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PRELIMINARY WORK IN OBTAINING SITE-DIRECTED MUTANTS OF HEN EGG WHITE LYSOZYME

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INTRODUCTION

Protein crystal growth studies are recognized as a critical endeavor in the field of molecular biotechnology. The scientific applications of this field include the understanding of how enzymes function and the accumulation of accurate information of atomic structures, a key factor in the process of rational drug design. NASA has committed substantial investment and resources to the field of protein crystal growth and has conducted many microgravity protein crystal growth experiments aboard shuttle flights. Crystals grown in space tend to be larger, denser and have a more perfect habit and geometry. These improved properties gained in the microgravity environment of space result largely from the reduction of solutal convection, and the elimination of sedimentation at the growing crystal surface. Shuttle experiments have yielded many large, high quality crystals that are suitable for high resolution X-ray diffraction analysis. Examples of biologically important macromolecules which have been successfully crystallized during shuttle missions include: lysozyme, isocitrate lyase, gamma-interferon, insulin, human serum albumin and canavalin. Numerous other examples are also available.

In addition to obtaining high quality crystals, investigators are also interested in learning the mechanisms by which the growth events take place. Crystallization experiments indicate that for the enzyme HEWL, measured growth rates do not follow mathematical models for 2D nucleation and dislocation-led growth of tetragonal protein crystals. As has been suggested by the laboratory of Marc L. Pusey, a possible explanation for the disagreement between observation and data is that HEWL tetragonal crystals form by aggregated units of lysozyme in supersaturated solutions. Surface measurement data was shown to fit very well with a model using an octamer unit cell as the growth unit. According to this model, the aggregation pathway and subsequent crystal growth is described by:

monomer <------> dimer <-------> tetramer <------> octamer <------> higher order

It is believed that multimer aggregation of lysozyme occurs by interaction at specific binding sites on the surface of the protein crystals. If the presence of discrete biding sites and the aggregation hypothesis is true, then it follows that the alteration of the binding site(s) should have significant effect on the measurements obtained during growth experiments.

Site-directed mutagenesis allows the specific alteration of proteins by replacement, deletion or addition of specific amino acid residues. This report outlines the approach for this strategy and the progress made thus far toward that end.

EXPERIMENTAL

I. Isolation of plasmid DNA. The HEWL gene was provided by the Kirsch lab (UC-B)

on a pAB24 circular double-stranded plasmid. This plasmid carries the gene for leucine synthesis, allowing for isolation and selection on leucine minus media. Figure 1. shows a map of this pDNA and the location of the genes and restriction sites.

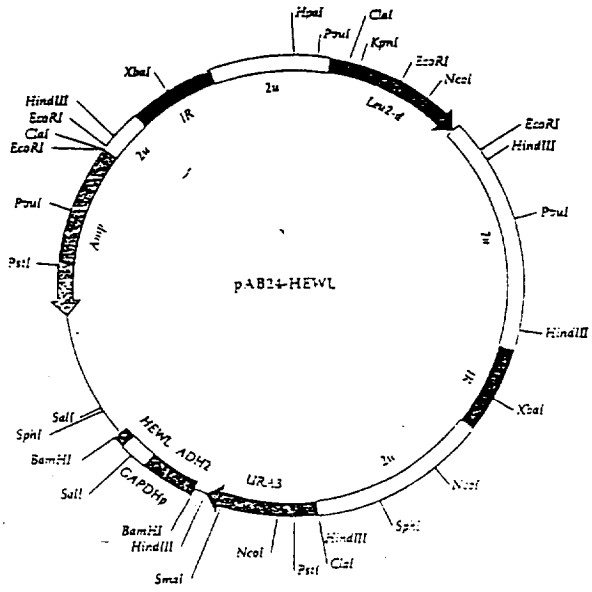

Figure 1.

The plasmid is contained in *E.coli* strain DH52. The plasmid was isolated and purified following three protocols:

a. Promega Wizard Mini-Prep Purification Systems. This approach was utilized to carry out small-scale purifications of pDNA (minipreps). An overnight 5 mL culture of *E.coli* in LB broth containing ampicillin was treated according to Promega protocol. Briefly, a cell pellet is lysed, neutralized and applied to a proprietary silica-based column resin. The plasmid DNA is then eluted with Wizard Column Wash Solution.

b. Plasmid DNA Purification by PEG Precipitation. Polyethylene glycol (PEG) precipitation is a rapid, reliable and convenient method for the purification of milligram quantities of highly purified pDNA. A culture of 500 mL in LB media containing ampicillin antibiotic is inoculated and grown overnight. the cells are harvested by centrifugation. The protocol calls for the initial preparation of a crude lysate pellet by alkaline lysis. Since plasmids are much smaller than chromosomal DNA, subsequent centrifugation permits separation of the plasmids from the chromosomal DNA and cellular debris. Although the resulting supernatant solution is enriched in plasmid, significant amounts of genomic DNA and RNA must be removed if the plasmids are to be used for mutagenesis. Finally, plasmid DNA is extracted from the supernatant with phenol/chloroform/isoamyl alcohol, followed by precipitation with PEG. This produces a DNA preparation free of any contaminants.

c. Promega Wizard Plus Maxipreps DNA Purification System. This is a fairly simple and rapid protocol for large-scale plasmid purifications. A 400 mL overnight culture will typically

yield 500 micrograms of pDNA. Plasmid yield will depend on several factors including volume of bacterial culture, plasmid copy number, culture medium and bacterial strain. An advantage of this system is that it does not require organic extractions or long overnight PEG precipitations. In brief, the protocol uses alkaline lysis to break open the cells. Genomic DNA and cell Debris is removed by precipitation and centrifugation. The Supernatant is applied to the Promega column and the pDNA is eluted by low speed centrifugation.

II. Identity and quantity of purified pDNA.

a. Identity. The double stranded plasmid DNA may be characterized by treatment with restriction endonuclease enzymes that recognize short DNA sequences and cleave at sites within or near these regions. We attempted to cleave the pAB24 plasmids by incubation with two restriction enzymes. SalI and BamHI were added to appropriate quantities of pDNA in restriction buffers and incubated for at least one hour at 37 ^{o}C. These incubations were then loaded onto a 1% agarose submarine gel for horizontal electrophoresis at approximately 10 volts/cm of gel. The restriction treatment should have produced DNA fragments that could be visualized by treatment with ethidium bromide at 254nm. Additionally, we experimented with another commercial fluorescent visualization (SYBER Green I Nucleic Acid Gel Stain from Molecular Probes, Inc.)system. Unfortunately, the restriction experiments appeared inconclusive. The digestions were incomplete. Possible sources of this apparent low enzyme activity include: 1) contamination with phenol extraction solvent 2) impure pDNA prep 3) denaturation of restriction enzyme. It was learned also that small amounts of BSA added to the restriction digest may improve enzyme performance.

b. Quantitation. The amount of plasmid DNA and the purity was assessed by UV absorption. A_{260} is a reliable measure of nucleic acid, while A_{280} indicates total protein. Although the A_{260} measurement cannot discriminate between pDNA, genomic DNA and RNA the ratio of A_{260}/A_{280} is used as an indicator of purity. A ratio of 1.8 to 1.9 indicates highly purified DNA. Protein contaminants will lower this number. Our pDNA preps had ratios ranging from approx. 1.1 to 1.9. The best prep was obtained by using the Promega Wizard Plus Maxipreps DNA Purification System (0.750 mg/mL).

III. Yeast Genetics.

a. Yeast cell cultures & electroporation. The aim was to transform liquid cultures of yeast cells (*S. cerevisiae*) with the plasmid DNA and induce the yeast to synthesis lysozyme enzyme. The yeast will excrete lysozyme to the liquid medium and then the protein may be purified. The purification of HEWL is routine business in the Pusey group, and should not be problematic. The concentration of lysozyme enzyme in the media will approach approx. 10milligrams/liter cell culture. Thermostatically controlled batch fermentations of yeast may be carried out. Alternatively, the Pusey lab may elect to have a single large culture prepared by contract at a facility such as the UAB Fermentation Center (Birmingham). Cultures of 500

mL of yeast cells were inoculated with 5 mL of overnight culture. The culture was grown in sterile, rich medium of yeast extract, peptone and dextrose (YPD) (30 ^{o}C, vigorous shaking). The cells were then harvested by centrifugation and made competent for uptake of plasmid DNA by electroporation. An *Invitrogen Electroporator II* instrument was used for transformation experiments. The protocol for yeast electroporation as recommended by *Invitrogen* was followed.

b. Isolation of transformed colonies. 100 microliters of the electroporated yeast cells were spread on plates containing solid selective dropout media. The media was prepared according to the recipe outlined in *Current Protocols*. Two types of media were used: a) complete b) minus leucine. The media also contained 1 molar sorbitol in some experiments. The addition of sorbitol to the solid media gave no visible improvement in colony survival and growth. The media also contained 8% glucose as according to the protocol of Shih et. al. A modification of the *Protocols* preparation included adjusting of the pH to 7.0. The specific protocol followed in this work is outlined:

1.3 g (plus or minus leu) dropout powder
1.7 g YNB (no aa, no ammonium sulfate)
5 g ammonium sulfate
300 mL water ----------adjust this 300 mL solution to pH 7/filter sterilize

80 g glucose
182 g sorbitol
20 g agarose
700 mL water ---------- 700 mL, autoclave

IV. Results of transformation experiments

Electroporation protocol is reported to result in transformation efficiencies approx. 10 to 100 times greater than chemical transformation methods. Repeated electroporation experiments did not yield any yeast transformants that survived on the (minus leucine) plates. Electroporated cells however, did grow and thrive nicely on complete media plates. These results indicate that the yeast did not take up the pAB24 plasmid via electroporation. Electroporation is expected to kill approx 70-80 percent of the treated cells. Cell counts taken before and after electroporation revealed that an expected fraction of the cells perished due to electroporation.

CONCLUSION AND ACKNOWLEDGMENTS

Molecular biology can be a powerful approach to studying the crystallization of biological macromolecules. Using the techniques of genetic manipulation, in principle, it is possible to alter the physico-chemical properties of enzymes and examine the effect on their crystallization behavior. Although this summer work did not achieve all goals hoped for at the outset, it did

serve as a beginning upon which continued work may build upon. The Pusey laboratory now has the a starting point to begin a more vigorous exploration into the application of molecular biology to the problem of protein crystallization.

This work was a result of teamwork with Laurel Karr (NASA) and Randy Wolfe (Visiting Summer High School Scholar). Their efforts toward making this work an educational and productive project is sincerely appreciated. Dr. Andy Britton (USRA) and Dr. Tom Prasthofer (NASA/ASEE Summer Faculty Fellow) are acknowledged for many helpful discussions, freely given instruction and personal time. A very special thanks is given to Dr. Marc Lee Pusey, my NASA host, who paid for this work and had the insight to understand its potential importance.

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