Lipase Deactivation at Gas–Liquid Interface and Its Subsequent Reactivation

N. S. Patil, R. S. Ghadge, S. B. Sawant, and J. B. Joshi
Dept. of Chemical Technology, University of Mumbai, Matunga, Mumbai 400 019 India

Proteins are exposed to the gas–liquid interface during their synthesis and purification. Moreover, some proteins (enzymes) may be exposed to the interface during their use as catalysts in bioreactors. At the interface, proteins generally undergo unfolding, which leads to undesirable denaturation. Sometimes, however, the air–water interface can be deliberately provided to deactivate proteins. For example, Caussette et al. (1999) recently suggested passing air through food products to deactivate enzymes present in them. If the enzymes are left in the food, the quality of the food product deteriorates over time, and if the enzymes are thermally deactivated, food quality is simultaneously altered due to high temperatures.

Several proteins, including cellulase (Kim et al., 1982), β-lactoglobulin (Reese and Robbins, 1981), interferon, ovalbumin, recombinant human growth hormone (Maa and Hsu, 1997), and lysozyme (Caussette et al., 1999), have been shown to undergo interfacial denaturation. Elias and Joshi (1997) have reviewed the effect of hydrodynamic shear on the deactivation of proteins and enzymes. Understanding the mechanism of interfacial deactivation would be useful in determining strategies for enhancing or suppressing the deactivation, depending on whether it is desired or undesired. Here, we suggest a mechanism for deactivation of lipase from *Aspergillus oryzae* at the air–water interface. Knowledge of the structural changes that accompany the deactivation is also desirable. The preliminary model suggested here could also be useful in interpreting the studies on the protein structural changes during deactivation when spectroscopic techniques are used.

Materials and Methods

Lipase from *Aspergillus oryzae* (Lipolase 100L) was obtained from Novo Nordisk A/S, Denmark. The enzyme was supplied in solution form. Tributyrin and gum accacia were purchased from HiMedia Laboratories Limited, Mumbai, India.

Correspondence concerning this article should be addressed to S. B. Sawant.

The enzyme was exposed to the air–water interface in a stirred-tank reactor. A baffled cylindrical glass vessel (internal diameter ~105 mm, height ~175 mm, total capacity ~1000 mL) with a three-port lid was used. Seven hundred and fifty mL of 100 mM phosphate buffer (pH ~7.0) and 250 μL of enzyme solution were added to the reactor. Agitation was provided by a 40-mm six-bladed disc turbine. The agitation was carried out with an AC motor equipped with a speed regulator. The desired temperature was maintained by immersing the reactor assembly in a constant-temperature bath. A noncontact-type tachometer was used to measure the speed of agitation.

Twenty mL aliquots were withdrawn from the reactor at various time intervals, and the activity of lipase was analyzed. The percentage of loss in residual activity was calculated by comparing with the initial activity.

Estimation of the lipase activity was carried out by the hydrolysis of tributyrin. Twenty mL of the enzyme solution withdrawn from the reactor were placed in a 50-mL conical flask. Six mL of 25% w/v gum accacia solution and 2 mL of tributyrin were added to the flask. The solution was stirred using a magnetic stirrer. The speed of agitation was kept low enough to avoid surface air entrapment and subsequent deactivation during the assay time. The hydrolysis of tributyrin was allowed to take place for 10 min. The liberated butyric acid was estimated by titration of the solution with 0.01 N NaOH, and the activity of lipase was calculated. Twenty mL of buffer was used as a control instead of the enzyme solution. The titration reading obtained for the control was subtracted from all the titration readings obtained for the samples.

Results and Discussion

Deactivation of lipase from *Aspergillus oryzae* at 1,000 rpm is shown in Figure 1. In earlier reports on deactivation of other enzymes in stirred tanks, the loss of enzyme activity has been attributed to shear stresses and/or the air–water interface. The lipase was stable for several hours ($>$15 h) in the absence of the air–water interface under otherwise similar conditions (Figure 1). To carry out the experiments in the

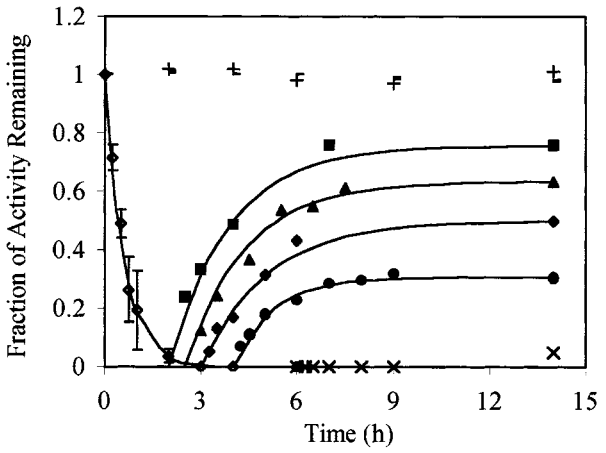

Figure 1. Deactivation followed by reactivation of lipase from *Aspergillus oryzae.*

Deactivation was carried out in a stirred-tank reactor equipped with a flat-blade impeller at 30°C and reactivation was examined after stirring for different times. Key: 0 rpm: + — deactivation (control); 1,000 rpm: ◇—deactivation; — —deactivation in the absence of air–water interface; ■ —reactivation after 2 h stirring; ▲—reactivation after 2.5 h stirring; ♦—reactivation after 3 h stirring; ●—reactivation after 4 h stirring; ×—reactivation after 6 h stirring. The curves indicate fits according to the equations in Table 1.

absence of the interface, a circular acrylic baffle was placed in the reactor so that it touched the liquid at its top surface (which was otherwise in contact with air). The result suggests that the deactivation occurred at the interface that was created by the surface entrainment of air into water. Without stirring the enzyme solution (0 rpm), the enzyme did not show any significant changes in its activity (Figure 1).

Reactivation of the enzyme was also studied after removal of the interface, which was done by stopping the agitation. This reactivation was determined after stirring the enzyme solution for different periods of time at 1,000 rpm (Figure 1). The reactivation behavior was similar in all cases, except that the extent of reactivation decreased when the stirring time was increased. For 2 h stirring time at 1,000 rpm, the reactivation was 75%, which decreased to about 35% at 4 h stirring time. The effect of the agitation speed on deactivation and reactivation of the lipase is shown in Figure 2. The rate of enzyme deactivation at 1,000 and 1,500 rpm was about the same. For 1,500 rpm and 2 h stirring time, the reactivation was less (~43%) than that at 1,000 rpm (~ 75%).

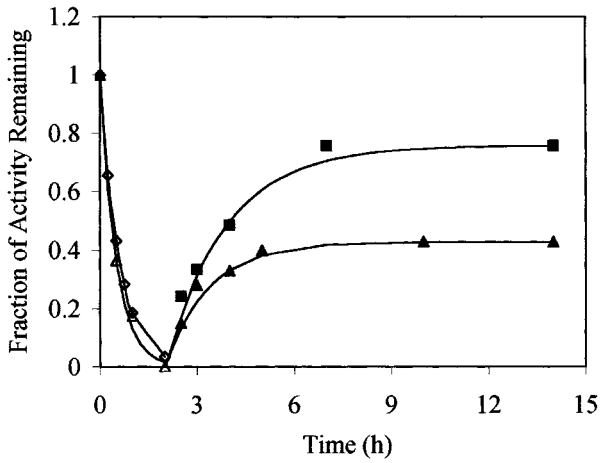

Figure 2. Effect of agitation speed on deactivation and reactivation of lipase from *Aspergillus oryzae.*

Deactivation was carried out in a stirred-tank reactor equipped with a flat-blade impeller at 30°C and reactivation was examined after stirring from 2 h. Key: 1,000 rpm: ◇—deactivation; ■—reactivation; 1,500 rpm: △—deactivation, ▲—reactivation. The curves indicate fits according to the equations in Table 1.

Based on the deactivation and reactivation behavior, the mechanism of the enzyme deactivation is suggested, as shown in Figure 3. E_N and E_N^* are the original forms of the enzyme in the solution and at the air–water interface, respectively. At the interface, E_N^* undergoes conformational changes leading to E_{d1}^*, which can change further, giving E_{d2}^*. However, E_{d1} and E_{d2} are the enzyme forms that are similar to those of E_{d1}^* and E_{d2}^*, respectively, in the solution. In the solution, E_{d1} can return to its original enzyme form, E_N. E_N and E_N^* are the active forms of the enzyme, while when the enzyme is in the E_{d1}, E_{d2}, E_{d1}^*, and E_{d2}^* forms, it is in an inactive state.

The rate constants for different reactions (k_1, k_{-1}, and k_2) are as shown in Figure 3. K_i are the equilibrium constants between different forms of the enzyme defined as

$$K_i = \frac{[E_i^*]}{[E_i]}, \tag{1}$$

where i can be N, $d1$, or $d2$, and $[E_i^*]$ and $[E_i]$ are concentrations of the enzyme in a certain form in the solution and at the interface, respectively. If $[E_i']$ is the apparent concentration of the enzyme in a certain form (that is, protein in a certain form per unit of liquid volume), we can write:

$$[E_i'] = [E_i] + [E_i^*]\left(\frac{a}{\epsilon_L}\right). \tag{2}$$

The rate of change of E_N' is given by following equation:

$$\frac{d[E_N']}{dt} = -k_1[E_N^*]\left(\frac{a}{\epsilon_L}\right) + k_{-1}[E_{d1}], \tag{3}$$

where a is the interfacial area per unit volume and ϵ_L is the

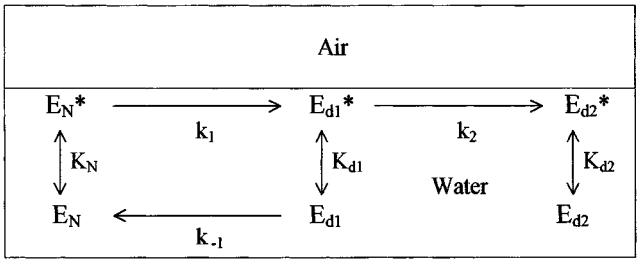

Figure 3. Mechanism of lipase deactivation at air–water interface.

E_N is the enzyme in its native form, E_{d1} is the enzyme in intermediate denatured form, while E_{d2} is the enzyme in final denatured form. * indicates the protein at air–water interface.

Table 1. Expressions for Concentrations of E'_N, E'_{d1}, and E'_{d2} During and After Stirring*

	E'_N or $E_0 \cdot a$	E'_{d1}	E'_{d2}
$t = 0$	E_0	0	0
$0 \le t \le t_s$	$E_0 \exp(-k_1 t)$	$E_0 \dfrac{k_1}{k_2 - k_1}\{\exp(-k_1 t) - \exp(-k_2 t)\}$	$E_0\left\{1 - \dfrac{k_2}{k_2 - k_1}\exp(-k_1 t) + \dfrac{k_1}{k_2 - k_1}\exp(-k_2 t)\right\}$
$t \ge t_s$	$E_0 \exp(-k_1 t_s) + E_0 \dfrac{k_1}{k_2 - k_1}\{1 - \exp(-k_{-1}(t - t_s))\} \cdot \{\exp(-k_1 t_s) - \exp(-k_2 t_s)\}$	$E_0 \dfrac{k_1}{k_2 - k_1}\exp[-k_{-1}(t - t_s)] \cdot \{\exp(-k_1 t_s) - \exp(-k_2 t_s)\}$	$E_0\left\{1 - \dfrac{k_2}{k_2 - k_1}\exp(-k_1 t_s) + \dfrac{k_1}{k_2 - k_1}\exp(-k_2 t_s)\right\}$

*Derived according to the equations given in the text.

liquid holdup. Assuming that the equilibrium between the different enzyme forms in the solution and at the interface takes place much faster than the enzyme deactivation and reactivation steps, Eq. 3 can be written as

$$\frac{d[E'_N]}{dt} = -k_1\left(\frac{\left(\frac{a}{\epsilon_L}\right)K_N}{1+\left(\frac{a}{\epsilon_L}\right)K_N}\right)[E'_N] + k_{-1}\left(\frac{1}{1+\left(\frac{a}{\epsilon_L}\right)K_{d1}}\right)[E'_{d1}], \quad (4)$$

where $[E'_{d1}]$ and $[E'_{d2}]$ can be expressed similarly to Eq. 4.

The first term in Eq. 4 represents deactivation of the enzyme at the interface, and the term represents reactivation in the solution. Assuming that $aK/\epsilon_L \gg 1$ while the stirring is going on, Eq. 4 becomes

$$\frac{d[E'_N]}{dt} = -k_1[E'_N]. \quad (5)$$

Similarly,

$$\frac{d[E'_{d1}]}{dt} = k_1[E'_N] - k_2[E'_{d1}] \quad (6)$$

$$\frac{d[E'_{d2}]}{dt} = k_2[E'_{d1}]. \quad (7)$$

Table 2. Different Kinetic Parameters for Deactivation and Reactivation of Lipase from *Aspergillus oryzae*

t_s* (h)	Agitation Speed (rpm)	k_1 (h^{-1})	k_{-1} (h^{-1})	k_2 (h^{-1})
2	1,000		0.54	0.21
2.5	1,000		0.54	0.25
3	1,000	1.68	0.49	0.29
4	1,000		0.81	0.36
6	1,000		—	—
2	1,500	1.75	0.74	0.62
Average ± standard deviation →			0.62 ± 0.14	0.35 ± 0.16

*t_s—time after which stirring was stopped.

During reactivation (that is, when the stirring is stopped), following equation holds

$$\frac{d[E'_N]}{dt} = -\frac{d[E'_{d1}]}{dt} = k_{-1}[E'_{d1}]. \quad (8)$$

Concentrations of E'_N, E'_{d1}, and E'_{d2} as a function of time, which were obtained using Eqs. 5–8, are given in Table 1.

The deactivation and reactivation data, shown in Figure 1, were fitted in expressions in Table 1. The suggested model gave satisfactory fits, which are shown in Figure 1 by different curves. The enzyme underwent first-order deactivation with a rate constant (k_1) of 1.68 and 1.75 h^{-1} at 1,000 and 1,500 rpm, respectively (Table 1). The fact that the enzyme underwent first-order deactivation suggests that the assumption $aK/\epsilon_L \gg 1$ is valid. If it was not valid, the deactivation would have been more complex. Moreover, the deactivation constant at both speeds is approximately the same (Table 2), which is expected if $aK/\epsilon_L \gg 1$. The values of k_2 and k_{-1} were obtained as 0.35 ± 0.16 and 0.62 ± 0.14 h^{-1}, respectively (Table 1). In order to compare reactivation in different cases,

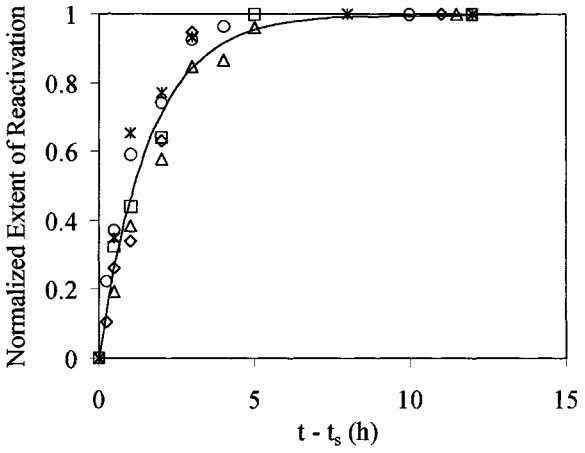

Figure 4. Normalized reactivation curves for different stirring times.

t is the time and t_s is the time for which stirring was carried out. Key: 1,000 rpm: □—reactivation after 2 h stirring; △—reactivation after 2.5 h stirring; ◇—reactivation after 3 h stirring; ○—reactivation after 4 h stirring; 1,500 rpm: *—reactivation after 2 h stirring. The curve is plotted using the average value of k_{-1} from Table 2.

the normalized extent of reactivation was plotted against $(t - t_s)$, where t_s is the stirring time (Figure 4). The plot shows that the reactivation behavior was similar in all cases. The curve in Figure 4 is plotted using the average k_{-1} value from Table 2.

In general, protein unfolding is expressed in terms of micro- and macrounfolding (Privalov and Tsalkova, 1979). Microunfolding leads to a protein that is either partially unfolded or has undergone local unfolding. Macrounfolding leads to the complete disruption of the protein structure. In our model, E_{d1} might be a result of the microunfolding of the lipase, while macrounfolding results in E_{d2}.

Conclusion

Lipase from *Aspergillus oryzae* underwent first-order deactivation at the air–water interface in a stirred tank. Before reaching the final denatured state, the enzyme arrived at an intermediate inactive form. In solution, the intermediate form was able to refold to its original form, with the simultaneous reactivation of the enzyme. The enzyme converted to the final denatured state was, however, permanently deactivated. This study would be useful in determining strategies for reducing or enhancing enzyme deactivation at the gas–liquid interface. Moreover, the model suggested here could form the basis for further investigation of the enzyme deactivation mechanism using spectroscopic methods.

Acknowledgment

This research work was supported by a grant from the Department of Biotechnology, Government of India (No. BT/12/11/PRO485/97-PID).

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Manuscript received Sept. 22, 1999, and revision received Jan. 3, 2000.