EFFECT OF I_E CRYSTAL WATER ON ENZYME ACTIVITY

LUDMILA CHISTOSERDOVA, MARY E. LIDSTROM

Originally published as *Physical, Chemical and Biological Properties of Stable Water Clusters,*
Proceedings of the First International Symposium. Reprinted here by permission of
World Scientific Publishing Company, 1998.

Activities of seven commercial enzyme preparations used in the laundry detergent industry (three proteases, two alpha amylases, one cellulase and one lipase) were compared using four preparations of I_E crystal water (D_S-20, D_S-50, D_S-93 and D_S-320). The only enzyme for which significant stimulation was observed was the cellulase (Celluzyme), which was stimulated up to 60% by D_S-50. Some enzymes showed significant inhibition with specific preparations of I_E crystal water. Our conclusion is that these preparations of I_E crystal water are not generally stimulatory to enzyme activity under the conditions used for the enzyme assays, but that moderate stimulation of cellulase apparently occurs with one of the preparations.

1. Introduction

Enzymes are the catalysts for cellular reactions. They usually increase reaction rates by at least a million fold and sometimes up to a factor of 10 to 12. Enzymes are active only when they have folded into the proper three-dimensional structure, which is determined by the sequence of the amino acid monomers and folding pathways.

A large global market exists for enzymes in commercial applications, which include food additives, detergents, production of small molecules such as alcohol and glucose, a variety of pharmaceutical applications, textile industry, etc. Therefore, stimulation of enzymatic activity with an additive has an obvious commercial benefit.

Stable I_E^{TM} water clusters (I_E water) were described recently by Lo[1] and Lo *et al.*[2] . Preliminary studies have revealed the biological activity of various I_E preparations *in vivo* and *in vitro*[3] . The purpose of this study was to look at the effect of different concentrations of D_S type preparation of I_E water on the activity of commercial enzymes used in the laundry detergent industry.

2. Methods

The tested enzymes represent five different commercially important classes: proteases (Alcalase, Esperase and Savinase.), α-amylases (Duramyl, Termamyl), cellulases (Celluzyme), and lipases (Lipolase). Additionally Soy Bean Lipoxidase (Sigma, St. Louis) was tested. The concentration of I_E water in activity assays was from 50 to 90 percent.

2.1 Protease Assay, Continuous (Alcalase, Esperase, Savinase)

The protease assay is based on measuring the formation of a colored compound as a result of the reaction of primary amino groups with a chromogen, trinitrobenzoic acid. Absorbency was measured at 420 nm. The assays were run at room temperature.

Reaction mixture contained in a final volume of 1 ml:
190 µl of 0.5 M Tris-HCl buffer pH8.5,
500 µl of 0.5% dimethylcaseine,
300 µl of 0.1% trinitrobenzoic acid,
10 µl of appropriately diluted enzyme.

2.2 Alpha Amylase Assay, Continuous (Duramyl, Termamyl)

The alpha amylase assay is based on determination of the release of a colored compound, 4-nitrophenol from a chromogenic alpha amylase substrate, 4-nitrophenyl-a-D-maltopentaoside. Absorbency was measured at 405 nm. The assays were run at room temperature.

Reaction mixture contained in a final volume of 1 ml:
970 µl of 50 mM K-Phosphate buffer pH7.0,
10 µl of 27 mM 4-nitrophenyl-a-D-maltopentaoside,
10 µl of α-glucosidase (Sigma), (5000U/ml)*,
10 µl of appropriately diluted enzyme.

* The addition of α-glucosidase to the assay mixture was not necessary, which might be due to contamination of the enzyme preparations with α-glucosidase.

2.3 Cellulase Assay, Discontinuous (Celluzyme)

The cellulase assay is based on determination of reducing capacity of low molecular weight glucose oligomers originating from carboxymethylcellulose in the ferricyanide reaction, by reversed colorimetry.

Reaction mixture contained in a total volume of 0.5 ml:
365 µl of 50 mM K-Phosphate buffer pH7.0,
125 µl of 0.75% carboxymethylcellulose,
10 µl of appropriately diluted enzyme.

The mixture was incubated at 40°C for 10 to 60 min. 250 µl of Stop Reagent (0.125 M Na_3PO_4 x 12 H_20, pH12) was added to stop the reaction. 250 µl of Ferry Reagent (0.16% $K3Fe(CN)_6$ in 1.4% Na_3PO_4) was added, and tubes incubated at 100°C for 10 min.

Absorbance was measured at 420 nm against individual blanks. The blanks were prepared as follows. Reaction mixture was prepared as above, and the Stop Reagent was added immediately. Then blanks were incubated at 40°C along with the samples.

2.4 Lipase Assay, Continuous (Lipolase)

The lipase assay is based on the hydrolysis of tributirin resulting in a pH change. The reactions were performed in a total volume of 50 ml at room temperature with constant stirring. Measurements of pH were performed using an Orion Research microprocessor pH/ millivolt meter 811.

The reaction mixture contained:

39 ml of H_20,

10 ml of Emulsifier,

1 ml of tributirin.

The pH was adjusted to 7.0 using 0.1 N NaOH. Then 10 µl of appropriately diluted enzyme was added. Immediately after the addition of enzyme, pH measurements were followed every 5 seconds for about 1 minute. Based on these time points, pH curves were built, and the linear segment of each curve was used for calculating the activity.

The emulsifier was prepared as follows:

NaCl, KH_2PO_4 and glycerol were dissolved in H_2O to the final concentration of, respectively, 1.7%, 0.04% and 54%. Under vigorous stirring, Gum Arabic was sprinkled into the solution to the final concentration of 0.6%.

2.5 Lipoxidase Assay, Continuous

The method is based on following an increase in absorbance at 234 nm (Sigma). The assays were run at room temperature.

The reaction mixture contained in a final volume of 1 ml:

90 µl of 1M Tris-HCl buffer pH8.5;

900 µl of Substrate,

10 µl of appropriately diluted enzyme.

Substrate was a 0.03% solution of linoleic acid, first emulsified with an equal volume of 100% ethanol, and then diluted with water to an appropriate volume with constant stirring.

3. Results and Discussion

Activities of seven commercial enzyme preparations used in the laundry detergent industry (three proteases, two alpha amylases, one cellulase and one lipase) were compared using four preparations of I_E crystal water (D_S-20, D_S-50, D_S-93 and D_S-320). For all seven enzymes, double distilled water was used for control measurements. In addition, a purified preparation of lipoxidase purchased from Sigma chemical company was tested in the same set of water preparations under two conditions, using optimal and non-optimal pH.

With the exception of the cellulase assay, which was discontinuous, enzyme activities were measured in continuous assays providing minimal assay error. All measurements were done at least in triplicate, and standard deviation was calculated for the average values obtained.

No pronounced effect was observed on the proteases (Alcalase, Esperase and Savinase). A slight inhibitory effect of D_S-50, D_S-93 and D_S-320 on Alcalase (in the range of 5-10%) was observed.

No pronounced effect was observed on alpha amylases (Duramyl and Termamyl). A slight inhibitory effect (in the range of 10%) on Duramyl by D_S-320 was observed.

D_S-20 caused slight stimulation of cellulase (Celluzyme) activity (in the range of 10-35%), while D_S-50 caused more pronounced stimulation of Celluzyme activity (in the range of 35-60%). The error for this assay is rather high, as a continuous cellulase assay is not available. Therefore, the range observed for this stimulatory effect was fairly wide.

No pronounced stimulatory effect was observed for lipase (Lipolase), while an inhibitory effect was observed with D_S-320 (up to 35%). Since the substrate for this reaction is highly hydrophobic, the apparent inhibitory effect may be due to changed substrate availability rather than altered enzyme activity.

In the case of purified lipoxidase (Sigma), a pronounced inhibitory effect of D_S-320 was observed for both optimal and non-optimal assay conditions, and it was more pronounced under optimal pH conditions (up to 74%). Under non-optimal

Table 1. Activities of commercial enzyme preparations in I_E crystal water compared to activities in double-distilled water (as percent of activity in double-distilled water)

Type of I_E	Type of Enzyme						
	Alcalase	Esperase	Savinase	Duramyl	Termamyl	Celluzyme	Lipolase
Control	100 +/-5	100 +/-3	100 +/-5	100 +/-6	100 +/-7	100 +/-5	100 +/-5
D_S-20	94 +/-12	86 +/-3	100 +/-7	97 +/-5	99 +/-12	122 +/-13	103 +/-7
D_S-50	88 +/-3	104 +/-5	108 +/-9	93 +/-7	105 +/-9	148 +/-13	87 +/-6
D_S-93	86 +/-7	93 +/-9	105 +/-8	91 +/-7	98 +/-7	100 +/-10	90 +/-4
D_S-320	88 +/-8	105 +/-12	98 +/-4	84 +/-4	112 +/-5	113 +/-14	65 +/-3

Table 2. Activity of purified lipoxidase (Sigma) in I_E crystal water compared to activity in double-distilled water (in percent)

Type of I_E water	pH 8.5 (optimal)	pH7.0 (non-optimal)
Control	100 +/- 5	100 +/-5
D_S -20	96 +/-3	75 +/-10
D_S -50	100 +/-7	80 +/-8
D_S -93	89 +/-14	84 +/-6
D_S -320	28 +/-2	77 +/-7

conditions, preparations D_S-20, D_S-50 and D_S-93 were also slightly inhibitory (in the range of 10 - 35%). As with lipase activity, the inhibitory effect on lipoxidase may be due to the hydrophobic nature of the substrate.

These results suggest that I_E water does not exert a general effect on enzymatic activity. This is an encouraging result for potential medical applications, because it may be possible to selectively increase activity of certain enzymes, while not affecting others. The results also indicate that activity of certain commercial enzymes, e.g. cellulases, can be stimulated with I_E preparations.

This preliminary study does not allow us to comment on mechanisms responsible for the described effects of I_E waters on the tested enzymes. However, it is unlikely that I_E structures have a direct effect on the active site of an enzyme, due to size constraints. The size of enzymes is on the order of 10 to 30 nanometers, which is comparable to the size of the smallest I_E structures[4]. The size of an enzyme active site is about an order of magnitude smaller. Enzyme molecules or their substrates (or both) might be absorbing on I_E structures, which might explain the observed phenomenon as a concentration effect. However, a more detailed study is required to confirm or to refute this hypothesis.

4. References

1. S.-Y. Lo, *Modern Phys. Lett.* B, 10 (19), 909 (1996).
2. S.-Y. Lo *et al., Modern Phys. Lett.* B, 10 (19), 921 (1996).
3. A.P. Sinitsyn *et al., Microbiology J.*, In preparation.
4. Berson, S.-Y. Lo, *Personal communication.*