temperature on Elution of Cellulase from Avicel by 6 M urea.

A. Elution of materials absorbing at 280 m μ
B. Elution of enzyme activity
o — o 0 $^{\circ}$ C; Δ --- Δ 25 $^{\circ}$; \square — \square 50 $^{\circ}$



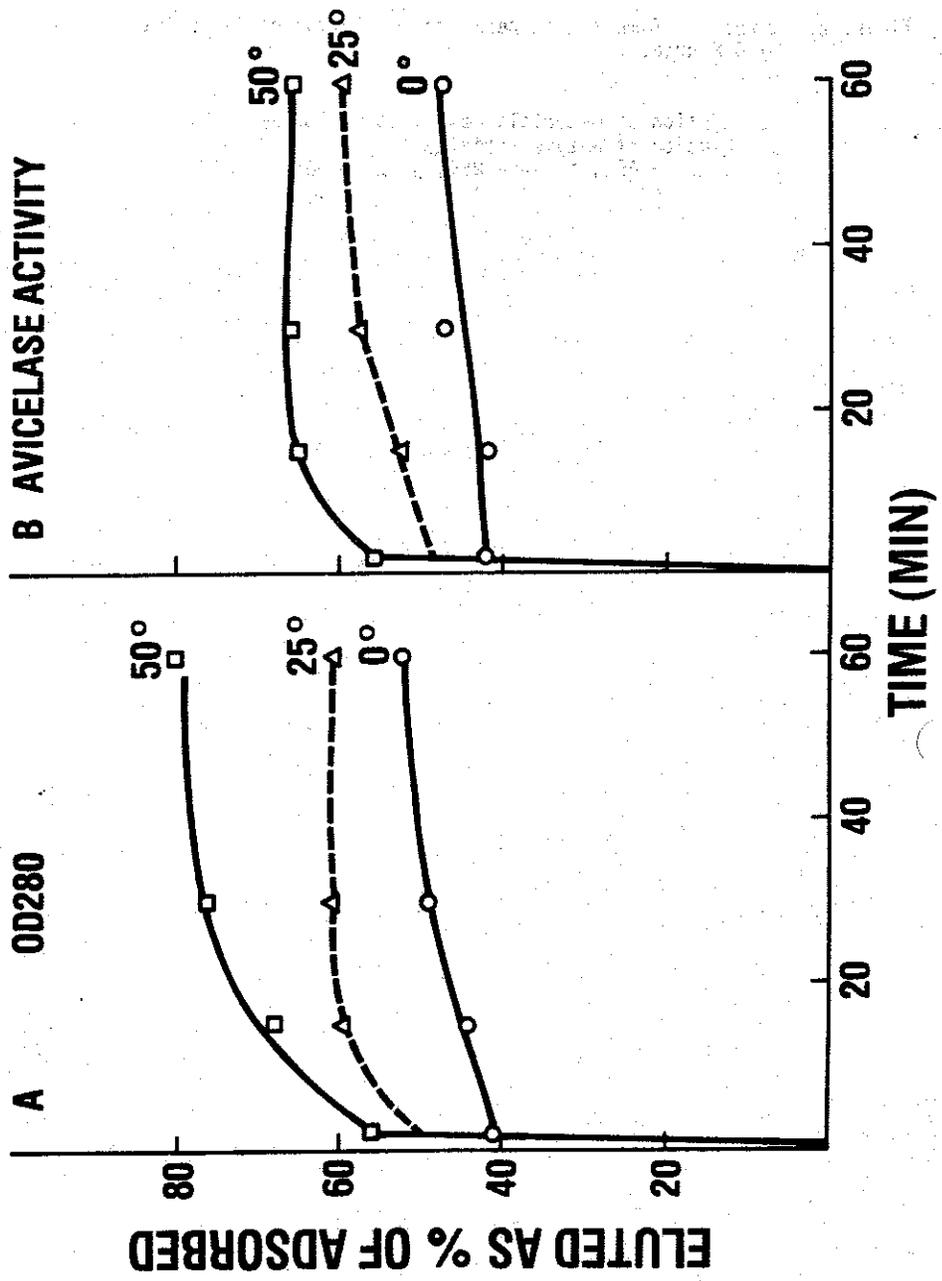


Figure 3. Effect of pH on Elution of Avicelase from Avicel.

U = 6 M urea (Δ — Δ); G = 4 M guanidine HCl (o — o);
K = 2 M KCl (\blacksquare — \blacksquare); B = 0.025 M Citrate (\bullet — \bullet)
All compounds in 0.025 M Citrate.



AVICELASE ELUTED AS % ADSORBED

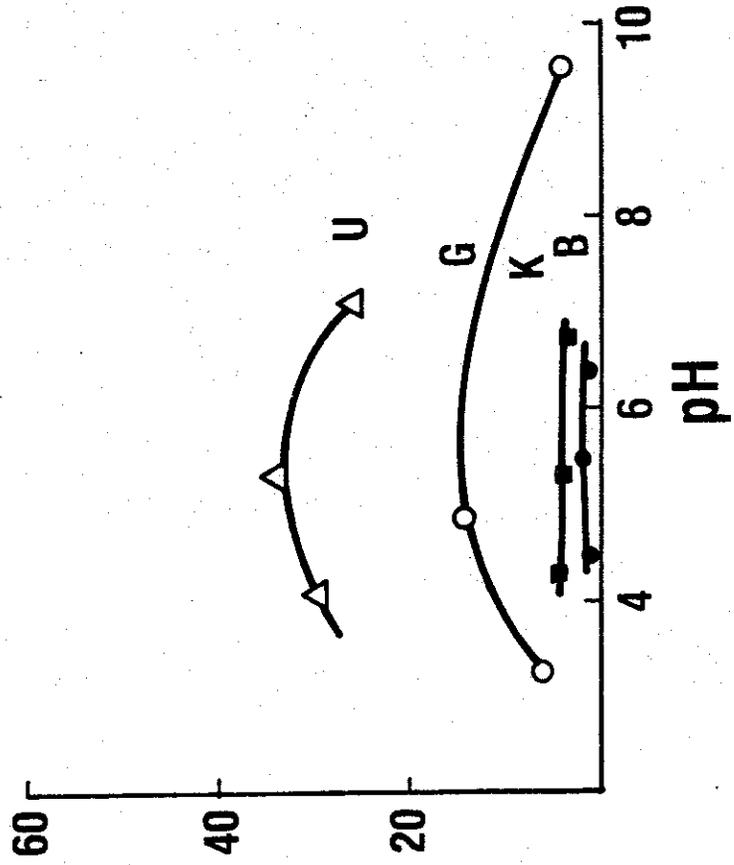


Figure 4. Effect of Concentration of Eluent on Elution of Cellulase Protein from Avicel (based on OD280).

o - - - - o Urea 0^o pH 5.0, 10 minutes
Δ - - - - Δ Urea 40^o pH 5.0, 15 minutes
● - - - - ● Guanidine 40^o pH 4.6, 15 minutes
All in 0.025 M Citrate pH 5.0



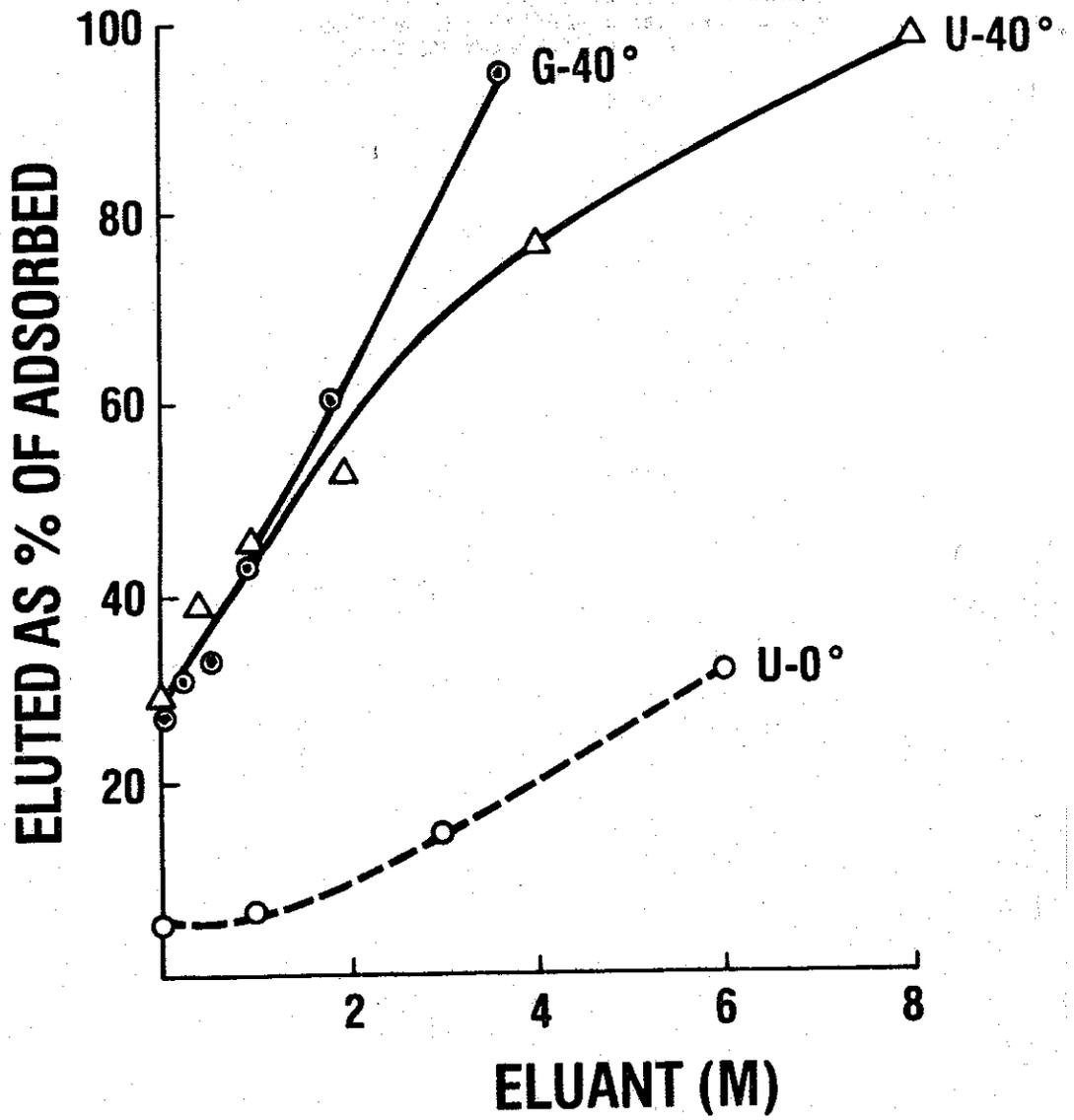
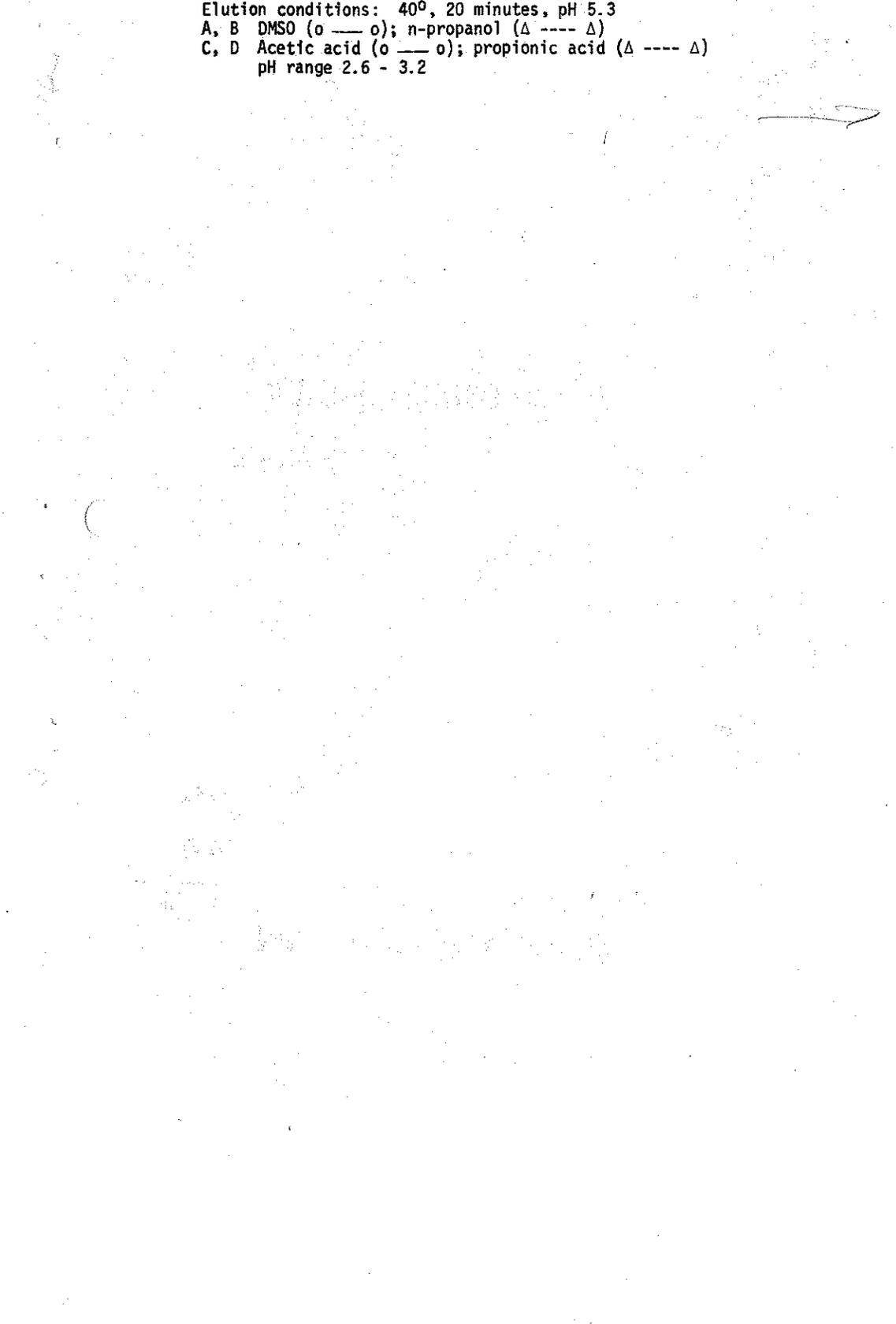


Figure 5. Effect of Eluent Concentration on Removal of Cellulase from Avicel.

Elution conditions: 40°, 20 minutes, pH 5.3
A, B DMSO (o — o); n-propanol (Δ ---- Δ)
C, D Acetic acid (o — o); propionic acid (Δ ---- Δ)
pH range 2.6 - 3.2



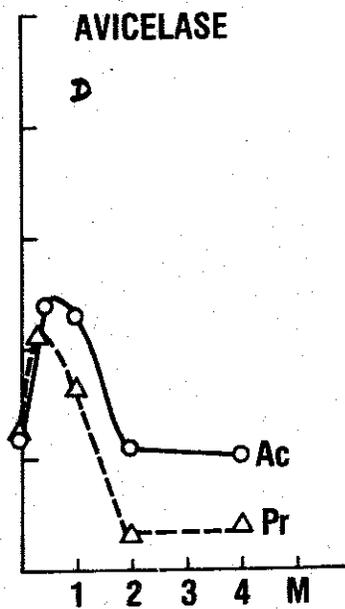
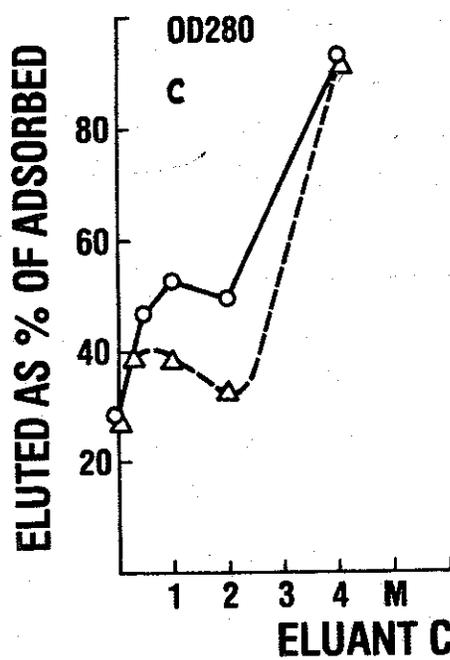
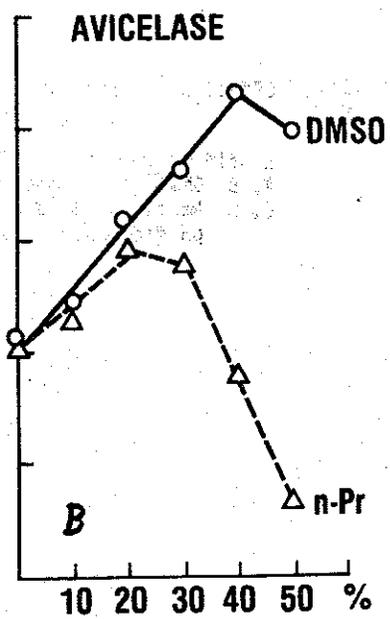
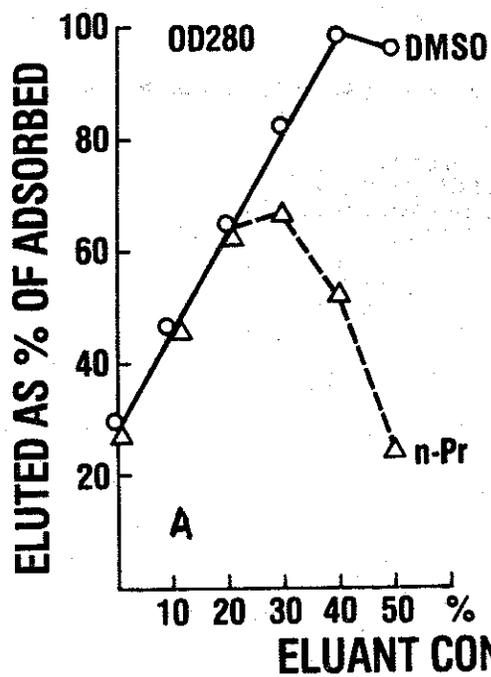


Figure 6. Elution of Avicelase from Avicel by $\text{Ca}(\text{OH})_2$.

Effects of Concentration and of pH. Elution at 0° , 15 minutes.
OD280 o — o; Avicelase (E) Δ ---- Δ .

