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Enzymatic activity in the presence of surfactants commonly used in dissolution media, Part 1: Pepsin

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ABSTRACT

The *United States Pharmacopeia* (*USP*) General Chapters Dissolution ⟨711⟩ and Disintegration and Dissolution of Dietary Supplements ⟨2040⟩ allows the use of enzymes in dissolution media when gelatin capsules do not conform to dissolution specifications due to cross linking. Possible interactions between enzymes and surfactants when used together in dissolution media could result in loss of the enzymatic activity. Pepsin is an enzyme commonly used in dissolution media, and in this work, the activity of pepsin was determined in the presence of different surfactants as usually found in case of dissolution tests of certain gelatin capsule formulations.

Pepsin enzymatic activity was determined according to the *Ninth Edition of the Food Chemicals Codex* (*FCC*) 9 method, in dissolution conditions: simulated gastric fluid, 37 °C and 50 rpm. Sodium dodecyl sulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), polysorbate 80 (Tween 80) and octoxynol 9 (Triton X100) in concentrations above and below their critical micellar concentrations were selected. Results showed a significant reduction in the activity of pepsin at all the concentrations of SDS assayed. On the contrary, CTAB, Tween 80, and Triton X100 did not alter the enzymatic activity at of pepsin any of the concentration assayed.

This data demonstrates a rational selection of the surfactant to be used when pepsin is required in dissolution test.

1. Introduction

The *United States Pharmacopeia* (*USP*) General Chapters Dissolution ⟨711⟩ [29] and Disintegration and Dissolution of Dietary Supplements ⟨2040⟩ allow the addition of enzymes to the dissolution medium when gelatin capsules and gelatin-coated tablets do not conform to the dissolution specification due to cross-linking of gelatin. Cross-linking entails the formation of strong chemical linkages between gelatin chains due to interactions with the filling material or between the gelatin and the environment during storage [32]. The covalent bonding produced with this type of cross-linking is, for all practical purposes, irreversible, and will eventually render the gelatin insoluble.

Cross-linking typically results in the formation of a pellicle on the internal or external surface of the gelatin capsule shell that prevents the capsule fill from being released. In vitro dissolution testing of cross-linked capsules can result in slower or incomplete release of the active ingredient or no release at all [14,7]. The degree of cross-linking is not usually uniform within one capsule or among different capsules. As consequence, dissolution results will have higher variability when gelatin capsules are cross-linked [10,16,5,7].

When the gelatin is no longer soluble in water, dissolution of the shell must involve the breaking of other bonds, e.g., by enzyme-mediated breaking of peptide bonds in protein chains. The pH of the dissolution medium determines the appropriate enzyme to be used according to ⟨711⟩ and ⟨2040⟩ [31].

Pepsin is the enzyme commonly used in acidic dissolution media (pH 1 to pH 4) to break peptide bonds in gelatin capsules that are affected by cross linking. Pepsin is a monomeric, two domain, mainly L-protein, with a high percentage of acid residues (43 out of 327) leading to its very low isoelectric point (IEP) ≈ 1. It catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids [1]. The catalytic site of pepsin is formed by two aspartate residues, Asp32 and Asp215 (pKa values about 1.4 and 4.5, respectively), one of which has to be protonated, and

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the other deprotonated, for the protein to be active. This occurs in the pH range between 1 and 5 [6].

The proteolytic activity of pepsin is affected by the conditions of the dissolution medium. In acidic dissolution media like simulated gastric fluid (SGF, pH 1.2, 37 °C), pepsin shows its maximum activity. In fact, porcine pepsin has optimal activity at pH approximately 2.2. At pH 4.5 it decays to about 35% [3]. Within a pH range of 2–4, the enzymatic activity of pepsin is not affected by temperature changes between 4 °C and 37 °C [13]. The denaturation temperature of pepsin in solution at pH 2 is 67.96 ± 0.015 °C [13]. The effect of ionic force (μ) on pepsin denaturation may be explained by changes in the ionizable groups and alterations of the electrostatic interactions. It has also been previously demonstrated that the alkaline denaturation rate constant is accelerated by the ionic strength [11].

There is widespread acceptance of the view that vagal stimulation evokes the secretion of acid gastric juice rich in pepsin. According to that, variability in human gastric secretion is high. The SGF is an artificial dissolution medium that is intended to represent a standardized way the stomach acid secretion in fasted state. The composition for SGF is given in the section Reagents: Test Solutions in USP-NF.

The dissolution medium may additionally contain a percentage of surfactants, dispersing agents, or solubility enhancers when either the capsule fill or the active ingredient (or both), are hydrophobic or water-insoluble. They may also be used if the media as described in ⟨1092⟩ [30] is ineffective in dispersing the capsule fill or in achieving proper sink conditions for the active ingredient.

The need for a surfactant and its particular concentration can be justified from drug substance solubility investigations, that include all common surfactant types, anionic (i.e., sodium dodecyl sulfate (SDS)), cationic (i.e., cetyltrimethyl ammonium bromide (CTAB)) and nonionic (i.e., polysorbate 80 (Tween 80) or (octoxynol 9 (Triton X100))) [17,25,28,8,9,26]. When a suitable surfactant has been identified, different concentrations should be investigated to identify the lowest concentration needed to achieve sink conditions for the dissolution test. Typically, such concentration is above the surfactant's critical micellar concentration (CMC).

When the concomitant use of surfactants and enzymes are required for dissolution tests, a rational selection of the surfactant to be used should be performed in order to reach reliable results. Among the reasons that should be considered with this selection are the interactions between the surfactants and enzymes. The interaction between enzymes and surfactants has been widely described in the literature [24]; however, this topic has not been discussed for its implication in dissolution tests and no systematic studies have been reported about the effect of surfactants on the activity of the pepsin under the conditions used in *USP* dissolution tests, i. e. temperature, pH, stirring and time. Besides, this interaction depends on both the type and the concentration of the surfactant used. The monomeric or micellar form of the surfactants, which is related with their CMC, is one of the main factors that can affect the type of interaction with the enzymes. For water-soluble proteins, interactions with surfactants can be broadly split up into two regions: below and above the CMC [24]. Depending on the concentration of the surfactant and its ionic character, an unfolding or denaturation process of the enzyme could occur as a consequence of their interaction [24].

The goal of this study was to evaluate the impact in pepsin activity produced by the concomitant presence of surfactants currently used in dissolution studies. USP-SGF was selected as dissolution medium for this study.

2. Materials and methodology

2.1. Reagents

USP Pepsin for assay RS, Lot FOM 228, having an activity of 7.7 U/mg (USP 2015 Pepsin Activity) was used. Pepsin from porcine gastric mucosa lyophilized powder, 3200–4500 U/mg protein from SIGMA-ALDRICH® (Lot. SLBL 1721V, 26.0 U/mg) was used as reagent. Milli-Q water was used for all solutions preparations. The SGF was prepared according to specifications described in *USP 38* (USP, 2015 Reagent, test solutions). The 4.0% *w*/*v* trichloroacetic acid (TCA) solution was prepared by diluting 5% TCA solution (RICCA®, reagent grade) with water, and the diluted hydrochloric acid solution (HCl Sol) was prepared by diluting 2.5 mL of 37% hydrochloric acid (Fisher®) to 1 l with water. The pH of the final solution was adjusted to 1.6 ± 0.1 with 37% hydrochloric acid.

The substrate solution consisted of 2% *w*/*v* hemoglobin protease substrate USP RS, Lot FOM 231 in HCl Sol. The pH of the final solution was adjusted to a pH of 1.6 ± 0.1 with 1 M HCl.

SDS (Spectrum®, Lot 2DH221), CTAB (MP Biomedicals, Lot MR31911), Tween 80 (Fisher®, Lot 132307) and Triton X100 (Fisher®, Lot 136597) were selected since they are the surfactants most commonly used un dissolution media [17,25,26,28,8,9].

2.2. Standard curve of pepsin RS activity

Enzymatic activity of pepsin was performed using the method "Activity of pepsin" described in the *FCC 9* [33]. In this assay, acidified hemoglobin is hydrolyzed by pepsin at 25 °C. This gives TCA soluble peptides, which are detected by UV absorbance at 280 nm. Enzymatic activity is expressed as U/mg protein.

In order to construct a calibration reference curve, fresh standard solutions (SS) of 0.7, 0.9, 1.0 and 1.30 U/mL (0.09–0.17 mg/mL) were prepared from a 0.2 mg/mL stock solution of pepsin RS in HCl Sol.

2.2.1. Enzymatic reaction

SS tubes: Tubes (in duplicate) containing 1.0 mL of each of the SS were placed into a water bath maintained at 25 ± 0.1 °C, added with 5.0 mL of the hemoglobin substrate solution and mixed by vortexing. Exactly 10 min after that, the reaction was stopped by the addition of 10.0 mL of the TCA Solution.

SS blank tubes (SSB): Tubes (in duplicate) containing 1.0 mL of each of the SS were placed into a water bath maintained at 25 ± 0.1 °C, added with 10.0 mL of TCA and mixed by vortexing. Then, an aliquot of 5.0 mL of the hemoglobin substrate solution was also added.

After 25 min, the content of SS and SSB tubes were filtered twice through a Whatman No. 41 ashless filter circle with a diameter of 150 mm. The absorbance of each of the filtrates was measured at 280 nm using an AGILENT single beam UV–visible spectrophotometer, in a 1-cm quartz cell. A blank solution was prepared by transferring 1.0 mL HCl Sol into a single tube and processed as described for SSB.

The net absorbance for the SS was calculated by subtracting the average absorbance of the SSB from the average absorbance of the corresponding SS. A standard curve of the net absorbance for each SS versus its concentration (mg/ml) was established. The calibration curve was performed six times, and the results of these curves were averaged in order to obtain an equation to be used to determine the activity of the pepsin.

The Pepsin RS enzymatic activity showed linearity in the concentration range between 0.7 and 1.3 U/mL (0.09–0.17 mg/mL), with a correlation coefficient of 0.9997 (R^2). The equation of the average adjusted curve was calculated as $y = 4.6647x - 0.00104$. This equation was used to calculate the activity of pepsin.