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Molecular Chaperone Tools for Use Against Neurodegenerative Diseases

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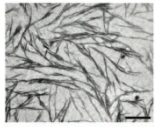
1. Abstract

A noted characteristic found in several neurodegenerative disorders, including Alzheimer's Disease, Parkinson's Disease, Huntington's Disease and bovine spongiform encephalopathy, is the accumulation of amyloid plaques in the brain. Amyloid plaques contain deposits of fibrillar aggregates of misfolded proteins that disrupt normal functionality in neurons. Certain variants of these misfolded proteins are self-replicating; these self-replicating amyloids are termed prions (for infectious protein). We are interested in how protein misfolding contributes to amyloid formation and how molecular chaperone proteins can change the formation of amyloid deposits. Chaperone proteins function by catalyzing the proper folding of other proteins, the refolding of misfolded proteins, and the disaggregation of protein aggregates. An example is the ATP-dependent chaperone protein Hsp104 from *Saccharomyces cerevisiae* (yeast). Overexpression of Hsp104 results in clearance of amyloid deposits of Sup35, a naturally occurring amyloidogenic protein. When expressed at low levels, Hsp104 is also required for propagation of Sup35 prion in daughter cells and prion inheritance.

Due to their amyloid disassembly activity, chaperone proteins may hold significant therapeutic potential for treatment of amyloid-associated neurodegenerative diseases. Therefore, we wanted to engineer a molecular chaperone protein with robust amyloid disassembly activity that can also degrade subunits to prevent them from reentering the pool of amyloidogenic precursors. We focused on ClpXP and Lon proteases from *Escherichia coli*. ClpX is a chaperone protein related to Hsp104 that is found in bacteria and the mitochondria of eukaryotes, which partners with the protease ClpP. Lon is also a related protease from the AAA+ domain family. We constructed plasmids for expression of ClpXP and Lon in yeast strains containing Sup35. We plan to test if expression of ClpXP or Lon in yeast is associated with clearance of the Sup35 prion phenotype, referred to as [PSI$^+$]. We also plan to perform random mutagenesis of ClpXP and Lon to engineer and select for chaperone-protease complexes that demonstrate enhanced clearance of amyloids in yeast via proteolysis. These studies will help to develop new molecular chaperone based tools that can be used promote *in vivo* clearance of toxic aggregates and amyloid deposits.

2. The [PSI$^+$] prion is a self-replicating amyloidogenic protein that forms a fiber

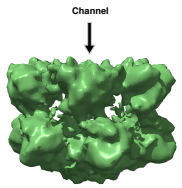


The Sup35 protein in yeast can aggregate into amyloid fibers

(Shorter and Lindquist, *Science*, 2004)

- *sup35* is a gene which encodes a translation termination factor, which is responsible for release of nascent proteins.
- Sup35 misfolds and forms amyloids *in vivo* and *in vitro*.
- Amyloids are formed from the conversion of normal proteins into misfolded prions; prions aggregate to form amyloid deposits.
- The presence of the Sup35p^{PSI+} conformation is referred to as the [PSI$^+$] phenotype and is associated with intracellular amyloid deposits (Liebman and Derkatch, *Journal of Biological Chemistry*, 1999).
- The [PSI$^+$] phenotype in yeast is used as a model system to investigate amyloid formation by prions.

3. Hsp104 is an ATP-dependent chaperone protein that disassembles amyloid fibers

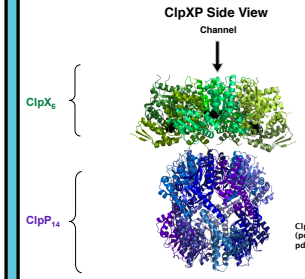


(Wendler, et al., 2007)

- Hsp104, a member of the Hsp chaperone family of proteins, is a hexameric AAA+ ATPase found in *Saccharomyces cerevisiae*.
- Hsp104 disaggregates, refolds and reactivates misfolded and aggregated proteins.
- Aggregated proteins are refolded by threading through the channel of hexameric Hsp104.
- This disaggregase activity is accomplished in conjunction with other Hsp co-chaperones, including Hsp40 and Hsp70. (Vashist, et al., *Biochemistry and Cell Biology*, 2010).
- Hsp104 is necessary for prion inheritance and propagation (Winkler, et al., *Journal of Structural Biology*, 2012).
- Elevated expression levels of Hsp104 cure the [PSI$^+$] phenotype in *S. cerevisiae* by refolding Sup35 from aggregates.

4. Bacterial chaperone proteases from *E. coli*

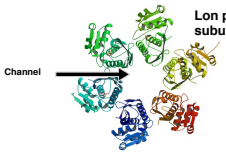
A) ClpXP is an ATP-dependent protease



ClpXP Model (pdb 3HWS, Glynn, et al., 2009; pdb 1TYF, Wang, et al., 1997)

- ClpXP is a comprised of the two subunits: ClpX and ClpP
- ClpX is an ATP-dependent molecular chaperone with unfoldase activity
- ClpX recognizes, unfolds and transfers substrates into the chamber of the bound protease ClpP
- ClpP forms a barrel-shaped protease with a central chamber
- ClpP degrades proteins in the chamber and releases small peptides
- ClpXP recognizes and degrades substrates with specific recognition signals

B) Lon is an ATP-dependent protease



Lon protease catalytic subunit top view

(Botos, et al., *Journal of Biological Chemistry*, 2004)

- Lon is an ATP-dependent serine protease found in *E. coli*, with a proteolytic barrel structure (Gur and Sauer, *Genes and Development*, 2008)
- Lon protease functions to degrade diverse protein substrates, as well as misfolded and aggregated proteins
- Lon protease is induced under stress conditions, such as heat or accumulation of abnormal proteins
- The presence of Lon is required for the prevention of aggregate build-up; Lon localizes to intracellular protein aggregates

5. Goals of Project

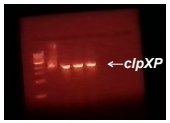
a) Express bacterial chaperone proteases in yeast

b) Test if bacterial chaperone proteases can cure the [PSI$^+$] phenotype by degrading amyloids

c) Generate ClpXP and Lon mutant proteins with enhanced amyloid recognition

6. Amplification and Cloning of Lon protease and ClpXP

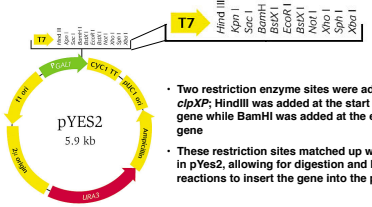
- To express the bacterial chaperone proteases in yeast, we isolated a genomic DNA from *E. coli*
- A set of primers, or small sequences of DNA with overlaps of the genes encoding for ClpXP and Lon, were designed to add restriction sites to the ends of either gene
- We amplified *clpXP* and *lon* gene sequences by polymerase chain reaction to add the desired restriction sites, enabling them to be inserted into a vector
- Amplification was confirmed via a agarose gel electrophoresis



Gel electrophoresis results for ClpXP cloning

7. ClpXP and Lon genes were cloned into yeast expression vector pYes2

A) *clpXP* genes were inserted between HindIII & BamHI



- Two restriction enzyme sites were added to *clpXP*; HindIII was added at the start of the gene while BamHI was added at the end of the gene
- These restriction sites matched up with sites in pYes2, allowing for digestion and ligation reactions to insert the gene into the plasmid

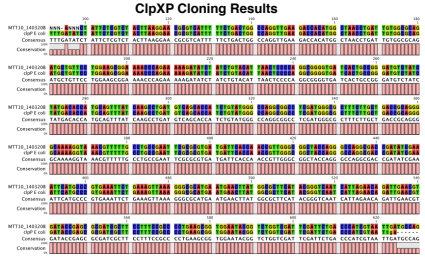
Forward primer (restriction site is HindIII)

atacaa**aagctt**ATGTCATACAGCGGC

Reverse primer (restriction site is BamHI)

ttgtat**ggatcc**tctTTATTCACCAGATG

ClpXP Cloning Results



B) *lon* gene was inserted between EcoRI & XbaI

- The restriction site EcoRI was cloned onto the start of the *lon* gene and XbaI was cloned onto the end of the gene
- These restriction sites matched up with sites in pYes2, allowing for digest and ligation reactions to insert the gene into the plasmid

Forward primer (restriction site is EcoRI)

aataag**gaattc**ATGAATCCTGAGCGT

Reverse primer (restriction site is XbaI)

aagaac**tctaga**ggaCTATTTTGCAGTCAC

8. Transformation of bacterial genes into yeast

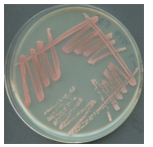
- Yeast was transformed with plasmids containing bacterial genes via electroporation.
- Yeast parental strains are both unable to synthesize uracil. The pYes2 vector contains the gene required for uracil synthesis.
- The absence of uracil in the growth media was used as a selective marker forcing the cells to maintain the pYes2 plasmids.

Strain	Plasmid	Genotype
783/4a PSI+	pYES2	*ura3-1, ade2-1*, PSI+
783/4a psi-	pYES2	*ura3-1, ade2-1*
783/4a PSI+	pHsp104[1]	*ura3-1, ade2-1*, PSI+ , *hsp104+*
783/4a psi-	pHsp104	*ura3-1, ade2-1, hsp104+*
783/4a PSI+	pClpXP	*ura3-1, ade2-1*, PSI+ , *clpXP*
783/4a psi-	pClpXP	*ura3-1, ade2-1, clpXP*

- *ade2-1* contains a nonsense allele which results in early termination of the transcript of an adenine biosynthesis gene

[1]pHsp104 expression vector 'pYS104' was constructed and described in Patino, et al., *Science*, 1996.

9. Visualizing the [PSI$^+$] Phenotype

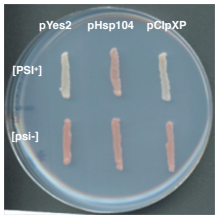


[psi-] phenotype

[PSI+] phenotype

- The red phenotype is caused by termination of the adenine biosynthesis gene *ade2*, which results in an accumulation of red pigment.
- The presence of [PSI$^+$] allows for a read-through of the nonsense codon in the *ade2-1* allele. This prevents the accumulation of the red pigment, which is a product of adenine biosynthesis, because Sup35 is unavailable.
- Clearance of [PSI$^+$] occurs when amyloids are removed, allowing native Sup35 to function in translation termination. This is detected by a color conversion from white to red colonies.

10. Test if expression of chaperones in yeast cures [PSI$^+$]



Transformed strains grown on media without uracil

- [PSI$^+$] cells expressing Hsp104 appear red, indicating that Hsp104 overexpression cures the [PSI$^+$] phenotype.
- [PSI$^+$] expressing ClpXP appear white, similar to the vector control, suggesting that this strain is not cured of [PSI$^+$].
- These results suggest that ClpXP does not recognize and degrade the [PSI$^+$] amyloid

11. Conclusions and Future Studies

- Expression of Hsp104 from a plasmid cures [PSI$^+$] in yeast, via disassembly of amyloid fibers
- ClpXP expression is not sufficient to cure [PSI$^+$] in yeast

Future Work:

- Perform random mutagenesis of *clpXP* to generate mutants with enhanced recognition of [PSI$^+$]
- Screen random mutants for enhanced prion curing by monitoring for a color change from white to red
- Express Lon in [PSI$^+$] and [psi-] yeast strains to determine if Lon degrades amyloids, then perform random mutagenesis of *lon* to generate and screen mutants with enhanced recognition of [PSI$^+$]

12. Acknowledgements

This research was supported by the Undergraduate Research Initiative at the University of Rhode Island.