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An alkali stable cellulase by chemical modification using maleic anhydride

Rajesh K. Bund, Rekha S. Singhal

Food and Fermentation Technology Division, University Department of Chemical Technology, Matunga, Mumbai 400 019, India

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Abstract

An alkali stable cellulase was obtained by chemical modification with 0.5 M maleic anhydride at 2–4°C and pH 8.0 for 25 min. The cellulase so modified showed a marginally better activity in alkaline range as compared to native cellulase. It had a half-life of 462.0, 433.13, 128.33 and 144.38 h vis-à-vis 13.08, 121.58, 126.0 and 187.30 for native unmodified enzyme at a pH of 8.0, 9.0, 10.0 and 11.0, respectively, at 50°C. These results could have promising applications in textiles and detergents.

Keywords: Akali stable cellulase; Chemical modification; Maleic anhydride

1. Introduction

Cellulases have been used in the textile industry worldwide for textile finishing and biopolishing (Sharma, 1993; Shukla, Sharma & Kulkarni, 2000). It is noteworthy that most of the processing technologies in textiles use an alkaline medium. However, commercially, only acidic and neutral cellulases are available, and are obviously not compatible with textile processing. It was on these considerations that attempts were made to chemically modify the acid cellulase, so that its activity and stability are enhanced under alkaline conditions of pH 8.0–11.0.

Maleic anhydride was chosen to prepare an alkaline stable cellulase by chemical modification of acid cellulase. Maleic anhydride is selective in action. It acts specifically on the lysine residues (Scheme 1). Previous reports on chemical modification of cellulase indicated an improved stability at pH 8.5 (Kajiuchi and Park, 1992; Park and Kajiuchi, 1994). However, for industrial applications in textiles and detergents, the pH conditions could be up to 10.0–11.0.

The reaction products of maleic anhydride and amino groups are stable at neutral pH, but rapidly hydrolyze on acidification to pH 3.5. The half-life of ϵ-maleyllysine is approximately 11 h at this pH and 37°C. Reaction of maleic anhydride with most other side chains appears to be reversed even more rapidly (Means and Feeney, 1971).

In this study, acid cellulase was modified with maleic anhydride to determine its effect on stability as a function of pH and temperature.

2. Materials and methods

2.1. Materials

Commercial acid cellulase obtained from *Trichoderma viridae* was a gifted sample from M/S Khandelwal Laboratory, Mumbai, India. Maleic anhydride AR grade was obtained from M/S Sisco Research Laboratories, Mumbai, India. 3,5-dinitrosalicylic acid (DNSA) AR Grade was obtained from M/S Spectrochem, Mumbai, India. Other reagents were purchased from M/S S.D. Fine Chemicals, Mumbai, India and were of analytical grade.

2.2. Methods

2.2.1. Enzyme assay (Ghose, 1987)

50 mg cut strip (1.0 × 1.57 cm) of Whatman No.1 filter paper was added in 25 ml test tubes to which 1.0 ml each, of well-diluted enzyme and 0.2 M acetate buffer, pH 4.8 was then added. This was then incubated at 50°C for 60 min. 1.0 ml DNSA was added after that to each test tube and the contents were mixed. The tubes were transferred to a boiling water bath and kept for exactly 5.0 min, after which the tubes were immediately transferred to a water bath at room temperature for 10–15 min. 9.0 ml distilled water was then added to the test tubes. The contents were mixed by inverting the tube several times. The pulp was allowed to settle down well and after 20 min the colour of the supernatant was measured at 540 nm. Activity in terms of international units (IU) was defined as amount of glucose

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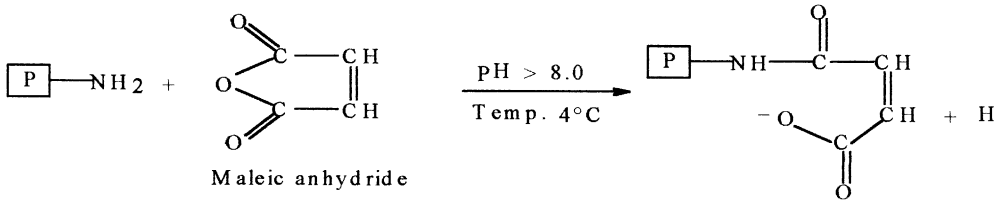

Scheme 1.

produced by 1.0 ml of enzyme in 1.0 min from a given substrate.

2.2.2. Method for chemical modification of cellulase using maleic anhydride as modifier

The procedure for the modification of maleic anhydride was as per previous reports (Kajiuchi and Park, 1992; Kajiuchi, Park & Moon, 1993) with slight modifications. 1.5 ml of native enzyme was diluted to 30 ml using phosphate buffer pH 8.0 in a reactor. The magnetic needle and pH probe were introduced into the container placed in an ice bath to maintain the temperature at 2–4°C. 3.8 ml of maleic anhydride (0.25–0.75 M) in dry dioxane was added slowly to the reaction mixture. pH was maintained at 8.0–8.2 by adding 0.2N NaOH. The reaction was carried out for 25 min.

Before finalizing the reaction conditions, the reaction pH (7.5, 8.0, 8.5) and maleic anhydride concentration (0.25–0.75 M) were varied, keeping all other reaction conditions constant. Reaction at pH 8.0 and maleic anhydride concentration at 0.5 M gave a modified cellulase with best activity at pH 4.8, 8.0 and 9.0. Hence these reaction conditions were fixed for further reactions. The modified cellulase so obtained is termed as Ma-cellulase henceforth.

2.2.3. Determination of pH and temperature optima of native and Ma-cellulase

The method as described above in Section 2.2.1 was used to determine pH and temperature optima of native and Ma-cellulase under identical conditions. For pH and temperature optima determination, the pH of buffer was varied from 4–11.0. The activity was determined for each pH at temperatures ranging from 30 to 70°C.

2.2.4. Determination of pH stability of native and Ma-cellulase

The results obtained from Section 2.2.3 indicated the temperature optima of 50°C for native cellulase to be unaltered on treatment with maleic anhydride. In textile industries, the cellulase treatment is generally carried out at 45–55°C. In detergents also, warm water at about 45°C is recommended for best results. Hence the stability of native and Ma-cellulase was carried out at 50°C. The cellulase was modified with the aim of using it in alkaline pH conditions. Therefore, native and Ma-cellulase were studied for stability up to 48 h at pH 4.8, 8.0, 9.0, 10.0 and 11.0. The degradation kinetics was studied by plotting a semi-log plot of cellulolytic activity vs time. A linear graph is obtained, confirming that the degradation follows first order kinetics. From the slope, k, $t_{(1/2)}$ or the half-life of the enzyme was calculated as $t_{(1/2)} = 0.693/k$.

3. Results and discussion

The cellulase sample used in the study was a complex of at least three enzymes, since it was of fungal origin. Generally, cellulolytic enzymes of fungal origin contain endoglucanase, cellobiohydrolase, exoglucanase and β-glucosidase (Bhat & Bhat, 1997). However, this is the form in which it is used commercially. The aim of the present work was to modify the stability of the enzyme in the alkaline range of pH, as is used commercially. Modification was not targeted at any particular enzyme, but rather at the cocktail which would be used industrially.

Initially, the reaction of native cellulase with 0.5 M maleic anhydride was carried out at pH of 7.5, 8.0 and 8.5 at 2–4°C for 25 min. The maximum activity of the Ma-cellulase was found to be at a reaction pH of 8.0. Native

Table 1
Effect of different reaction pH on activity (results are mean±SD of three determinations) of native (N) and Ma-cellulase (M)

pH	Activity at different reaction pH					
	7.5		8.0		8.5	
	N	M	N	M	N	M
4.8	22.56±0.56	12.49±0.41	**23.53**±0.65	**13.2**±0.27	22.41±0.56	9.26±0.40
8.0	5.9±0.26	5.74±0.23	6.21±0.28	6.9±0.29	6.09±0.31	6.56±0.29
9.0	5.97±0.27	6.17±0.49	6.71±0.43	7.4±0.19	6.71±0.37	7.17±0.25

Table 2
Effect of concentration of maleic anhydride on the activity (results are mean±SD of three determinations) of native (N) and Ma-cellulase (M)

pH	Activity at different concentration of modifier			
	N	0.25 M M	0.5 M M	0.75 M M
4.8	23.68±0.47	9.18±0.40	12.94±0.25	9.06±0.18
8.0	6.09±0.20	6.13±0.28	6.98±0.31	5.86±0.15
9.0	6.59±0.23	6.75±0.19	7.25±0.20	6.25±0.18

cellulase had an activity of 23.53, 6.21 and 6.71 units/ml at pH 4.8, 8.0 and 9.0, respectively, Ma-cellulase prepared at a reaction pH of 8.0 had an activity of 13.2, 6.9 and 7.4 units/ml at pH 4.8, 8.0 and 9.0, respectively, given in Table 1.

The initial activity of native cellulase was found to be 23.68, 6.09 and 6.59 units/ml at pH 4.8, 8.0 and 9.0, respectively. Effect of modifier concentration on the cellulolytic activity is illustrated in Table 2. 0.5 M maleic anhydride gave the best activity of 12.94, 6.98 and 7.25 units/ml at pH 4.8, 8.0 and 9.0, respectively. This modification decreased the activity of the native enzyme by 45% at pH 4.8, while it increased the same by 15 and 10% at pH 8.0 and 9.0, respectively. Thus, for all further work, 0.5 M maleic anhydride was chosen for chemical modification of cellulase.

Results from Table 3 showed native cellulase to have a pH optima of 4.8 and second pH optima of 9.0 in alkaline range of pH. The native cellulase has the temperature optima of 50°C. After modification with maleic anhydride both pH and temperature optima remained unaltered. The activity of Ma-cellulase decreased by around 42% at pH 4.8 whereas the activity in alkaline range of pH from 8.0–11.0 showed a considerable rise of 9–20% in activity. Literature reports indicate a decrease in specific activity of many enzymes on modification with maleic anhydride. For instance, *Escherichia coli* asparginase on succinylation and acetylation showed considerable decrease up to 63% in activity (Sadana and Henley, 1986). This decrease in activity may be attributed to the modification of some amino acids of importance for the activity of the enzyme. Since maleic anhydride specifically reacts with the lysine residues, and the results obtained show decrease in Ma-cellulase activity at pH 4.8. These results indicate that lysine residue must be an important residue either in catalytic or CBD domain of one or the entire enzyme from cellulase complex. At alkaline pH 8–11, the lysine residues may denature rapidly. Further, the assay temperature used is also quite high, i.e. 50°C. These may be responsible for very low activity of native cellulase in this range of pH. Modification of lysine residue in cellulase with maleic anhydride may have increased its pK_a resulting in improvement of stability in alkaline pH of 8–11 (Tomazic, 1991).

Comparative pH stability of native and Ma-cellulase is displayed in Table 4 Ma-cellulase showed better stability than native enzyme at pH 4.8, 8.0, 9.0 and 10.0, whereas the native enzyme showed better stability than the Ma-cellulase at pH 11.0. After 96 h, the percentage activity retained by native cellulase was around 9, 21, 22, 33 and 44% at pH 4.8, 8.0, 9.0, 10.0 and 11.0, respectively. The corresponding values for Ma-cellulase were 30, 64, 62, 35 and 36%. These results showed that the stability of cellulase, improved substantially in the pH range of 8–10 after chemical modification with maleic anhydride.

An idea about the comparative stability of native and Ma-cellulase can be obtained from the degradation kinetics. This is obtained from a semi-log plot of cellulolytic activity vs time. Linear plot obtained in all cases (figures not shown) indicated first order degradation kinetics. The time required for the enzyme to come to 50% of its initial activity is indicated by $t_{(1/2)}$ that can be calculated from the slope (k) of the semi-log plot as $t_{(1/2)} = 0.693/k$. Fig. 1 shows comparative data on the $t_{(1/2)}$ of the native and Ma-cellulase at different pH. The $t_{(1/2)}$ of Ma-cellulase improved by 131, 3434, 256 and 2% at pH 4.8, 8.0, 9.0 and 10.0, respectively, over the native cellulase. The $t_{(1/2)}$ of Ma-cellulase decreased by 23% at pH 11.0 as compared to native cellulase.

Enzymes may be inactivated by a variety of conditions such as temperature, pH, ionic strength, denaturing agents, pressure, or mechanical forces. They occur naturally in the

Table 3
Comparative pH and temperature optima studies (results are mean±SD of three determinations) between native (N) and Ma-cellulase (M)

pH	Activity (units/ml)									
	30°C		40°C		50°C		60°C		70°C	
	N	M	N	M	N	M	N	M	N	M
4	1.0±0.11	0.19±0.03	1.66±0.13	0.39±0.07	2.97±0.25	1.23±0.13	1.43±0.27	1.04±0.06	1.31±0.18	0.62±0.11
4.8	12.54±0.51	8.26±0.38	19.9±1.05	9.72±0.44	23.72±1.18	13.85±0.58	17.78±0.81	9.14±0.55	16.59±0.65	7.68±0.42
5	11.53±0.28	7.95±0.51	18.13±0.49	9.53±0.59	21.72±1.01	10.8±0.41	16.59±0.59	8.68±0.44	16.51±0.42	6.87±0.55
6	0.89±0.15	0.5±0.11	1.08±0.09	0.54±0.06	1.04±5.86	0.5±0.12	0.81±0.10	0.42±0.06	0.69±0.09	0.27±0.06
7	4.67±0.38	4.32±0.47	4.9±0.20	4.44±0.36	5.86±0.35	4.78±0.20	4.4±0.32	3.82±0.29	4.24±0.24	3.74±0.37
8	5.25±0.45	5.71±0.44	5.67±0.30	6.48±0.27	6.09±0.38	7.02±0.47	5.21±0.36	5.94±0.33	5.13±0.39	5.94±0.41
9	5.36±0.43	5.98±0.56	5.94±0.39	6.6±0.44	6.6±0.36	7.25±0.45	5.86±0.24	6.29±0.31	5.71±0.55	6.02±0.36
10	4.32±0.24	4.67±0.26	4.47±0.39	5.09±0.49	4.78±0.37	5.71±0.36	4.17±0.36	4.59±0.34	4.44±0.43	4.63±0.29
11	4.47±0.47	4.63±0.36	4.63±0.43	5.21±0.46	5.21±0.39	5.86±0.37	4.32±0.30	4.82±0.35	3.63±0.40	4.32±0.37

Table 4
Comparative stability (results are mean±SD of three determinations) of native (N) and Ma-cellulase (M)

Time (h)	pH					
		4.8	8.0	9.0	10.0	11.0
0	N	21.37±0.71	5.79±0.49	6.25±0.35	4.71±0.22	4.83±0.35
	M	11.07±0.30	6.89±0.44	7.28±0.47	5.1±0.41	5.19±0.36
12	N	5.94±0.35	2.03±0.24	2.78±0.31	4.15±0.27	4.07±0.36
	M	5.36±0.24	5.13±0.34	5.37±0.53	4.55±0.34	4.23±0.24
24	N	4.44±0.45	1.97±0.27	2.47±0.31	3.47±0.21	3.53±0.34
	M	5.35±0.31	5.08±0.35	5.25±0.39	4.01±0.33	3.7±0.35
36	N	3.82±0.25	1.74±0.17	2.14±0.17	2.99±0.29	3.09±0.23
	M	5.27±0.43	5.06±0.3	5.19±0.27	3.59±0.31	3.2±0.31
48	N	3.28±1.21	1.70±0.14	1.83±0.13	2.32±0.25	2.76±0.38
	M	5.04±0.23	4.9±0.20	5.02±0.38	2.62±0.28	2.51±0.28
60	N	2.97±0.38	1.51±0.15	1.72±0.22	2.1±0.26	2.49±0.29
	M	4.61±0.33	4.83±0.19	4.94±0.27	2.26±0.26	2.34±0.19
72	N	2.28±0.19	1.35±0.12	1.51±0.07	1.72±0.13	2.39±0.25
	M	4.17±0.27	4.69±0.29	4.77±0.54	1.99±0.18	2.24±0.16
96	N	1.85±0.21	1.2±0.22	1.39±0.16	1.54±0.24	2.12±0.21
	M	3.36±0.35	4.4±0.20	4.48±0.25	1.76±0.25	1.85±0.14
Residual activity (%)	N	8.66	20.67	22.22	32.79	44.0
	M	30.33	63.87	61.54	34.47	35.69

folded form, which on inactivation causes reversible unfolding of enzyme. This is true especially at higher temperature and alkaline pH. Reversible unfolding occurs when the non-covalent forces responsible for the three-dimensional structure of the native enzyme are disrupted. These interactions include hydrogen bonds, ionic and van der Waals interactions and hydrophobic forces. Subsequent to the disruption of such interactions, irreversible inactivation processes occur.

Chemical modification of enzyme is one of the methods to overcome this problem. The resulting stabilization if it occurs, is attributed to a rigidification of the protein's conformation such that reversible unfolding is slowed or prevented. In this case after modification the enzyme shows better stability at a temperature of 50°C that is quite high, and in alkaline and acidic range as well (pH 4.8, 8.0, 9.0,10.0). This can be explained on the basis of ionic interactions or hydrophobic interactions.

Ionic interactions are important for maintaining the three-dimensional structure of a protein. Moreover, it has been observed that in comparing some highly stable proteins with their mesophilic counterparts, a replacement of histidine (His) and lysine (Lys) residues by arginine (Arg) residues frequently occurs. The pKs for the side-chains of His and Lys (6.0 and 10.8, respectively) are much lower than for Arg (pK = 12.5) hence, on increase of temperature at neutral and alkaline pHs, the ionogenic groups of His and Lys will become increasingly neutral, resulting in disruption of a stabilizing salt bridge. On the other hand arginine remains positively charged at highly alkaline pH values, and also at higher temperatures (compared with lysine). Thus, stabilization may be induced by the introduction of new or additional electrostatic interaction. In this case, chemical modification of lysine residues with maleic anhydride created an Arg-like side chain. This may possibly be the reason for the better stability of Ma-cellulase than native cellulase at pH 4.0, 8.0, 9.0, 10.0.

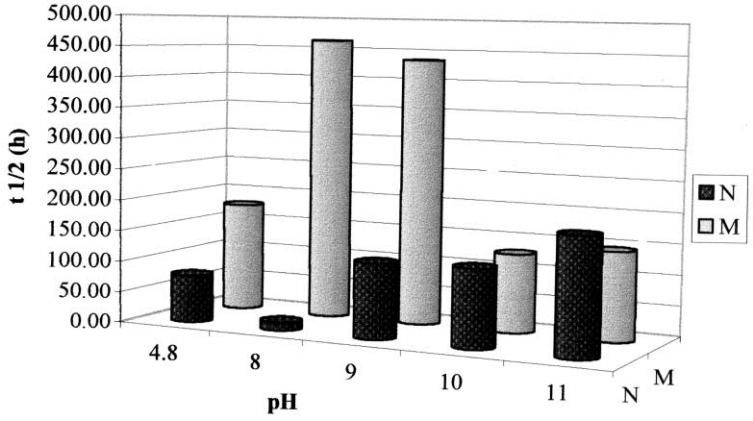

Fig. 1. Comparison of $t_{(1/2)}$ of native (N) and Ma-cellulase (M).

Hydrophobic interactions are often important contributors to the overall stability of a protein. The presence of hydrophobic residues on the surface of protein, in contact with the aqueous medium creates a thermodynamically unfavourable situation. Therefore, to lower the free energy of the system, the regions around hydrophobic associate, resulting in conformational changes, which may irreversibly denature the biocatalyst (Tomazic, 1991). Succinylation has been used to increase the stability of many enzymes such as alanine amino transferase (O'Fagain, O'Kennedy & Kilty, 1991), chymotrypsin (Mozhaev, Siknis, Melik-Nubarov, Galkaite, Denis, Batkus et al., 1988) and trypsin (Murphy & O'Fagain, 1998). A similar effect was observed in the present study when cellulase was treated with maleic anhydride. Most of the lysine residues of a protein molecule are present on the surface of molecule. Lysine residues contain two hydrophobic methylene groups in addition to the charged moieties. These hydrophobic groups tend to destabilize the protein structure when exposed to aqueous environment. Maleylation screens these hydrophobic moieties from unfavourable contact with water and hence brings about stabilization.

These two mechanisms explain the increase in stability of Ma-cellulase at pH 4.8, 8.0, 9.0 and 10.0. However, Ma-cellulase showed a decrease in stability at pH 11.0 vis-*à*-vis native enzyme. This could be because of very high alkaline pH. Table 4 shows that the activity of Ma-cellulase was more than that of the native cellulase upto 36 h, after which it decreased very rapidly as compared to native cellulase. This could be due to reversing of modification; or at this high pH, the modified lysine residues must denature rapidly because of rupture of the salt bridge.

4. Conclusions

Modification of acid cellulase obtained from the industry with 0.5 M maleic anhydride under the specified conditions improved its activity in an alkaline pH range of 8.0–11.0 as well as stability at pH 8.0–10.0. However, there was a substantial decrease in activity at pH 4.8. These results are promising and warrant applications of Ma-cellulase in textile industry as well as in detergents.

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