Final Project Report of UGC Major Research Project MRP-MAJOR-BIOT-2013-32040

"Stability and folding mechanism of alpha amylase"

for the period 01 .07.2015 to 30.06.2018

F.NO 43-82/2014 (SR)

By

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Annexure – IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project
Stability and folding mechanism of alpha amylase

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR

Dr. Rajesh Mishra, Associate Professor, School of Biotechnology, Jawaharlal Nehru University, New Delhi-110067

3. NAME AND ADDRESS OF THE INSTITUTION
Jawaharlal Nehru University, New Delhi-110067

4. UGC APPROVAL LETTER NO. AND DATE
MRP-MAJOR-BIOT-2013-32040, F.No.43-82/2014 (SR), Dated 28 Sep 2015

5. DATE OF IMPLEMENTATION
01-07-2015

6. TENURE OF THE PROJECT
01-07-2015 to 30-06-2018 (3 years)

7. TOTAL GRANT ALLOCATED
Rs. 13,72, 500

8. TOTAL GRANT RECEIVED
Rs. 12,42,500

9. FINAL EXPENDITURE
11,76010

10. TITLE OF THE PROJECT
Stability and folding mechanism of alpha amylase

11. OBJECTIVES OF THE PROJECT: The project aims to understand the mechanism of the stability of alpha amylases from *Bacillus amyloliquefaciens* (BAA) and *Bacillus licheniformis* (BLA) with the following specific objectives:

1. Unfolding kinetics by Urea and GdmCl.

2. Effect of polyols during thermal unfolding.

3. Effect of polyols in the refolding and prevention of aggregation.

12. WHETHER OBJECTIVES WERE ACHIEVED **YES**

(GIVE DETAILS)

Objective 1: The work in this objective involves understanding the stability and folding of BAA and BLA by unfolding in the presence of GdmCl and urea. The experiments were focused mainly on the differential effects of urea and GdmCl on the unfolding and the refolding of BAA and BLA. These studies gave insight into the nature of forces which are responsible for the stabilization of these proteins.

Objective 2: Second objective deals with the stability of BAA and BLA in the presence of cosolvents like polyols. In this study we have used a series of polyols including glycerol and sorbitol for improving the stability of both the enzymes. Mechanistic analysis reveals that sorbitol and glycerol has significant role in increasing the thermal stability of bacterial α-amylases.

Objective 3: Polyols have been successfully used in the refolding and prevention of aggregation for several aggregation prone proteins. However very few studies have focused on understanding the differential effect of polyols during mesophilic and thermophilic protein refolding. We have successfully used a series of polyols which has significantly prevented the aggregation of alpha amylases during their refolding and also increased the refolding yield.

13. ACHIEVEMENTS FROM THE PROJECT

α-amylases catalyze the hydrolysis of α, 1-4 glycosidic bonds in most of the polysaccharides. This catalytic power of α-amylase makes it one of the most potential candidates for enzyme industry. More than ninety percent industrially important α-amylases are obtained from bacterial sources. In this study, we have selected homologous bacterial α-amylases (BAA, *Bacillus amyloliquifaciens* and BLA, *Bacillus licheniformis*) to understand the comparative stability and folding of these industrially important enzymes. The current project deals with comparative analysis of two bacterial α-amylases which differ from each other in terms of their thermal stability. The different perspectives of this study are unfolding and stability of α-amylases, effect of cosolvents on thermal stability of α-amylases and refolding of bacterial α-amylases in the presence of cosolvents. The detailed unfolding pathway of both α-amylases in the presence of chemical denaturants was thoroughly investigated by equilibrium and kinetic unfolding experiments. Our study is focused on the comparative analysis of stability and unfolding of

BAA and BLA, to ascertain the origin of differential stability of mesophilic (BAA) and thermophilic like (BLA) α-amylases. Majority of previous studies regarding the comparative thermal stability of globular proteins were revolving around the amino acid sequence and structural comparison only. In this study, in addition to amino acid sequence analysis and structural comparison studies, we have also tried to understand the basis of the different stability in terms of their differential unfolding pathways in the presence of chemical denaturants. Our results shows that in GdmCl and urea, BAA exhibit less resistance to the unfolding in comparison to BLA under similar experimental conditions. This indicates that the prevalence of ionic, hydrophobic and hydrogen bonding in BLA is more prominent than the BAA which results in the higher stability of BLA. In the second objective of this project, a series of polyols has been employed for the enhancement of thermal stability of α-amylases. Among the various polyols, glycerol and sorbitol were found to be the most effective. The relative increase in the T_m of α-amylases in the presence of both polyols is almost the same, but the reason behind this outcome is still not known. This might be due to the similarity in the amino acid sequence and structural properties of two α-amylases. In the third objective, we have explored the polyol effects on the refolding yield of BLA. Similar to thermal stability of α-amylases, refolding of the same was successfully improved by the glycerol and sorbitol. Increase in the refolding yield was more for BAA than BLA which might be due to higher aggregation propensity of BLA than BAA which results in the low refolding yield at the same protein concentration. The findings of the present project may further provide new insights into the stability and the mechanism of unfolding/refolding of bacterial α-amylases and also add to the knowledge in the field of cosolvents effect on thermal stability and refolding of α-amylases.

14. SUMMARY OF THE FINDINGS (IN 500 WORDS)

Bacterial α-amylases are the leading industrial enzymes among all the members of amylolytic enzyme family. They generally catalyzes the hydrolysis of internal α, 1-4 glycosidic linkage in most of the polysaccharides, convert them into simpler form which can be used for wide industrial applications. Stability of industrial enzymes is one of the most desirable qualities for their application in industry. In the present research project, we have worked with homologous bacterial α-amylases BAA and BLA which have more than 80% sequence and structural similarity. In spite of very high sequence identity in their amino acid composition and structural

similarity, there is a significant variability in the conformational stability of both enzymes. What is the origin of differential conformational stability in bacterial α-amylases? In the first part of this project we have answered this question by comparing the unfolding pathway of α-amylases in the presence of chemical denaturants. Protein unfolding in urea and GdmCl provides the quantitative information about the relative contribution of hydrophobic and ionic interactions in the conformational stability in the form of $\Delta G_{unf,}$ as the urea mainly breaks hydrophobic interactions while GdmCl perturb both hydrophobic and ionic interactions during the unfolding process. According to our results, BLA has greater stabilization of its native conformation by a combination of hydrophobic and ionic interactions rather than BAA.

We have used a series of polyols for improving the thermal stability of α-amylases. Various cosolvents are known to stabilize protein conformation in aqueous environment. In general most of the cosolvents exert their effect by making native protein more compact thus preventing its partial or complete unfolding. Polyols are most widely used cosolvents for enhancing the stability of the proteins. The current study is based on the relative effect of various cosolvents on the thermal stability of bacterial α-amylases. The extent of stabilization by cosolvents depends upon the physico-chemical properties of protein and cosolvent and also on the concentration of the cosolvent. So it is quite interesting to find out the differential behavior of various polyols in respect of thermal stability of two homologous α-amylases, which have different physico-chemical properties. Among all the polyols being used in this particular study, sorbitol and glycerol significantly increases the thermal stability of bacterial α-amylases.

The refolding of α-amylases was performed in the presence of various polyols. The efficient refolding of multidomain and multimeric proteins always remains a major challange during recombinant protein production. During refolding of multidomain and multimeric proteins, aggregation is the main reason behind their low refolding yield. It has been reported that bacterial α-amylases undergo irreversible unfolding transition during chemical and thermal denaturation. We have explored the polyol series for improving the refolding of α-amylases but only sorbitol and glycerol were found to be effective for BAA, while none of them seems to be effective for BLA refolding. This contrasting behavior of polyol effect on α-amylases might be explained by the fact that BLA being more stable and therefore having higher tendency towards

aggregation during refolding than BAA. This study would provide new insights into the protein stability and refolding related research on multidomain proteins in general.

15. CONTRIBUTION TO THE SOCIETY (GIVE DETAILS)

Alpha amylase is an industrially important enzyme therefore findings of the project will helpful for the industrial applications. The project helped in man power training in the form of one continuing Ph.D. student. One Master thesis has been completed on the project topic. The UV-Visible spectrophotometer procured under this project is used for research and training the students.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT

Yes. Mr. Aziz Ahmad has been working on this project and enrolled in the Ph.D. programme of School of Biotechnology, Jawaharlal Nehru University, New Delhi.

17. NO. OF PUBLICATIONS OUT OF THE PROJECT

Conference Presentation:

Aziz Ahmad and Rajesh Mishra, UNFOLDING THE STABILITY OF ALPHA AMYLASE. Poster Presentation in the Annual symposium of Indian Biophysical Society (IBS), 22-03-2017 to 25-03-2017 held at IISER Mohali.

Aziz Ahmad and Rajesh Mishra, Understanding the Stability of Alpha Amylase from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. Poster Presentation on National Science Day "Science and Technology for Sustainable Future" held on 28-02-2018 at JNU, New Delhi

Aziz Ahmad and Rajesh Mishra, Conformational Stability and Folding of Bacterial α-Amylases. Poster Presentation on National Science Day "Science for the People and the People for Science" held on 28-02-2019 at JNU, New Delhi

Manuscripts under Preparation:

1. Aziz Ahmad and Rajesh Mishra: A comparative analysis of the stability and folding of *Bacilus amyloliquifaciens* alpha amylase (BAA) and *Bacilus licheniformis* alpha amylase (BLA).
2. Aziz Ahmad and Rajesh Mishra: Improving the refolding yield of *Bacilus amyloliquifaciens* alpha amylase (BAA) and *Bacilus licheniformis* alpha amylase (BLA) in the presence of polyols.

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(PRINCIPALINVESTIGATOR)

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प्रतिहस्ताक्षर/Countersign

2/9/2019

(REGISTRAR/PRINCIPAL)

(Seal)

28/8/2019
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