Characterization and Stabilization of Pepsin- A Systematic Approach in Formulating Therapeutic Proteins

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Summary

The major hindrance to the development of protein therapeutics is their physical and chemical instability as well as the propensity to form aggregates and as a result poor storage capability, immunogenicity and loss of bioactivity. The objective of this study is to observe the stability of our model protein pepsin at different physicochemical conditions (pH, temperature, ionic strength, exposure to air-water interface) and to evaluate the effect of added stabilizer and their abilities to retain bioactivity. Pepsin activity has been measured by using haemoglobin as a substrate and measuring its UV absorbance. Protein aggregation before and after excipient stabilization, has been observed by dynamic light scattering (DLS) and by turbidity measurement. To study the denaturation temperature and the enthalpy of heat denaturation, the protein has been studied by using differential scanning calorimetry (Nano-DSC). Biacore ® technology (Surface plasmon resonance analysis) was used to study the Antibody binding capacity of pepsin. Our results suggest that pepsin has high enzyme activity at pH 2 and the stability of pepsin in solution is highly affected by higher pH (pH 6.4) and by agitation (exposure to air-water interface) but not by change of temperature (up to 37 ºC). Formation of aggregates in solution is not directly associated with the loss of enzyme activity. Polysorbate 80 has been proved to be the excipient of choice to stabilize pepsin in solution at higher pH and upon agitation (exposure to air-water interface) but Sucrose and β-Cyclodextrin failed to stabilize pepsin in solution.
Introduction

General Introduction to therapeutic proteins

The importance of Protein and peptide drugs for the diagnosis, prevention and treatment of life threatening diseases is increasingly recognized. Protein drugs are used in Diabetes (e.g. insulin, Somatokine), Cancer (e.g. monoclonal antibodies), HIV/AIDS (e.g. Interferon), Heart attacks, Strokes, Cystic fibrosis, (e.g. Enzymes, Blood factors), Dwarfism (Growth factors) etc. Research is also going on for antimicrobial peptides (Strömstedt et al. 2008).

These therapeutic proteins possess a lot of benefits over the small molecule organic drugs. Protein drugs are metabolized to endogenous amino acids and therefore, generate fewer side effects (Bryant Miles, 2003); whereas, the small organic molecules are metabolized to active or inactive metabolites and trigger side effects. However, there are problems regarding the stability as well as immunogenicity of protein drugs. Unlike the small organic molecules, proteins have primary, secondary, tertiary and quaternary level of structures. Therefore, the degradation of protein and peptide are not a single-step reaction (Ajay K. Banga, 2nd edition 2006). Proteins are highly susceptible to denaturation and aggregation. Aggregates in protein therapeutics are totally undesirable because small aggregates may cause immunogenic reaction and particulates may lead to problem even on administration. (Cromwell et al. 2006).

Thus the aggregation of proteins during the formulation, shipping, storage leads to reduced biological activity, and can give rise to adverse reactions including immune responses. Therefore, to assure the integrity and stability of protein in the dosage form during manufacturing, administration as well as during shipping and storage is the prerequisite for the approval of marketing of Protein drugs (Ajay K. Banga, 2nd edition 2006). To devise suitable way to prevent protein aggregation is a great challenge.

Structural features of protein

As we know, Proteins are complex molecules with various functional groups. They possess primary and delicate secondary and tertiary structures. The primary structure is simply the amino acid sequence where the amino acids are linked to each other via peptide bonds. This primary structure is determined by the nucleotide sequence of Deoxyribonucleic acid (DNA). Thus the primary structure of a protein is determined
genetically. The peptide bonds are so strong that they are not usually broken by the environmental factors (e.g. temperature, pH, agitation etc.). This peptide chain through hydrogen bonding interactions forms the secondary structure. Two most common secondary structures are $\alpha$-helix and $\beta$-sheet, while others are $\beta$-turn, random coils and small loops. Through this hydrogen bonding in secondary structure protein neutralizes its polar atoms. The secondary structures are then folded into three dimensional tertiary structures that determine the function of the protein. The specific folding of protein is determined by its amino acid sequence of the primary structure. The binding interactions which are involved in stabilizing the tertiary structure are covalent interaction, e.g. Disulfide bridges and noncovalent interactions, e.g. hydrogen bonding, salt bridges, hydrophobic interactions etc. In the tertiary structure, the hydrophobic side chains are buried inside the protein and the hydrophilic groups are exposed to the surrounding solvent. The quaternary structure is a well-defined structure which is formed by the self association (by noncovalent bindings like hydrophobic interactions, hydrogen bonds, or vander waals interactions) of subunits or monomers (tertiary structure of protein). Specific protein structures are essential for its specific activity. State of the primary and secondary structure ultimately affects the tertiary structure of the protein. Surrounding environmental factors also influence the protein structures (Ajay K. Banga, 2nd edition. 2006).

**Instability problems of therapeutic proteins**

The degradation of peptides and proteins can occur through several physical and chemical pathways. Physical instability may include aggregation, denaturation, precipitation, or adsorption to surfaces. While the chemical instability is due to hydrolysis, deamidation, oxidation, disulfide exchange, $\beta$-elimination, and racemization (Manning et al., 1989).

Denaturation of proteins can be reversible or irreversible which typically involves unfolding of the protein (disruption of higher-order structure). In the denaturation process the protein undergoes a sharp transition from its native structure to the unfolded state at the melting temperature $T_m$. The denaturation of protein is followed by the formation of protein aggregates. Denaturation of protein leads to the exposure of buried hydrophobic amino acids to the aqueous phase. These hydrophobic residues then undergo self association that leads to the formation of protein aggregates. The aggregates can be soluble or may be insoluble. The macroscopic equivalent of the aggregates is known as
the precipitates. During formulation, handling, storage, or administration of protein or peptide drugs, any type of energy input from the microenvironment of these proteins and peptide can lead to denaturation, as well as aggregation. Therefore, the factors, which can cause denaturation of protein and ultimately formation of aggregates, involve extremes of pH, thermal stress, presence of denaturing chemicals, shear stress (exposure to air-water interface) etc.

Extremes of pH and temperature of the environment can alter the intramolecular attractive forces (hydrogen bonding, dipole-dipole interaction, hydrophobic interaction etc.) of the protein (e.g. enzyme). This can alter the active site of the enzyme rendering it inactive. Agitation may expose the protein to the air-water interface. At the interface protein unfolds and exposes the hydrophobic amino acids which are normally buried inside the protein. This cause denaturation and/or aggregation of protein (Ajay K. Banga, 2nd edition. 2006).

**How to stabilize protein**

In order to stabilize proteins or to prevent denaturation and aggregation of protein or peptide drugs in the formulation, proteins can be engineered or the microenvironment of the protein in the formulation can be controlled. To control the environmental factors of protein formulation, proteins can be lyophilized or excipients can be used. The commonly used excipients for stabilization include, sugars and polyols, amino acids, amines, salts, polymers, surfactants etc. But the conditions influencing protein aggregation are dependent on unique structural and physicochemical properties of proteins; therefore, the choice of suitable excipient to prevent aggregation will also be highly protein specific. (Poon et al. 2005)

Electrolytes are known to exert stabilizing effects on proteins. The salts which are used for this purpose include, NaCl, Na₂SO₄, CaCl₂, MgCl₂ etc. Due to increased surface tension of water, salts are excluded from the protein-water interface and become available to stabilize the protein. Again salts can also form weak bonds to the charges on the protein surface. Depending on the nature of ions the balance between these two forces are changed. However higher concentration of salt can lead to salting out effect or decreased solubility of protein (Middaugh et al. 1992).

Polyoxyethylene sorbitan monooleate or Polysorbate 80, commercially known as Tween 80, is a surfactant. Its molar mass is 1310 g/mol. It is the most widely used
surfactant in the pharmaceutical field. The exact mechanism of protein stabilization by surfactant is not clear. It is believed that surfactants are preferentially adsorbed at the interface and prevent protein to be adsorbed at the interface and to unfold. There is also proof that surfactants interact directly with the protein and reduce its available hydrophobic surface area for self association of the protein. However, Tween 80 may accelerate the degradation of protein as it may contain a trace amount of peroxide (Ajay K. Banga, 2nd edition. 2006). Surfactant is active above its critical micelle concentration (CMC). The CMC of Tween 80 is 0.012 mM (Chou et al. 2005).

Cyclodextrins are also used to stabilize protein. They are carbohydrates but their mechanism to stabilize protein is different from that of other carbohydrates. They are capable to solubilize, stabilize and to promote the delivery of protein and peptide drugs (Brewster et al.). Cyclodextrins can be α, β and γ. But the most commonly used Cyclodextrin is the β-Cyclodextrin. Its molecular weight is 1315 Dalton. It is a cyclic oligosaccharide with seven glucopyranose units. It can form inclusion complex with proteins by using its hydrophobic cavity but the problem is that β-Cyclodextrin has very low water solubility (Duchene et al. 1990, Harada et al. 1993).

\[
\begin{align*}
\text{CH}_3\text{CH} &= \text{CH (CH}_2\text{)}_{14} \text{ COO} - \text{C} - \text{O (CH}_2\text{CH}_2\text{O)}_n \text{ H} \\
\text{H}_2\text{C} &\quad \text{H} \\
\text{O} &\quad \text{CH} - \text{O (CH}_2\text{CH}_2\text{O)}_n \text{H} \\
\text{CH}_2 &\quad \text{O (CH}_2\text{CH}_2\text{O)}_n \text{H}
\end{align*}
\]

Here, \(X+Y+Z=20\)

Polysorbate 80 (Tween 80)

**β-Cyclodextrin**

\[
\begin{align*}
\text{OH} &\quad \text{OH} \\
\text{H}_2\text{C} &\quad \text{H}_2\text{C} \\
\text{OH} &\quad \text{OH} \\
\text{OH} &\quad \text{OH} \\
\text{OH} &\quad \text{OH}
\end{align*}
\]

Sucrose

Figure 1: Some excipients used to stabilize therapeutic proteins (A. Biwer et.al. 2002)
Sucrose is another carbohydrate which is widely used in pharmaceutical field to stabilize proteins. Sucrose is a disaccharide with its molar mass 342.3 g/mol. The mechanism of protein stabilization by sucrose is not well established. It is believed that due to higher cohesive force of sucrose and water, sucrose is preferentially excluded from the protein domain. This leads to protein stabilization because in the solution containing sucrose the unfolded state of protein is thermodynamically unfavorable (Lee et al. 1981).

There are also lot more stabilizers but all stabilizers are not suitable for all proteins.

Previous works in this field

Numbers of attempts have been made in few years to find out the mechanism of protein aggregation and also suitable excipients to prevent protein aggregation in formulation. An attempt has been made to explain the mechanism of protein stabilization bestowed by detergents. Circular dichroism and native gel electrophoresis were used to investigate the effect of Tween 80 on aggregation of bovine serum albumin (BSA). The results suggest that Tween 80 alters the aggregation behaviour of BSA rather than inducing significant stabilization of the native state (Arakawa et al. 2000).

Another study was performed with LEA (late embryogenesis abundant) proteins. This protein is present in both plant and animal. In case of desiccation and cold shock this LEA protein is associated with tolerance to water stress. In this study the effect of LEA on Citrate synthase and Lactate dehydrogenase has been examined. The results showed that LEA proteins suppress protein aggregation in water stress through desiccation and freezing and also proved the synergistic effect of LEA and Trehalose in suppressing protein aggregation (Goyal et al. 2005).

Again, study was performed to stabilize recombinant human serum albumin. The effect of sugar lyoprotectants on the stability of lyophilized rHSA was assessed. For the experiment, rHSA was formulated with sucrose and trehalose, respectively alone or in combination with mannitol. They were lyophilized and stored at 35°C. The results suggest that sucrose and trehalose can protect rHSA protein from lyophilization and can also stabilize the protein in the solid state during storage (Han et al. 2007).

An attempt has been made to assess the effect of structural features on the physicochemical stability of proteins. In this study, the effect of pH, temperature, and ionic strength on the physical stability of EC5 domain of E-Cadherin protein have been studied. The effect of disulfide bond on its physical stability has also been studied. The
results showed that ionic strength and the presence of the disulfide bonds are crucial for the stability of EC5 domain (Zheng et al. 2009).

In another study the mechanisms of aggregate formation and excipient stabilization of a recombinant human monoclonal antibody in freeze dried formulations was evaluated. The result suggests that the addition of carbohydrate excipients sucrose or trehalose to the formulation provided improved solid-state protein structure and reduced protein aggregation during storage (Andya et al. 2003).

Another study was performed using human growth hormone (hGH) as a model protein. In this study, the changes in secondary structure, aggregation, and loss of the magnitude of the heat of denaturation upon scanning to and partially through the temperature range of the thermal denaturation peak of the protein was studied. Here formulations of pure protein (with a trace of phosphate buffer) and another formulation with trehalose in a 3:1 trehalose: hGH weight ratio were studied. The results showed that denaturation of freeze dried hGH occurs in the dry state at temperatures above the glass transition temperature of the system, and roughly 100°C above the denaturation temperature of the aqueous solution state and trehalose can protect the protein from denaturation (Pikal et al. 2008).

**Research objective**

In our experiment we have used pepsin as a model protein because, it is a well characterized enzyme and there is an established method to study its enzyme activity. Pepsin is a hydrolase enzyme produced in the stomach mucosa. It has molecular weight 37400 Daltons (Boyer et al. 1960) and isoelectric point near 3.1 (pdb.org). The nominal enzyme activity of pepsin is 3500-+300 units/mg protein according to the unit definition of pepsin (An absorbance change of 0.001 per minute at 280 nm during digestion of haemoglobin at pH 2.0 and 37°C) (Kokufuta et al. 1991). We have studied the stability of pepsin at different conditions (extremes of pH, change of temperature and Agitation). Then we have examined the effect of excipients (Polysorbate 80, Sucrose and β-Cyclodextrin) on the stability of pepsin in aqueous solution and have investigated whether its bioactivity remains intact. The ultimate objective is to devise a systematic approach to the formulation of therapeutic proteins.
2. Materials and methods

2.1. Material

For this experiment, Pepsin from Porcine gastric mucosa was obtained from Sigma-Aldrich, Haemoglobin from Bovine blood was obtained from Sigma, Tween80 (Polyoxyethylene sorbitan monooleate) was obtained from Sigma Ultra. β-Cyclodextrin (Cycloheptaamylose) was obtained from Sigma, Sucrose (α-D-Glucopyranosyl β-D-Fructofuranoside; Saccharose) was obtained from Sigma. Polyclonal antibody (rabbit) to pepsin from porcine stomach mucosa was obtained from Nordic Immunology, Netherlands. CM5 sensor chip for Biacore study was obtained from GE Health Care AB, Uppsala, Sweden. All the other chemicals were of analytical grade and were obtained from commercial suppliers.

3.2. Methods

3.2.1. Enzymatic Assay

Enzymatic activity of Pepsin was determined by established method (Enzymatic Assay of Pepsin (EC 3.4.23.1)). In this assay acidified haemoglobin is hydrolysed by pepsin at 37ºC. This gives TCA (Trichloro acetic acid) soluble peptides, which is detected by UV absorbance at 280 nm. Enzyme activity is expressed as Units/mg protein.

For this at first the reagents were prepared. Reagent A (enzyme diluent), 100 ml of 10 mM Hydrochloric acid, was prepared by dissolving hydrochloric acid in deionised water. Reagent B, 100 ml of 300 mM Hydrochloric acid solution, was prepared by dissolving hydrochloric acid in deionised water. Reagent C, 100 ml of 2.5% (w/v) Haemoglobin solution, was prepared in deionised water by mixing vigorously and filtering through the glass wool filter. Reagent D, 2% (w/v) Haemoglobin solution was prepared by adding 20 ml of Reagent B to 80 ml of Reagent C. Reagent E, 100 ml of 5% (w/v) Trichloroacetic Acid Solution (TCA) was prepared by dissolving Trichloroacetic Acid solution (6.1 N, approximately 100% (w/v)) in deionised water. Reagent F, Enzyme Solution (containing 0.01-0.05 mg/ml of pepsin in cold Reagent A), was prepared immediately before use. After that 5 ml of Reagent D was taken as test sample and another 5 ml of it was taken as blank. They were equilibrated at 37ºC Then 1 ml of Reagent F was added to the test sample. They were mixed by swirling and were incubated for exactly 10 minutes. Then
10 ml of the Reagent E was added to both the test sample and the blank. And 1 ml of Reagent F was added to the blank. They were mixed by swirling and were incubated at 37°C for 5 minutes. The solutions were filtered through 5 µm syringe filter. The solutions were transferred to quartz cuvettes and the A280 nm was recorded for both the test and the blank.

The enzyme activity was calculated by using the following formula—

\[
\text{Units/ml enzyme} = \frac{(A_{280 \text{ nm Test}} - A_{280 \text{ nm Blank}}) \text{df}}{0.001 \times 10 \times 1}
\]

\( \text{df} = \text{Dilution Factor} \)

\( 0.01 = \text{Change in absorbance at 280 nm per unit of Pepsin (Unit Definition)} \)

\( 10 = \text{Time of assay (in minutes) as per the Unit Definition} \)

\( 1 = \text{Volume (in millilitres) of enzyme used.} \)

Units/mg solid was calculated by using the following formula—

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]

Units/mg protein was calculated by using the following formula—

\[
\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}
\]

Unit Definition: One unit will produce a \( \Delta A_{280 \text{ nm}} \) of 0.001 per minute at pH 2.0 at 37°C measured as TCA-soluble products using haemoglobin as substrate. (Final volume = 16 ml. Light path = 1 cm).

**3.2.2. UV spectroscopy**

UV spectrometer (Unicam UV/Vis spectrometer UV4, ATI unicam) has been used to make calibration curve for aqueous solutions of pepsin at different concentrations (1 mg/ml, 2 mg/ml) and to study aggregation index and the enzyme activity of pepsin. The instrument was blanked with the respective solvents of the pepsin solutions. The absorbance was recorded in a UV spectrum in a range of 220-440 nm. To make calibration curve, maximum UV absorbance of pepsin solution in 0.01 M HCl (pH 2) with/without 0.9% NaCl has been plotted against their respective concentrations 1 mg/ml (0.027 mM) and 2 mg/ml (0.053 mM).

The aggregation index value is a relative measurement of the degree of protein aggregation and was calculated according to the following equation

\[
\text{AI} = 100 \times \frac{\text{LS max}}{\text{Abs max} - \text{LS max}} \quad \text{(equation 1)}
\]

Where, LS max is the light scattering interference at maximum absorbance wavelength determined by linear regression extrapolation through absorbencies at 340, 370 and 400 nm (Dunn et al. 2005), and Abs max is the maximum absorbance.
3.2.3. Dynamic light scattering (DLS)

Protein aggregation has been studied by Dynamic light scattering (DLS) (Zetasizer 4000, Malvern). Measurements were performed at 25°C using a refraction index of 1.330 and viscosity of 0.47 cP. Sample time was 0.5 µs for a 1 second run duration with a total of 10 accumulations. The intensity of scattered light, expressed as the photon count rate in counts per second (Cps) was noted. The Cps is related to the number of particles and particle size. Here, one formulation containing 0.027 mM (1 mg/ml) concentration of pepsin solution of pH 2 with 0.9% NaCl and another formulation containing only 0.027 mM (1 mg/ml) concentration of pepsin solution of pH 2 without any electrolyte have been studied. Both of the formulations were filtered through 0.2 µm pore size filter to remove any extraneous substances.

3.2.4. Nano-Differential scanning calorimetry (Nano-DSC)

To determine the denaturation temperature and the enthalpy of thermal denaturation of pepsin without any excipient and also after addition of an excipient Tween 80 (Polyoxyethylene sorbitan monooleate), Nano-Differential Scanning Calorimetry has been performed. The test was performed using VP-DSC Micro Calorimeter, MicroCal Incorporated, America. Both the sample (0.027 mM pepsin solution) and the reference (solvent of the pepsin solution) were scanned at a pressure of 29 psi and at 90°C/hr scan rate. The scanning was started from 25°C and was up to 100°C.

3.2.5. Biacore ® (Surface plasmon resonance)

To study the antibody binding capacity of pepsin Biacore® X100 (GE Health Care AB, Uppsala, Sweden) was used. The antibody to pepsin was immobilized on the CM5 sensor chip of the Biacore instrument. For immobilization Acetate buffer (10 mM NaCH3COOH, 0.15 M NaCl, 3 mM EDTA) of pH 4 was used. Pepsin solution (2µM) of pH 4 containing 0.9% NaCl was prepared. A series of 4 dilutions (with dilution factor 2) of the pepsin solution were performed. Pepsin solutions were injected into the Biacore machine. As running buffer HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3M EDTA, 0.005% surfactant polysorbate 20) was used. The same buffer was also used as regeneration buffer. The contact time of pepsin with the antibody was 20 sec and the dissociation time was 60 sec. Real-time molecular interactions were monitored from the
changes of the optical refractive index from a prior reading point. The signals were generated as resonance unit in a sensogram. The experiment was repeated for the pepsin solution of pH 2 upon agitation (15 RPM) for 1 hr. The dilution of the sample was made by using acetate buffer of pH 4.

3.2.6. Test for pH and temperature effects

To study the effect of pH and temperature on the stability of pepsin, 1 mg/ml (0.027 mM) Pepsin solution in 0.01 M HCl containing 0.9% NaCl was prepared and were adjusted to different pHs (2, 4, 5.5, 6.1 and 6.4) using 1 M NaOH. pH was measured with a Metrohm pH meter (Swiss made) and a Metrohm electrode. The pH meter was pre-calibrated using a 3-point calibration, against pH 4, 7 and 10 standards. The solutions with pH 2, and pH 4 were incubated for 72 hours and 96 hours respectively and the solutions with pH 5.5, pH 6.1 and pH 6.4 were incubated for 24 hours at 37°C and samples were collected at different time intervals to study the enzymatic activity and the aggregation index. As a control a fraction of each of the respective solutions were kept at 4°C.

3.2.7. Agitation studies

Pepsin at a concentration of 0.027 mM was formulated without excipient and with Tween 80 at an amount that corresponds to the molar ratio of 0.1:1, 10:1, 30:1, 50:1 and 100:1 of Tween 80 to pepsin where the concentration of Tween 80 was 2.8 µM, 0.28 mM, 0.76 mM, 1.42 mM, and 2.84 mM respectively.

Samples containing 7.5 mL of each of the formulations were placed in to 14 mL falcon tube to provide enough head space facilitating bubble entrainment into the solution from the air-liquid interface during agitation. Samples were oriented horizontally and agitated at 15 RPM on a Stuart Rotator STR4 (Bibby Sterlin, UK) at room temperature for 80 mins. Sample tubes were removed for testing at 0, 20, 40, 60 and 80 mins. The experiment has been repeated for pepsin solution containing Sucrose at different concentrations (2% (60mM), 10% (292mM)) as stabilizer and also for pepsin solution containing β-Cyclodextrin at different amount that corresponds to the molar ratio of 33:1 and 65:1 of β-Cyclodextrin to pepsin (0.881 mM conc. of β-Cyclodextrin).

3.3. Statistics
All the assessments have been made from three individual replicate measurements. The mean values and standard deviations have been calculated.

3. Results and Discussion

3.1 Calibration curve

For the experiment, a calibration curve has been made (Fig. 2). For this purpose two experiments have been performed with 0.053 mM (2mg/mL) concentration of pepsin and one experiment has been performed with 0.027 mM (1mg/mL) concentration of pepsin. For each of the experiments pepsin solution has been made with/without electrolyte (NaCl). The pH of the solutions was 2. For each sample four sets of dilution were made.

![Effect of NaCl on Pepsin Absorbance](image)

**Figure 2**: Calibration curve. Maximum UV absorbancies have been plotted against the respective concentration of pepsin solutions. In three experiments two concentrations (1 mg/mL and 2 mg/mL) of pepsin with/without electrolyte (NaCl) have been studied. For each experiment four sets of dilution of the samples have been performed. The plotted values are showing linear correlation with around ±10% deviation in values among different experiments.

The maximum UV absorbancies have been plotted against their respective concentrations. The plotted values are nicely linear. There is around ±10% deviation in values among different experiments. This may be due to lack of homogeneity of pepsin in pepsin powder. The amount of pepsin in pepsin powder is only 47%. Other proteins are also mixed with it as impurities. As for different experiments the samples have been
collected independently, the concentration of pepsin may not be consistent which shows up as deviations in maximum UV absorbencies.

### 3.2 Effect of NaCl on aggregation index of Pepsin

To examine the effect of NaCl on the aggregation index of Pepsin solution the measured aggregation index has been plotted against the respective concentration (Fig. 3). Different concentrations (1mg/mL and 2 mg/mL) of pepsin solutions with/without NaCl have been made and the absorbencies have been recorded in a UV spectrum in a range of 220-440 nm. The light scattering interference at maximum absorbance wavelength has been determined by linear regression extrapolation through absorbencies at 340, 370 and 400 nm. The aggregation index has been calculated according to the equation 1. It is shown in figure 3 that the pepsin solutions containing NaCl have lower aggregation index compared to their counter solutions containing no NaCl.

![Effect of NaCl on Aggregation Index](image)

**Figure 3: The effect of NaCl on aggregation index of Pepsin.** The aggregation index of pepsin solution of different concentration formulated with or without electrolyte (NaCl) has been plotted against their respective concentrations

Aggregation Index has also been studied by Dynamic Light Scattering (DLS). According to the DLS result in pepsin formulation without NaCl average particle size is $64 \pm 67$ (standard deviation) nm and in pepsin solution with NaCl average particle size is $4 \pm 5$ (standard deviation) nm. These results are fairly correlated to the previous results of Aggregation Index. It reveals NaCl as an agent to protect the protein from aggregation.
However, electrolytes are generally known to stabilize protein (Ajay K. Banga, 2nd edition 2006).

3.3 The effect of pH and temperature on enzyme activity

Temperature and pH are important factors to influence the activity of enzyme. To study the effect of pH and temperature on the stability of pepsin, 0.027 mM Pepsin solution (containing 0.9% NaCl) of different pHs (2, 4, 5.5, 6.1 and 6.4) were incubated at different temperatures (4°C, 25°C and 37°C) for certain time points (Figure 4 A, B). In figure 4A the pepsin solution of pH 2, preserved at cold (4°C) temperature (serving as positive control) showing the enzyme activity within the range of 1041-1680 U/mg protein. The enzyme activity has been observed up to 72 hours and samples have been collected at 2hr, 24 hr and 72 hr time points. The pepsin solution of pH 2, preserved at 37°C temperature showing the enzyme activity within the same range as that of the positive control. These results indicate that at pH 2, increase in temperature to 37°C, the pepsin stability (observed as enzyme activity) is not affected.

**Figure 4: The effect of pH and temperature on enzyme activity as well as pepsin stability**

(A) Here, the symbol - ♦- is showing the enzyme activity (expressed as U/mg protein) at pH 2 and upon incubation at 37°C temperature; - ■- is showing the enzyme activity of pepsin (expressed as U/mg protein) upon storage on cold environment (4°C temperature); - ▲- is showing the enzyme activity (expressed as U/mg protein) at pH 4 and 37°C temperature. (B) Here, the symbol - ♦- is showing the enzyme activity of pepsin (expressed as U/mg protein) at pH 6.4 and upon incubation at 25°C temperature; - ■- is showing the enzyme activity of pepsin (expressed as U/mg protein) at pH 6.1 and upon storage at 25°C temperature; - ▲- is showing the enzyme activity of pepsin (expressed as U/mg protein) at pH 5.5 and upon incubation at 37°C temperature.
Again, the pepsin solution of pH 4, preserved at 37°C temperature maintains its enzyme activity within the range of 1005-1365 U/mg protein which is almost comparable to that of positive control. Here the enzyme activity has been observed up to 96 hours. This result indicates that these pH and temperature are quite favourable for the pepsin stability.

Again in Figure 4B, the pepsin solution of pH 5.5, preserved at 37°C temperature shows the enzyme activity within the range of 827-1058 U/mg protein. Here the enzyme activity is fairly lower compared to the positive control. The pepsin solution of pH 6.1, preserved at 25°C temperature, the enzyme activity is within the range of 550-585 U/mg protein. Whereas, in the pepsin solution of pH 6.4, preserved at 25°C temperature, the enzyme activity gradually decreased from 620 to 36 U/mg protein within 3.5 hours. These results suggest that up to 37°C enzyme activity of pepsin is not affected by temperature. Even change in pH up to 4 is causing no change in enzyme activity of pepsin. However at pH 2 pepsin is showing its maximum enzyme activity which is already established (Schlamowitz et al., 1959). But above pH 6.1 even at lower temperature (25°C) the enzyme activity is very low and at pH 6.4, there is a drastic fall in enzyme activity of pepsin which reaches to almost zero within few hours (3.5 hours). This finding agrees with the previous finding which says, pepsin is unstable above pH 6 (Worthington Enzyme Manual).

3.4 The effect of pH and temperature on Aggregation Index (AI)

To study the effect of pH and temperature on Aggregation Index of pepsin and to observe their correlation with the enzyme activity, samples were collected from pepsin solutions (0.027 mM and containing 0.9% NaCl) of pH 5.5, 6.1 and 6.4 which were incubated for 3.5 hours at 37°C, 25°C and 25°C temperature respectively. Samples were collected at different time intervals (0 hr, 1.5 hr, 2.25 hr and 3.5 hr) and were analysed by UV spectroscopy. AI was calculated and has been plotted against respective time points (hrs) (Fig. 5). According to figure 5 at pH 5.5 and 37°C the aggregation index is within the range of 4 to 6. At pH 6.1 and 25°C the aggregation index of pepsin is within the range of 4 to 8. And at pH 6.4 and 25°C the aggregation index of pepsin is within the range of 4 to 9. These values are within the limit as we have seen in figure 3. There is no significant change in aggregation index with these pH changes and there is no correlation of the aggregation index with the changes in enzyme activity.
Figure 5: The effect of pH and temperature on Aggregation Index of pepsin.

Here, the symbol -♦- is showing the Aggregation Index of pepsin at pH 5.5 and upon incubation at 37°C; -■- is showing the Aggregation Index of pepsin at pH 6.1 and upon incubation at 25°C; -▲- is showing the Aggregation Index of pepsin at pH 6.4 and upon incubation at 25°C.

Thus, these results suggest that protein aggregation is not directly related to the loss of enzyme activity of pepsin. There may be other factors (e.g., conformation change at the active site) involved in loss of enzyme activity of pepsin.

3.5 The effect of agitation on aggregation index and enzyme activity of pepsin

Figure 6: Effect of agitation on enzyme activity and aggregation index of pepsin. (A) The enzyme activity of pepsin (U/mg protein) upon agitation of pepsin solution (pH2) at a speed of 15 RPM on a rotator has been plotted against time (0 min, 20 min, 40 min, 60 min and 80 min). (B) The aggregation index of pepsin, upon agitation of pepsin solution (pH2) at a speed of 15 RPM on a rotator, has been plotted against time (0 min, 20 min, 40 min, 60 min and 80 min).

Protein formulations may experience agitation during manufacturing, shipping, or handling. To examine the effect of agitation or the exposure to air-water interface on
pepsin stability, pepsin solution of pH 2 containing 0.9% NaCl was rotated for 80 mins. The solutions were filled up to 50% of volume in falcon tube for rotation. And samples were collected for enzyme assay and to measure the aggregation index. According to figure 6A the enzyme activity of pepsin in a solution at pH2 upon agitation is decreasing gradually over time following the first order kinetics. And after 80 minutes the enzyme activity is almost zero. According to figure 6B the aggregation index is within the range of 6 to 10. Thus there is no correlation between change in Aggregation Index and the change in enzyme activity.

3.6 Excipients in stabilizing pepsin

There are number of excipients that can be used to stabilize proteins. Different stabilizers work in different mechanisms. To find out a suitable excipient to stabilize the pepsin in solution upon agitation or exposure to air-water interface and also at higher pH (pH 6.4) some excipients have been tried. They were Tween 80 (Polyoxyethylene sorbitan monooleate), Sucrose and β-Cyclodextrin.

3.6.1 Tween 80 (Polyoxyethylene sorbitan monooleate) in stabilizing pepsin in solution upon agitation

To examine the effect of Tween 80 in stabilizing pepsin in solution upon agitation, pepsin has been formulated with different concentrations of Tween 80. For these formulations enzyme activity (Fig. 7) and aggregation index (Fig.8) have been studied upon agitation. According to Figure 7, all the formulations, containing 10:1, 30:1, 50:1 and 100:1 molar ratio of Tween 80 to pepsin, maintain the enzyme activity at very good range; even better than the enzyme activity at starting point of the pepsin formulation containing no stabilizer.
Figure 7: Effect of different concentration of Tween 80 on enzyme activity of pepsin in solution upon agitation. Here, the symbol -♦- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 0.1:1 molar ratio of Tween 80 to pepsin; -■- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 10:1 molar ratio of Tween 80 to pepsin; -▲- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 30:1 molar ratio of Tween 80 to pepsin; -×- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 50:1 molar ratio of Tween 80 to pepsin; -٭- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 100:1 molar ratio of Tween 80 to pepsin; -•- is showing the enzyme activity (U/mg protein) of pepsin in solution without Tween 80.

For the pepsin formulation containing 0.1:1 molar ratio of Tween 80 to pepsin, the enzyme activity is lowered but still much better than the formulation containing no stabilizer.

According to figure 8, for almost all the formulations containing Tween 80 and the formulation without Tween 80, there is no observable change in aggregation index upon agitation over time. There is only one exception with pepsin formulation containing 100:1 molar ratio of Tween 80 to pepsin, which may be due to experimental error.

Figure 8: Effect of different concentration of Tween 80 on Aggregation Index of pepsin in solution upon agitation. Here, the symbol -♦- is showing the Aggregation Index of pepsin in solution containing 0.1:1 molar ratio of Tween 80 to pepsin; -■- is showing the Aggregation Index of pepsin in solution containing 10:1 molar ratio of Tween 80 to pepsin; -▲- is showing the Aggregation Index of pepsin in solution containing 30:1 molar ratio of Tween 80 to pepsin; -×- is showing the Aggregation Index of pepsin in solution containing 50:1 molar ratio of Tween 80 to pepsin; -٭- is showing the Aggregation Index of pepsin in solution containing 100:1 molar ratio of Tween 80 to pepsin; -•- is showing the Aggregation Index of pepsin without Tween 80.
Index of pepsin in solution containing 100:1 molar ratio of Tween 80 to pepsin; -•- is showing the Aggregation Index of pepsin in solution without Tween 80.

These results suggest Tween 80 as a very good stabilizer of enzyme activity of pepsin in solution upon agitation. And 10:1 molar ratio of Tween 80 to Pepsin (0.28 mM conc. of Tween 80) is preferable for preserving the enzyme activity of pepsin but Tween 80 at any concentration doesn’t exert any significant change in aggregation index of pepsin.

3.6.2 Sucrose in stabilizing pepsin in solution upon agitation

Sucrose is a disaccharide and is used to stabilize protein in stressful condition like heating, Lyophilization and also in solution (Back et al.1979, Lee et al. 1981, Carpenter et al. 1987). To examine the role of Sucrose in stabilizing pepsin upon agitation, pepsin formulation with different concentration of sucrose (60 mM (2%) and 292 mM (10%)) have been examined for enzyme activity (Fig. 9).

![Effect of sucrose on enzyme activity of pepsin upon agitation](image)

**Figure 9: Effect of different concentration of Sucrose on enzyme activity of pepsin in solution upon agitation.** Here, the symbol -♦- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 60 mM (2%) Sucrose; -■- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 292 mM (10%) Sucrose; -▲- is showing the enzyme activity (U/mg protein) of pepsin in solution without stabilizer.

According to Figure 9, Sucrose at any concentration (2% or 10%) could not exert any significant change in enzyme activity of pepsin compared to that of the formulation
without any stabilizer. Therefore, Sucrose is not suitable to stabilize the enzyme activity of pepsin in solution upon agitation.

### 3.6.3 β-Cyclodextrin in stabilizing pepsin in solution upon agitation

To examine the role of β-Cyclodextrin in stabilizing pepsin upon agitation, pepsin formulation with different concentration of β-Cyclodextrin (65:1 and 33:1 molar ratio of β-Cyclodextrin to pepsin (0.881 mM conc. of β-Cyclodextrin)) have been examined for enzyme activity (Fig. 10). According to figure 10 there is no significant change in enzyme activity of pepsin in formulations containing different concentration of β-Cyclodextrin compared to that of the formulation containing no stabilizer. At some points the enzyme activity of pepsin in formulation containing β-Cyclodextrin is even lower than that of the formulation containing no stabilizer.

**Figure 10: Effect of different concentration of β-Cyclodextrin on enzyme activity of pepsin in solution upon agitation.** Here, the symbol -♦- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 65:1 molar ratio of β-Cyclodextrin to pepsin; -■- is showing the enzyme activity (U/mg protein) of pepsin in solution containing 33:1 molar ratio of β-Cyclodextrin to pepsin; -▲- is showing the enzyme activity (U/mg protein) of pepsin pepsin in solution without stabilizer.

These results suggest that β-Cyclodextrin is not a suitable excipient to stabilize pepsin in solution upon agitation.

### 3.6.4 Tween 80 (Polyoxyethylene sorbitan monooleate) in stabilizing pepsin in solution at higher pH (pH 6.4)
To examine the effect of Tween 80 in stabilizing pepsin in solution at pH 6.4 pepsin has been formulated with different concentrations of Tween 80 and has been adjusted to pH 6.4. For these formulations enzyme activity have been studied up to 3.5 hours (Fig.11). According to Figure 11, the formulation, containing 10:1, molar ratio of Tween 80 to pepsin (0.28 mM conc. of Tween 80), maintain the enzyme activity within the range of 856-750 U/mg protein, which is much better than that of the formulation containing no stabilizer (maintained at pH 6.4). In case of this formulation, the enzyme activity of pepsin was gradually decreased from 620 to 36 U/mg protein. Whereas, in case of the pepsin formulation containing 0.1:1 molar ratio of Tween 80 to pepsin, there is no significant change in enzyme activity compared to that of the formulation containing no stabilizer (maintained at pH 6.4).

**Figure 11: Effect of different concentration of Tween 80 on enzyme activity of pepsin in solution at pH 6.4.** Here, the symbol - ■ - is showing the enzyme activity (U/mg protein) of pepsin in solution containing 10:1 molar ratio of Tween 80 to pepsin; - ♦ - is showing the enzyme activity (U/mg protein) of pepsin in solution containing 0.1:1 molar ratio of Tween 80 to pepsin ; - ▲ - is showing the enzyme activity (U/mg protein) of pepsin in solution without stabilizer.

According to these results 10:1, molar ratio of Tween 80 to pepsin (0.28 mM conc. of Tween 80) is capable to stabilize pepsin (maintain enzyme activity) in solution at higher pH (pH 6.4).

### 3.6.5 Sucrose and β-Cyclodextrin in stabilizing pepsin in solution at higher pH (pH 6.4)

To examine the effect of sucrose and β-Cyclodextrin on enzyme activity of pepsin in solution at higher pH (pH 6.4), formulations of pepsin containing different concentration
of sucrose (60 mM (2%) and 292 mM (10%)) and different amount of β-Cyclodextrin (65:1 and 33:1 molar ratio of β-Cyclodextrin to pepsin (0.881 mM conc. of β-
Cyclodextrin)) have been studied (results not shown). Our study results suggest that neither sucrose nor β-Cyclodextrin can stabilize pepsin in solution at higher pH (pH 6.4).

3.6.6 Nano-Differential Scanning Calorimetry

Nano-Differential Scanning Calorimetry has been performed to study the denaturation temperature and the enthalpy of thermal denaturation of pepsin in solution at different conditions (without agitation and no added stabilizer, upon agitation without stabilizer, and upon agitation with Tween 80 (Polysorbate 80)) at pH 2. According to the result (Fig. 12), the denaturation temperature (Tm) of pepsin in solution at pH 2 is 67.96ºC ± 0.015. This is very close to the previous finding, which says the denaturation temperature of pepsin is around 70º C (Kopelmank et al. 1983). And the enthalpy of heat denaturation of pepsin in solution at pH 2 is 3.74 E5 ± 1.41 E3 kcal/mol/ºC (corrected for the amount of pepsin in pepsin powder).

![Figure 12: Nano-Differential Scanning Calorimetry of ca 27 µM stirred pepsin (pH 2). The denaturation temperature of pepsin is 70ºC and the enthalpy of heat denaturation is 1.760E5± 1.41 E3 kcal/mol/ºC (uncorrected for the amount of pepsin in pepsin powder.](image)

However, upon agitation (15 RPM) of pepsin solution at pH 2 for 80 min, there was no change in denaturation temperature of pepsin. Even after addition of Tween 80 in pepsin
formulation and then agitation, there was no significant change in denaturation temperature and enthalpy of denaturation of pepsin (result not shown).

**Biacore® (Surface plasmon resonance)**

Immobilization of antibody to pepsin on CM5 sensor chip was successful (Fig. 13). In the Figure 13, the baseline shift from around 20000 RU to 28000 RU is confirming the immobilization of antibody on the chip. But the assay for antibody binding of pepsin was not successful (Fig. 14 A, B). According to Figure 14 A, B for both of the pepsin solutions (without agitation and upon agitation) there is insignificant binding signals. Here, the pH of the Running buffer (7.4) was not suitable for pepsin. Maybe pepsin is destroyed during study. Further optimization of the Biacore study condition is required and the running buffer should be of lower pH which is compatible with pepsin.

![Baseline shift](image)

**Figure 13:** Immobilization of polyclonal antibody to pepsin on sensor chip CM5. The shift in baseline from around 20000 RU to around 28000 RU indicating the antibody immobilization on chip.
Figure 14: Sensogram for antibody binding of pepsin from Biacore (Surface plasmon resonance analysis). (A) Sensogram for antibody binding of pepsin without agitation. (B) Sensogram for antibody binding of pepsin upon agitation.

4. Conclusion

The stability of a protein can be hampered by variety of factors like concentration of enzyme, concentration of substrate, pH, temperature, shear stress etc. Therefore, to stabilize protein in formulations is a challenge. The choice of suitable excipient is highly protein specific. Here, I have used pepsin as a model protein and have made an attempt to find out the critical conditions (such as, extremes of pH, effect of high temperature and also exposure to air-water interface during agitation) for the stability of pepsin in solution. The aim of this attempt was to provide a systematic approach to characterize and stabilize therapeutic proteins in formulations. My study results suggest that pepsin solution with NaCl has lower aggregates compared to that of the solution containing no NaCl. And pepsin has its maximum enzyme activity at pH 2 and also maintains high enzyme activity up to pH 4. Within this pH range enzyme activity of pepsin is not affected by temperature change (4°C-37°C). However, the enzyme activity of pepsin is highly affected by higher pH (pH 6.4). The enzyme activity reaches almost to zero within 3.5 hours of pH adjustment of the pepsin solution. The enzyme activity of pepsin is also affected by agitation. It diminishes almost to zero within 80 mins of rotation (15 RPM). From my study results Tween 80 (Polysorbate 80) at a concentration of 0.28 mM (10:1, molar ratio of Tween 80 to pepsin) has been proved to be the stabilizer of choice to protect the
enzyme activity of pepsin in solution upon agitation. Tween 80 in this concentration can also protect the enzyme activity of pepsin in solution at pH 6.4. But Sucrose and β-Cyclodextrin can not protect the enzyme activity of pepsin in solution either at high pH or upon agitation. Surprisingly, there is no change in aggregation index of pepsin in solution, coupled with the change in enzyme activity, regardless of different conditions (change of pH and temperature, agitation, presence or absence of stabilizer). Aggregation of pepsin in solution is not directly involved with the loss of its enzyme activity. The denaturation temperature (Tm) of pepsin in solution at pH 2 is 67.96ºC ±0.015 and the enthalpy of denaturation is 3.74 E5 ± 1.41 E3 kcal/mol/ºC. There is no change in denaturation temperature and enthalpy of denaturation of pepsin at different conditions. So it cannot be sure that there is any irreversible denaturation of pepsin at the critical conditions at which pepsin has been exposed to. There may be conformation change at the active site of pepsin.

However, further study is required to find out the exact mechanism by which the enzyme activity of pepsin is lost. For this purpose, the pepsin sample will be purified by gel chromatography and then by using circular dichroism (CD) the change in secondary structure of pepsin at different conditions will be studied. The structural changes can then be correlated to the change in enzyme activity of pepsin. Again by immobilizing inhibitor of pepsin on the Biacore chip I can study the binding affinity and the kinetics of inhibitor binding of pepsin. This study will confirm if there is any conformation change at the active site of pepsin. After that a study can be performed with real therapeutic protein. The scheme to characterize and stabilize a real therapeutic protein for formulation will be, bioassay to study the bioactivity of the protein, DSC (Differential Scanning Calorimetry) to study the denaturation temperature and the enthalpy of heat denaturation, circular dichroism (CD) to study secondary structure and the Biacore® (Surface plasmon resonance) to study the substrate binding capacity of the protein.

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