

Step by Step Preparation and Optimization of Lysozyme Hydrophobic Ion Pairing Complex

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Introduction

Clinical applications of therapeutically attractive peptides/proteins have been delayed due to several challenges including chemical and physiological barriers. Different approaches were employed to overcome these barriers, for example, by improving the encapsulation of proteins into nanocarriers by hydrophobic ion pairing (HIP) complexation. In this approach, ionizable functional groups of proteins are engaged in electrostatic interactions with a counterion containing one or more hydrophobic moieties resulting in an increased hydrophobicity of such hydrophilic macromolecules and therefore, improving their encapsulation into nanocarriers using, for example, emulsification techniques (Ristroph et al., 2021). A naturally occurring cationic single chain polypeptide lysozyme (LYZ), which is known for its antimicrobial activity, has been commonly employed as a model peptide in nonparenteral dosage forms. In different studies, HIP complex of LYZ with surfactants such as sodium dodecyl sulphate (SDS) was prepared. Variable complexation efficiencies were obtained in these reports as different levels of factors affecting the complexation process were used. For instance, variable pH values of the used mediums were proposed for the optimum complexation (Asuman Bozkır, 2015; Yoo et al., 2001). This study aimed to prepare and optimize the HIP complex of LYZ and SDS with the help of the quality by design (QbD) approach. Risk assessment (RA), as one of the QbD tools, was applied to identify and prioritize different parameters influencing the preparation of such complex.

Materials and methods

Lyophilized powder of LYZ, SDS and lyophilized cells of *Micrococcus lysodeikticus* bacteria were purchased from MedChem Express, EGIS and Sigma-Aldrich, respectively.

Risk assessment based on published reports and previous experience and with the aid of LeanQbD Software was used to identify and prioritize different factors affecting the preparation of the HIP complex.

Solutions of LYZ and SDS in PBS adjusted to the required pH values were mixed and added into Eppendorf tubes and the final peptide concentration was 2 mg/ml. The preparations were centrifuged, and the precipitated complex was dried after removal of the supernatant.

The optimum molar ratio of LYZ to SDS at pH 4, 6, 8 and 10 was determined using Műtek PCD02 particle charge detector (PCD) and it was obtained when a zero-charge point of the streaming potential was reached.

0.1, 0.5, 1.0 and 1.5 M NaCl solutions in PBS were added to dried HIP complexes and incubated for 24, 48, 72 and 168 hours. The enzyme activity and dissociated LYZ amount were determined after centrifugation of these solutions.

The complexation efficiency (%) and the dissociated LYZ (%) were indirectly determined by measuring the concentration of the free LYZ in the supernatant using UV spectrophotometric method at $\lambda_{max} = 281$ nm.

The enzymatic activity (%) of the dissociated LYZ in comparison to a reference solution was measured using lyophilized *Micrococcus lysodeikticus* bacterial suspension in PBS adjusted to the pH 6.24 at 25°C. The

enzymatic assay is based on the spectrophotometric rate determination of absorbance at $\lambda = 450$ nm vs time.

Results and discussion

Risk assessment: Firstly, the quality target product profile (QTPP) and the critical quality attributes (CQAs) were determined. Increased lipophilicity and preservation of the enzymatic activity were set as QTPP and high values of complexation yield, enzyme stability and dissociable enzyme were the CQAs. The effects of the critical process parameters (CPPs) and critical material attributes (CMAs) were identified using fishbone diagram as one of the RA tools (Fig.1). This was followed by the prioritization of these factors in a form of Pareto chart (Fig. 2) constructed based on the severity score. From this assessment, peptide/protein type, surfactant type, protein: surfactant molar ratio and pH of the medium were identified as high-risk factors. As LYZ and SDS are protein and surfactant, respectively, in this study, the investigations were focused on the latter two factors.

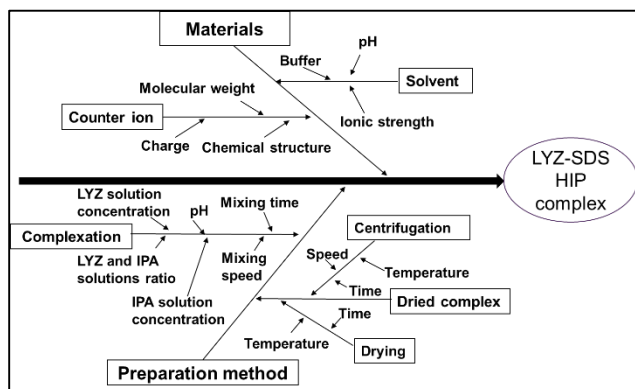


Fig. 1. CPPs and CMAs affecting the HIP preparation

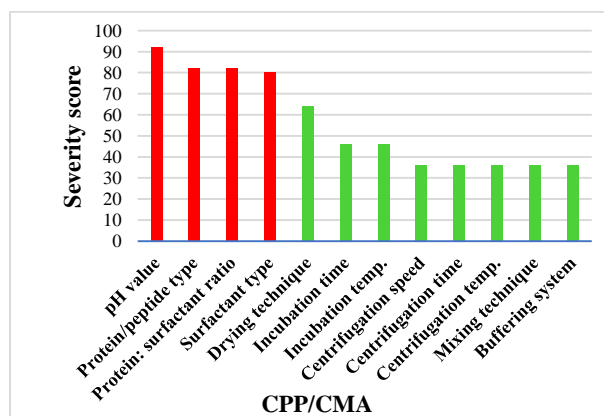


Fig. 2. CPPs and CMAs severity score of HIP preparation

Determination of the optimum molar ratio: The obtained optimum LYZ: SDS molar ratios at pH 4, 6, 8 and

10 were 1:14.57, 1:9.25, 1:7.95 and 1:7.50, respectively. As the pH increases the SDS ratio required to neutralize the ionized groups decreases. These ratios were employed to formulate the HIP complex and high complexation efficiencies were achieved at pH 4, 6 and 8 (99.0%, 98.5% and 95.0% respectively), and a lower value of 66.0% at pH 10. Consequently, pH 10 was excluded from further investigations.

Enzymatic activity and amount of the dissociated LYZ: The dissociable amount of the complexed LYZ and its enzymatic activity were determined at pH 4, 6 and 8. The maximum dissociable amounts from pH 8 and 6 complexes were 28% and 17% respectively, while only 5% was recovered from pH 4 complex. Factorial ANOVA analysis showed that all effects, namely pH, incubation time and NaCl solution concentration, were significant on both the enzymatic activity and dissociation. Generally, higher NaCl concentration, longer incubation and higher pH resulted in higher dissociation and enzymatic activity.

Conclusion

Quality by design principles were employed to develop and optimize LYZ HIP complex. Variable ratios of LYS:SDS were required to achieve high complexation efficiency at different pH values and pH 8 was the best choice.

References

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