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Lysozyme inactivation and aggregation in stirred-reactor

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Abstract

Mechanisms of enzyme inactivation and aggregation are still poorly understood. In this work, we are considering the characterisation of both inactivation and aggregation in stirred tank reactor, with lysozyme as the model enzyme.

The inactivation kinetics are first order. For stirring speeds in the range of 0–700 rpm, the kinetic constant is found to be proportional to the power brought by the impeller. It suggests that inactivation depends on collisions between enzyme molecules. Efficient collisions between native and inactive molecules induce native molecules to turn into inactive molecules and lead to lysozyme aggregation.

During inactivation, enzymes are found to aggregate as shown by light scattering measurements. The structure of aggregates was studied on samples treated for chemical denaturation and reduction. The aggregates are supramolecular edifices, mainly made up of inactivated enzymes linked by weak forces. But aggregates are also made up of dimers and trimers of lysozyme, linked by disulfide bridges. Dimers and trimers are 18% and 5%, respectively, of the total amount of lysozyme aggregates.

Whatever the stage of aggregate formation and the initial enzyme concentration are, these aggregates are irreversibly inactivated. Enzyme activity is definitely lost even if stirring is stopped and/or temperature decreased.

This study points out the importance of hydrodynamics in bioreactors and highlights the nature of the aggregates resulting from the interactions between native and inactive enzymes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Enzyme inactivation; Lysozyme; Protein aggregation; Stirring; Kinetics

1. Introduction

Usually, enzymes have to be in stable and active conformation for their production and their storage. In order to produce active enzymes and to use them in industrial processes, we need to prevent their thermal and physical denaturation. To avoid the denaturation of proteins, it is important to elucidate the mechanisms of enzyme degradation so that stabilisation strategies can be specifically planed.

High temperature and mechanical stresses are the main causes enhancing enzyme inactivation in industrial systems. Thermal denaturation of enzyme is a well-studied phenomenon [1–3]. Less is known about mechanical constraints and physical denaturation of enzyme. Irreversible inactivation of enzyme by adsorption on liquid–liquid interfaces and dynamic gas–liquid interfaces have been reported [4–9]. Inactivation of lysozyme by stirring have been observed in our laboratory and reported in a previous article [10]. This work focuses on kinetics of lysozyme

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inactivation and properties of the enzymes aggregates formed in a stirred reactor. The system was designed to achieve, under controlled conditions, a rapid inactivation of lysozyme, chosen as the model enzyme.

2. Materials and methods

2.1. Proteins and chemicals

Chicken egg white lysozyme supplied by Sigma (St. Louis, MO) was used without further purification. Its specific activity was 52,000 Sigma U mg^{-1} protein. *Micrococcus lysodeikticus* dried cells were supplied by Sigma. All the salts were of analytical grade. Water was purified by reverse osmosis.

2.2. Enzyme activity and concentrations

The native enzyme concentration was determined by lysozyme activity measurements at 25°C by following the decrease in absorbance at 450 nm of a *M. lysodeikticus* 0.16 g l^{-1} suspension in 0.1 M potassium phosphate buffer, pH 6.2. One activity unit is equivalent to an absorbance decrease of 0.001 absorbance unit per minute.

Total enzyme concentration, meaning native and inactive enzyme, was measured according to the bicinchoninic acid (BCA) Protein Assay Kit (BCA Protein Assay Reagent, Pierce, France). Cu^{2+} was reduced to Cu^{+} by protein in alkaline medium and the coloured complex product formed with BCA exhibited absorbance at 562 nm, which is linear with increasing protein concentrations in the working range 0.02–2 g l^{-1}.

2.3. Reactor with Rushton turbine (Holland and Chapman type reactor)

This reactor has been assayed to discriminate the effect of interfaces from the one of stirring in lysozyme inactivation. The entire surface was made of glass, even the baffles avoiding vortex effect and air bubble formation. The internal diameter of the reactor and its height were 60 mm and the assays were done with a 170-ml lysozyme solution. The reactor was thermostated at 72°C by a double-coated envelope. The aqueous solution was stirred by a six-bladed impeller (diameter 20 mm) located 20 mm from the bottom of the reactor. This way of stirring in the range of the stirring speed does not introduce bubbles in the solution.

2.4. Inactivation experiments

0.1 M phosphate buffer supplemented by 1 M bromide salt, pH 6.5, was filtered through 0.2 μm Millipore GS filter, degassed and pre-equilibrated at 72°C. Lysozyme was solubilized at 5 g l^{-1} in the same solution at 25°C and then, a given volume, allowing the final concentration of lysozyme (0.1 g l^{-1}), was transferred into the reactor. Mechanical stirring was applied and the first sample was withdrawn from the solution. During inactivation, samples were periodically removed and immediately cooled in ice bath in order to stop the mechanical and thermal inactivation. The lysozyme activity and concentration were measured according to the previously reported methods.

2.5. Chemical treatments of aggregates

In order to dissolve aggregates to study their structure, enzyme solutions containing aggregates were chemically treated, either by denaturing agent or by denaturing and reducing agents. Denaturation of aggregates by guanidinium chloride (GuCl) 6 M or urea 8 M was carried out for 4 h at room temperature. To denature and reduce aggregates, 2-mercapto-ethanol (2-ME) 50 μl ml^{-1} was added into samples before incubation. Samples were then dialysed extensively against pure water and analysed by electrophoresis and chromatography.

2.6. Electrophoresis experiments

Gels of electrophoresis and materials were supplied by Sebia. Denaturant and non-denaturant electrophoresis were performed through agarose gels (Kits Hydragel). For sodium dodecyl sulfate (SDS) denaturant electrophoresis, proteins were stained with

Coomassie Brillant Blue, which provided high sensibility. Calibration curve obtained with standards proteins (MW-SDS-70L, Sigma) allowed the estimation of the relative molar weight of lysozyme.

2.7. Size-exclusion chromatography (SEC)

Apparatus for Fast Protein Liquid Chromatography (FPLC) was obtained from Pharmacia (Germany). Separation and quantification of lysozyme oligomers were carried out using SEC on a 1-m column (diameter 16 mm, XK 16/100). The stationary phase, prepared with cross-linked polyacrylamide beads Bio-gel P30 (BIORAD), had a 40,000-Da exclusion limit. Equilibration, calibration and elution of the column were running at room temperature, with a 0.15 ml min^{-1} flow rate of 0.1 M phosphate buffer supplemented by 1 M bromide salt, pH 6.5. The protein detection was monitored by the absorbance at 280 nm.

2.8. Light scattering

Static Light Scattering (SLS) analysis used a photogoniodiffusometer Fica (France) apparatus. Measurements were made at wavelength $\lambda = 546$ nm, at a 90° fixed scattering angle. Lysozyme solution was prepared at 1 g l^{-1} and filtered through 0.1 μm Millex filters. Non-aggregated and aggregated lysozyme molecules behaved as Rayleigh scatterers (i.e., particles much smaller than the wavelength λ).

3. Results and discussion

Lysozyme inactivation is enhanced by stirring. At 72°C, the stirred solution (740 rpm) of lysozyme at 0.1 g l^{-1} concentration is inactivated in 8 h (Fig. 1). Initially, there is a flat period of steady state followed by a sloping period of activity indicating lysozyme inactivation. The same lysozyme solution without stirring does not decrease so quickly.

3.1. Inactivation kinetic order

Kinetics studies in which the initial lysozyme concentrations were varied in the 0.1–1 g l^{-1} range showed similar behaviour. For stirred and unstirred solutions, the inactivation rates (AU/min^{-1}) are determined on experimental curves and plotted versus initial lysozyme concentrations, in logarithmic scales (Fig. 2). For unstirred solutions, all points are on a straight line the slope (0.97) of which represents the kinetic order. Consequently, lysozyme inactivation in unstirred solution is a first-order reaction. For stirred solutions, proportionality is also observed with a slope value near 1 (0.914) in the 0.1–0.8 g l^{-1} lysozyme concentration range. Nevertheless, the slope tends to deviate from 1 for high initial lysozyme concentrations, from 0.8 to 1.1 g l^{-1}.

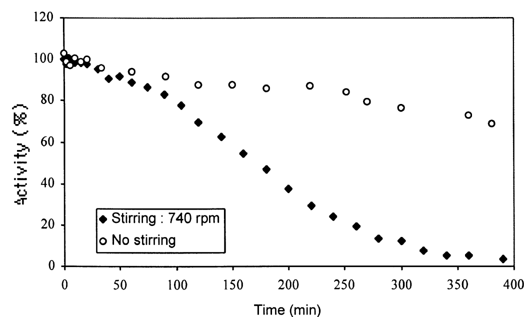

Fig. 1. Effect of stirring on lysozyme inactivation. The experiments were performed at 72°C with 0.1 g l^{-1} lysozyme solution in 0.1 M phosphate buffer (pH 6.5), supplemented by KBr 1 M.

These results suggest that lysozyme inactivation is a first-order kinetic reaction when initial lysozyme concentration is less than 0.8 g l^{-1}; but another mechanism, such as aggregation, disturbs this law at higher initial lysozyme concentrations. First-order kinetic is well in agreement with denaturation mechanisms previously described [11]. Although first-order kinetics are commonly observed during protein denaturation [12], in some cases, this kinetic order is in fact an apparent first-order kinetic concealing additional phenomena to denaturation-like peptide decomposition or protein aggregation [13,14].

As we observed, a first-order kinetic, the inactivation constant k (min^{-1}), is defined as the slope of the inactivation curve (when activity is plotted in logarithmic scale versus time). For the various initial concentrations of lysozyme, the inactivation constant k_1 observed in unstirred solutions is equal to 0.9×10^{-3} min^{-1}. In a 700 rpm stirred solution, the inactivation constant k_2 is 3.6×10^{-3} min^{-1}. The

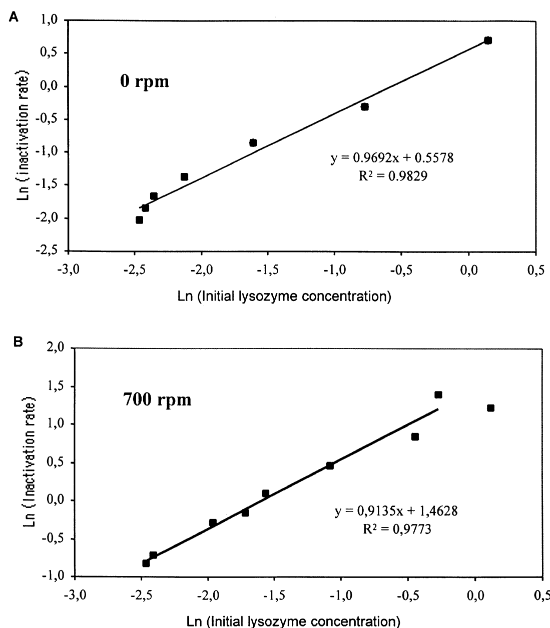

Fig. 2. Kinetic order determination for unstirred solutions (A) and for 700 rpm stirred lysozyme solutions (B) in phosphate–KBr buffer, pH 6.5. Temperature: 72°C.

ratio of these inactivation constants, k_2/k_1, is four. It represents the "destabilising effect" due to stirring.

3.2. Proportionality between inactivation constant and stirring speed

Inactivation experiments were studied in varying stirring speed, in the range of 0–700 rpm, with a 0.1-g l^{-1} initial lysozyme concentration. The inactivation constants increase with the stirring speed. More precisely, the inactivation constants determined for each experiment are proportional to w^3, with w the stirring speed (Fig. 3).

For a Rushton reactor, the following expression links the power brought by the impeller P (W) and the stirring speed w (s^{-1}):

$$P = N_p \rho w^3 D_A^5$$

where N_p is the a dimensional power number, ρ the fluid density (10^3 kg m^{-3}) and D_A the diameter of the impeller (0.02 m). In our geometry vessels, the power number N_p is constant and equal to 5.6 as we observe high Reynolds number (Re) [15]. Consequently, if P is proportional with w^3, the inactivation constant is proportional to the power brought by the impeller.

3.3. Lysozyme aggregation

During inactivation, cloudy solutions appeared and enzyme was found to form aggregates visible to the naked eye after total inactivation. Moreover, the shape of the inactivation curves — a flat portion followed by a slightly sigmoïdal curve — was consistent with aggregation.

For small particles [16] (until their radius are less than $\lambda/20$, no angular dependence is available), light scattering intensity (I_o) is proportional with the apparent molar weight of species (M_w) in solution:

$$\frac{1}{M_w} = K\frac{C}{I_o}$$

where K is a constant linked to the experimental conditions of apparatus and calibration:

$$K = 0.506.I_v^{90°}\left(\frac{500}{463}\right)\left(\frac{d\tilde{n}}{dc}\right)$$

($d\tilde{n}/dc$) is the refractive index increment (0.18 for lysozyme solutions) and C the initial lysozyme concentration (g ml^{-1}).

Light scattering experiments on lysozyme solution during the inactivation process showed increasing light scattering intensity after an initial grace period.

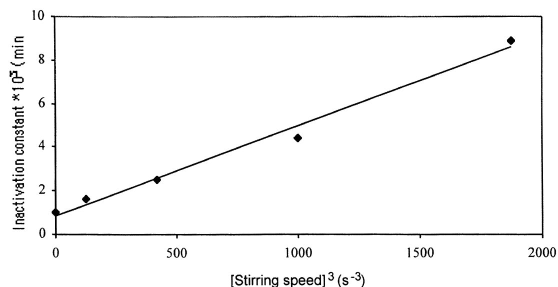

Fig. 3. Lysozyme inactivation depending power brought by the impeller. The experiments were performed at 72°C with 0.1 g l^{-1} lysozyme solution in 0.1 M phosphate buffer (pH 6.5), supplemented by KBr 1 M.

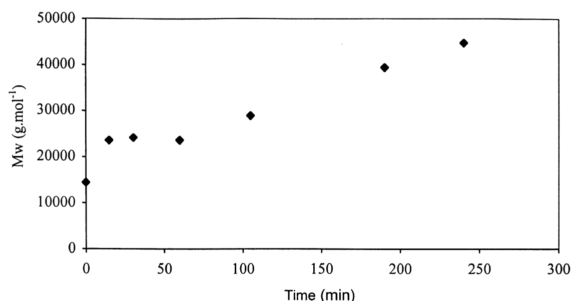

Fig. 4. Apparent molar weight versus time from static light scattering intensity experiments performed with a 1 g l^{-1} lysozyme concentration, in unstirred solution of phosphate buffer 0.1 M, with 1 M KBr, pH 6.5, temperature: 74°C.

The same curve shape is observed for the apparent molar weight plotted versus time (Fig. 4). The initial molar weight value (14,400 g mol^{-1}) is consistent with the native lysozyme molar weight. Early, before 20 min, the molar weight rapidly increases to steady at $M = 24{,}000$ g mol^{-1} until 60 min. After this steady state, molar weight slightly increases for 3 h, showing lysozyme aggregation. We notice that the curve shape of aggregation is correlated with the inactivation curve, so we suggest that lysozyme aggregation occurred simultaneously with enzyme inactivation. This result is in keeping with the denaturation process leading to an increase of surface hydrophobicity and to a protective effect by self-association of inactivated enzymes.

3.3.1. Aggregates structure

Aggregates removed from inactivated lysozyme solution after a 6-h stirring period (700 rpm, 72°C) were analysed qualitatively by electrophoresis and quantitatively by FPLC according to the methods described above. Gel electrophoresis in the presence of the SDS detergent was widely used to determine the molar weights of proteins whereas the use of non-denaturing gel electrophoresis allowed to analyse the protein conformations.

Aggregates were treated for chemical denaturation by concentrated solutions of 6 M guanidine hydrochloride (GuCl) or 8 M urea to disrupt non-covalent interactions before electrophoresis migration. These samples revealed three bands of protein (Table 1) the molar weights of which were evaluated by the calibration curve. The lightest and the most intense band matches the molar weight of the native lysozyme monomer. The two other bands match much less intense the molar weights of the dimer and the trimer of lysozyme, respectively. These two oligomers — dimer and trimer — are not disrupted by the denaturing treatment, so intermolecular covalent bonds link two or three molecules of lysozyme. These covalent bonds are suggested to be disulfide bridges. This hypothesis was confirmed by the electrophoresis analysis of the same samples treated for chemical denaturation and reduction by 2-mercapto-ethanol. Only one band appeared in the lysozyme monomer position, then intermolecular disulfide bonds were reduced. Consequently, aggregates are made up of monomers, dimers and trimers of lysozyme — the two last ones are linked by intermolecular disulfide bridges.

According to the molecular mechanism of irreversible thermal inactivation of lysozyme [17], disulfide exchange is probably responsible for the dimers' and trimers' formation. This reaction occurrs at neutral and alkaline pH in proteins containing cystine. Oxidation of free thiols groups (SH) of cysteine amino acids could also form disulfide bonds.

Analyses of aggregates without chemical treatment by denaturant electrophoresis revealed a large band corresponding to molar weights between 14,400 until 80,000 g mol^{-1} (Table 1). We suggest that

Table 1
Aggregates analysis by SDS-electrophoresis

Denaturant (SDS) electrophoresis	Molar weight (g mol^{-1}) estimated from calibration curve
Native lysozyme (0.5 g l^{-1})	12,100
Aggregates without chemical treatment	12,100 < ... < 80,000
Aggregates chemically denatured by GuCl 6 M or Urea 8 M	12,100; 24,400 and 34,700
Aggregates chemically denatured by GuCl 6 M or Urea 8 M and reduced by 2-mercapto-ethanol (50 μl ml^{-1})	14,600

aggregates are not totally denatured by SDS during migration and non-covalent interactions were not yet disrupted. Moreover, experiments by non-denaturant electrophoresis showed one band corresponding to the monomer and another band, higher and larger, attributed to the aggregates. These qualitative observations showed that non-covalent interactions maintain the aggregates.

The structure of the lysozyme aggregates is mainly made of weak and non-covalent interactions with some dimers and trimers linked by disulfide bonds.

Electrophoresis qualitative observations were finished off by a quantitative study using SEC. To determine the ratio of oligomers in the aggregates, the same sample as electrophoresis analysis was analysed on a size-exclusion FPLC column prepared according to the process described above. The sample was treated for chemical denaturation only (not reduction) and the chromatograph obtained showed three peaks, whereas only one peak was observed for native lysozyme.

The surface area of each of the three peaks observed represented the amount of species in the aggregates. The ratio (wt/wt) is: 77% of monomers, 18% of dimers and 5% of trimers.

3.4. Irreversibility of inactivation

Characterisation of stability and reversibility of lysozyme aggregates was studied, modifying two parameters, stirring speed or/and temperature of inactivation experiments. On the one hand, stirring was stopped at a given time, for instance 3 h 30 min, during thermal inactivation and experiment was carried on (Fig. 5). Activity lost was not restored after the stirring stopped. The flat period of steadying activity after the change time was significant with the irreversibility of lysozyme aggregates. On the other hand, the same inactivation experiment was started and at 3 h 30 min, temperature was decreased from 72°C to 25°C while stirring was maintained (Fig. 5). The result was similar, activity remained constant after the change time. This result confirms that aggregates are irreversibly formed, and even if one of the destabilising parameter is stopped, activity is irremediably lost. At least, for the same experiment, both temperature and stirring were modified at 3 h 30 min. Stirring was stopped and temperature was decreased from 72°C to 25°C. The same profile of the residual lysozyme activity was observed: the activity stopped to decrease and remained stable for several hours; the initial activity was not restored.

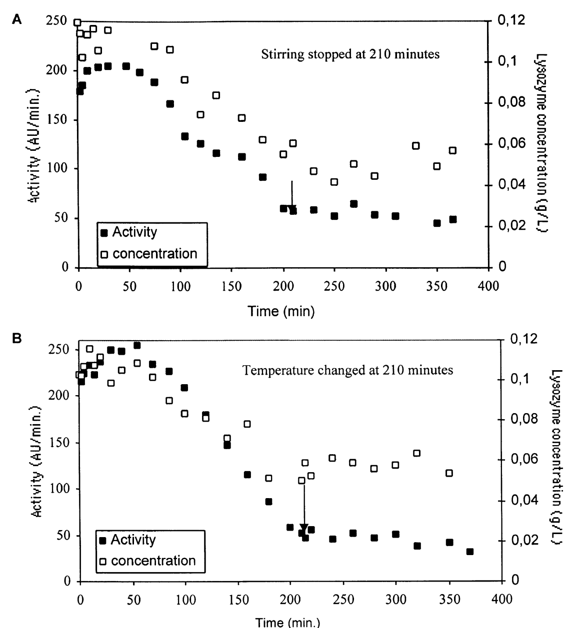

Fig. 5. Irreversibility of inactivated lysozyme aggregates with regard to stirring denaturation (A) and thermal denaturation (B) of 0.1 g l^{-1} lysozyme phosphate–KBr solutions initially thermostated at 72°C and stirred at 740 rpm.

For these three inactivation experiments, total enzyme concentration decreased slightly during inactivation and, like activity, steadied just after the change of parameter(s). The decrease of total enzyme concentration was due to the insolubility of the aggregates. Reactants of the chemical assay did not access into the agglomeration of proteins. This observation suggested that aggregates remained stable and insoluble after changing stirring and/or thermal conditions. We conclude that aggregation and inactivation of lysozyme are irreversible.

This result was observed whatever the change time, 1 h 30 min, 2 h 30 min and 3 h 30 min, then inactivated aggregates were irreversibly formed whatever their age and their size. Moreover, we obtained the same results for 1 g l^{-1} initial lysozyme concentration; irreversibility of inactivation is inde-

pendent of initial lysozyme concentration in the range of 0.1 to 1 g l^{-1}.

4. Conclusion

Mechanical stirring by a Rushton turbine in reactor vessel enhances lysozyme inactivation and aggregation. The inactivation follows a kinetic law of an apparent order 1 and the inactivation constant is proportional to the power brought by the impeller.

This result suggests that inactivation depends on collisions between native and inactive enzymes. These collisions are particularly efficient in order to turn the native enzyme into inactive ones as we have already reported [10].

Literature reported that inactivation could be induced by adsorption onto interfaces and especially onto hydrophobic interfaces [9,18,19]. With the reactor vessel used, we have reduced until a minimum (limited) the adsorption of lysozyme on air interfaces and on solid interfaces (the entire biorector is made up of glass, hydrophilic, and Teflon surfaces — hydrophobic — are avoided). Consequently, the lysozyme inactivation process studied is probably an interfacial mechanism involving the surface of enzyme themselves, as hydrophobicity of lysozyme increases during inactivation.

Once an enzyme has been denatured, the exposed hydrophobic surfaces tend to avoid interaction with the aqueous solvent. Then unfolded enzymes became insoluble and formed aggregates because of the amphiphilicity of the surface of the disordered enzyme [1]. The aggregates formed during inactivation are made up of 77% of monomers, 18% of dimers and 5% of trimers. Most of the interactions in the aggregates are non-covalent bonds, but dimers and trimers are linked by disulfide bonds. Stirring does not induce cleavage of enzyme molecules during inactivation but inactivated lysozyme aggregates were irreversible. The irreversibility was shown whatever the initial lysozyme concentration, the size of the aggregate and the denaturant parameter — stirring speed or temperature — stopped.

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