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Formulation and Production of a Novel Pharmaceutical Substance for Treatment of Infected Wounds - a Chitosan Chymopsin Complex

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Abstract

Wound infection represent a serious threat to wounded patients all over the world. Untreated wounds may result in bacterial wound infections, such as gas gangrene and tetanus. These in turn may lead to long term disabilities, chronic wound or bone infection, and, ultimately, death. Appropriate management of infected wounds is important to reduce the likelihood of life-threatening complications.

Chitosan chymopsin complex (CCC) is a novel pharmaceutical substance consisting of chymopsin (a combination of two proteolytic enzymes: trypsin and chymotrypsin) and acid-soluble chitosan (a natural high molecular weight polysaccharide). This substance, either alone or together with antiseptic and/or anesthetic agents, can be used in treatment of infected wounds of different etiology.

Synthesis, and production of active pharmaceutical ingredients (API) using biotechnological methods are the most demanding and costly stages of drug manufacturing. Therefore, a thoughtfully performed formulation can significantly reduce production costs, make manufacturing less labour-intensive, and, of course, improve API performance and quality.

The paper describes the process of formulation of a novel API which possesses complex therapeutical activity, allows for faster wound healing and effective clearance of wound surface from pus and necrotic debris.

Keywords: active pharmaceutical ingredient, chitin deacetylation, chitosan chymopsin complex (CCC), crab hepatopancreas, infected wounds treatment, polyelectrolyte complex (PEC), proteolytic complex (PC), proteolytic unit (PU).

INTRODUCTION

Wound treatment, especially complicated ones, is one of the most serious issues of modern medicine. Pyogenic infection is considered the most severe complication, both in terms of mortality and treatment costs. For the last several years, the mortality from pyogenic infections remains almost constant. It is favoured by several factors, including primary wound infections, suture material which may cause aseptic inflammation in surrounding tissues, etc [1, 2, 3].

How an infection enters the wound and overall body reactivity are also important for wound infection development. Cross-infections comprise about 30% of all pyogenic hospital infections and increase mean length of stay by 15-18 days [3, 4, 5].

After a series of experiments, chitosan (chitin deacetylation product) was selected as optimal carrier for chymopsin complex (Fig.1) [6].

During deacetylation, about 60% of chitin becomes acid-soluble and turns into chitosan. The process occurs at pH below 5 by protonation of free amino groups.

Several fuctional groups, such as -OH, $NHCOOCH_3$, and $-NH_2$ are present in chitosan. Amino groups confer chitosan properties of cationic polyelectrolyte (pKa ≈ 6.5) with unique features. Chitosan-based materials have positively-charged NH^{3+} groups and are able to retain on negatively-charged surfaces or form an interpolyelectrolyte complexes (IPECs) with biomolecules with low isoelectric point. Reaction of complexation in aqueous solutions is a fully reversible process. There are two types of regions that can be found within such complexes: ordered regions (A) which are formed by the opposingly-charged chains of both polyelectrolytes linked to each other and regions (B) in which defects and loops formed by non-linked polyelectrolyte chains are found. Hydrophilic properties of defect regions B allows IPECs to swell in water, whereas hydrophobic regions (A) limit this property to a certain extent. Due to its chemical nature chitosan can form all main types of chemical bonds (covalent, ionic, hydrogen, and hydrophobic) and coordinate bond, in which chitosan acts as a complexing agent [7,8].

Thus, chitosan makes an ultimate carrier for the novel drug complex. Moreover, chitosan itself has wound healing properties which can be explained by activation of immune response by macrophages stimulation and use of acetyl glucosamine as predecessor of mucopolysaccharides which, in turn, stimulate fibroblast proliferation, increase secretion of immune response mediators and directly participate in biological stucture formation. Mechanisms of immunogenesis-stimulating effect of chitosan is attributed to adjuvant action of polymers, their ability to affect processes occurring at the early stages of immunogenesis, possibly at the step of machrophage antigen capture and antigen information transduction to B-cells. Therefore, as a result of series of experiments chitosan was chosen as the optimal carrier for chymopsin.

The aim of this study was to formulate a novel pharmaceutical substance – the CCC complex - which can be used as API in drug products for treatment of infected wound and develop its manufacturing technology.

MATERIALS AND METHODS

A series of experiments was carried out in order to select proteolytic component of the novel complex substance. The choice of APIs should be both theoretically and experimentally justified [9, 10, 11, 12, 13]. It is important not only to obtain drug product with high specific activity, but also to preserve this activity throughout the shelf life of a drug [7,14]. Structural modification of enzyme can be performed in two ways: chemical modification of separate functional groups of protein macromolecule resulting in change in their charge or their hydrophilicity and cooperative interaction between enzyme and carrier matrix. The latter result in formation of multile bonds (which often have different nature) which leads to an increased structural rigidity and native structure changes. Such modifications can affect protein stability during denaturation [10,12,15].

Modification of enzyme stability can be associated with either changes in denaturation activation parameters (kinetic stability) or changes in equilibrium parameters (thermodynamic stability), or with both types of changes. It is also important to know which factor – enthalpy or entropy – contribute most to the changes in activation and equilibrium denaturation parameters. Modification of enzyme and formation of saline and other bonds

with carrier matrix results in increased protein stability due to significant decrease in activation entropy. Moreover, an observed enthalpy decrease is compensated by changes in entropy [16, 17, 18].

RESULTS

There are three main mechanisms of interaction between chitosan and proteins: polyelectrolyte complex (PEC) formation, encapsulation of proteins in chitosan gel, and adsorption of proteins on chitosan aggregates (Fig.2). The type of interaction is defined by chitosan concentration. PEC formation occurs at low concentrations, whereas incapsulation and adsorption – at higher and highest chitosan concentrations, respectively [19, 20].

Interaction between chitosan solutions and several enzymatic substances (trypsin, chymotrypsin, and proteolytic complex from crab hepatopancreas) was examined in order to select proteolytic enzyme, which will act as an active component of a novel substance [6, 13].

The concentration of chitosan can also affect drug product performance. Therefore, a number of chitosan solutions with different concentrations (0.5-1.5 wt%) were prepared. Solutions of enzymes used in the experiment were also prepared in different concentrations (0.01-0.03%). Duration of reaction and temperature were considered as optimization parameters. The aim of the experiment was to determine optimal ratio between carrier and enzyme.

Three solutions of chitosan (0.5, 1, and 1.5%) with addition of 0.5% acetic acid were prepared at room temperature using magnetic stirrer. After complete dissolution the solutions were left at room temperature for 24 hours for further structure formation, followed by immobilization of enzymes on structured carried.

Different quantities (100, 200, and 300 mg) of proteolytic enzymes (trypsin, chymopsin, and proteolytic complex from crab hepatopancreas) were dissolved in chitosan solutions by mixing on a magnetic stirrer for two hours.

Intermediate assay of proteolytic activity was carried out before drying stage (Table 1).

An optimal drying conditions, in terms of inactivation of enzymes, were selected during the study.

A thin, brittle film is formed when the composition is dried at room temperature. In order to obtain chitosan-enzyme films, after full enzyme dissolution about 100 µl of the obtained gel were placed on a polyethylene support. A piece of support with dried gel was cut out and placed into a test tube containing substrate in order to assay enzymatic activity. A series of experiments have shown that the support is completely inert and does not affect activity of the enzymes.

Freeze-drying option was chosen because of its ability to preserve API. During this process, the substance is frozen followed by vacuum sublimation of the solvent. Freeze-dried samples can be readily reconstituted which simplifies laboratory experiments due to the fact that the volume of the dried mixture remains almost the same owing to microcrystalline structure formation.

The choice of freeze-drying for obtaining experimental substances is additionally justified by the fact than no microbiological contamination and oxidation of thermolabile compounds can occur at low temperatures. Residual moisture content in freeze-dried products is about 1%, therefore it is possible to achieve prolonged shelf-life.

Freeze-drying also helps to remove excess of acetic acid used for chitosan dissolution. Freeze-dried material keeps is structure integrity and biological activity to a greater degree that in case of films obtained by drying at room temperature. Reconstituted material retains its original properties.

Freeze-drying of gel-like solutions of enzymes in chitosan is performed under vaccum conditions; the water is removed from the frozen substance by direct sublimation of ice.

Gel-like solution is poured into containers (100 ml per container), freezed at -40ºC and placed in a vacuum chamber. Total load volume is 0.5 l (500 g). Two freeze-drying cycles are performed, each is about 20-24 hours. As a result, a lyophilized mass is obtained (14.8 g per container).

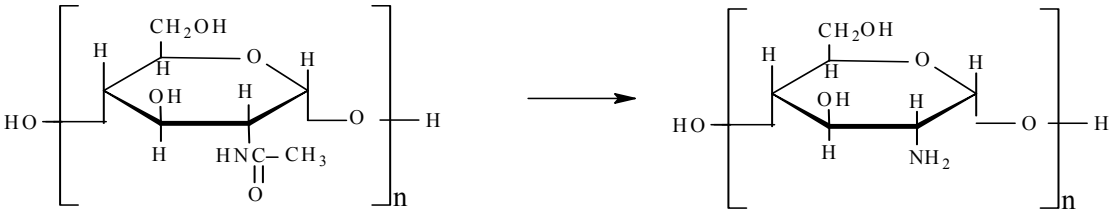
Proteolytic activity assessment was performed both in films and in lyophilizates (Table 2).

Table 1 - Comparative evaluation of the proteolytic activity of immobilized proteases on a step of obtaining a chitosan-based gel.

Sample	Concentration of enzyme	Proteolytic activity, PU / ml
Chitosan – trypsin Chitosan 0,5 wt%	0,1 %	1,50
	0,2 %	1,60
	0,3 %	1,65
Chitosan – trypsin Chitosan 1,0 wt%	0,1 %	1,55
	0,2 %	1,67
	0,3 %	1,65
Chitosan – trypsin Chitosan 1,5 wt%	0,1 %	1,80
	0,2 %	2,50
	0,3 %	2,55
Chitosan – PC Chitosan 0,5 wt%	0,1 %	1,80
	0,2 %	1,90
	0,3 %	1,95
Chitosan – PC Chitosan 1,0 wt%	0,1 %	1,80
	0,2 %	2,50
	0,3 %	2,60
Chitosan – PC Chitosan 1,5 wt%	0,1 %	1,90
	0,2 %	1,85
	0,3 %	1,95
Chitosan – chymopsin Chitosan 0,5 wt%	0,1 %	1,90
	0,2 %	2,00
	0,3%	2,25
Chitosan – chymopsin Chitosan 1,0 wt%	0,1 %	2,00
	0,2 %	2,70
	0,3%	2,85
Chitosan – chymopsin Chitosan 1,5 wt%	0,1 %	2,10
	0,2 %	2,65
	0,3 %	2,75

Table 2 - Comparative evaluation of proteolytic activity of samples in the form of lyophilizate.

Sample	Concentration of enzyme	Proteolytic activity, PU / ml
Chitosan – trypsin Chitosan 0,5 wt%	0,1 %	1,50
	0,2 %	1,56
	0,3 %	1,60
Chitosan – trypsin Chitosan 1,0 wt%	0,1 %	1,55
	0,2 %	1,65
	0,3 %	1,60
Chitosan – trypsin Chitosan 1,5 wt%	0,1 %	1,80
	0,2 %	2,30
	0,3 %	2,50
Chitosan – PC Chitosan 0,5 wt%	0,1 %	1,70
	0,2 %	1,90
	0,3 %	1,90
Chitosan – PC Chitosan 1,0 wt%	0,1 %	1,81
	0,2 %	2,40
	0,3 %	2,60
Chitosan – PC Chitosan 1,5 wt%	0,1 %	1,75
	0,2 %	2,50
	0,3 %	2,55
Chitosan – chymopsin Chitosan 0,5 wt%	0,1 %	1,90
	0,2 %	2,00
	0,3 %	2,00
Chitosan – chymopsin Chitosan 1,0 wt%	0,1 %	1,90
	0,2 %	2,65
	0,3 %	2,80
Chitosan – chymopsin Chitosan 1,5 wt%	0,1 %	2,10
	0,2 %	2,60
	0,3 %	2,75



Chitin **chitosan**

Figure 1. Chitin deacetylation product.

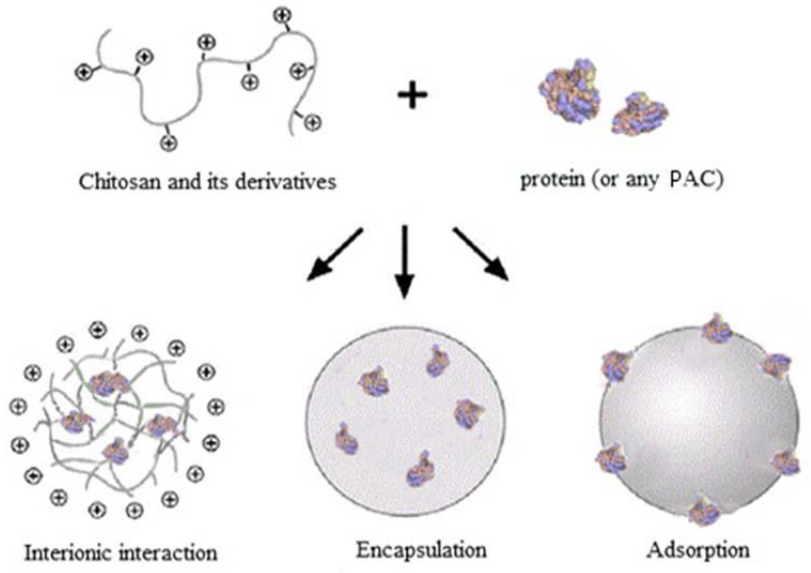


Figure 2. Main mechanisms of interaction between chitosan and proteins.

Proteolytic activity of the samples after drying by lyophilization remained practically unchanged.

Optimal conditions for obtaining of immobilized drugs were chosen based on the effect of mode of immobilization and storage on the biological activity of the developed carrier-enzyme complexes.

Enzyme inactivation kinetics which occur during immobilization, drying, and storage, is described by a complex equation, characteristic for hydrolases:

$$\boldsymbol{A/A_0 = x_1 e^{-k(1)\cdot t} + x_2 e^{-k(2)\cdot t}} \quad (1)$$

Effective inactivation rate constant (k_{in}) was determined as the slope of the linear plot of natural logarithm of the residual activity value versus time (similarly in semilog coordinates $\ln(A/A_0)$-τ). A specially performed tests have shown that the amount of enzyme in a sample does not affect residual enzymatic activity (both for immobiziled and non-modified proteolytic complex or chitosan).

Quantitative measure of stabilization during thermal inactivation is the temperature stabilization value (Θ_t) which represents ratio of inactivation rate constants of native and immobilized enzyme at a given temperature. If $\Theta_t>1$ then immobilization enhances thermal stability of enzymes. Calculation of Θ value of immobilized products should be performed using k_2.

Pharmaceutical substance thermostability was assessed at different pH values. Test tubes with samples were placed in a preheated water bath. The samples were regularly withdrawed and the residual activity of the enzyme was measured.

A CCC manufacturing process includes the following stages:

1. Preparation of chitosan water solution. 10.0 g of chitosan are placed into the reactor, 5.7 ml (6.0 g) of 0.5% acetic acid solution (d=1.05 kg/l) are added to the reactor, then the content if thoughtfully mixed and left for 5-10 minutes for wetting and swelling of chitosan. After that, purified water is slowly added to the reactor under constant mixing, up to a 1.0 l volume. Obtained suspension was intensively mixed for 1.5-2.0 hours until chitosan is almost completely dissolved and was left at room temperature for 10-12 hours until homogenous gel-like solution is obtained. pH of the obtained solution is 4.5-5.5.
2. Immobilization of enzyme on chitosan. 2.0 g of chymopsin are addedd to the prepared chitosan solution. The mixture is constantly mixed for 0.5-1.0 hour until chymopsin is completely dissolved. Immobilization is carried out at room temperature. Mass ratio of chitosan and chymopsin is 5:1, pH of the solution is 4.5-5.5. A 1000 g of gel-like CCC solution is obtained.
3. CCC freeze-drying. CCC substance is obtained by freeze-drying of gel-like solution under vaccum. Water vapour is evacuated from the chamber by vacuum pump and is condensed on a coil of low-temperature condenser. In order to prevent loss of lyophilized mass during packaging,

Gel-like solution is poured into containers (100 ml per container), freezed at -40ºC and placed in a vacuum chamber. Total load volume is 0.5 l (500 g). Two freeze-drying cycles are performed, each is about 20-24 hours. As a result, a lyophilized mass of CCC substance in form of thin plates is obtained (14.8 g per container).

DISCUSSION

Thus, as a result of series of experiments the properties of a novel pharmaceutical substance, its quantitative and qualitative composition were assessed and its manufacturing technology was trialed.

The following conclusions can be drawn from a series of experiments aimed at selection of proteolytic enzyme, its concentration and concentration of chitosan, and optimization of composition formulation and its performance parameters:

- physical immobilisation followed by freeze-drying is the optimal method for obtaining chymopsin-containing composition;
- chitosan is the polymer of choice for carrying enzyme;
- optimal mass ratio of chymopsin and chitosan is 1:5;
- Preferred CCC compositoon (amount, in wt%):
  Chymopsin – 0.2
  Chitosan – 1.0
  Acetic acid – 0.6
  Purified water – up to 100.0
- Quality control. Residual moisture content is determined in obtained substance (according to State Pharmacopoeia XI ed. should not be exceed 10%). Qualitative reaction for chitosan was also performed: sample under test should produce violet colour after addition of iodine solution containing potassium iodide in a 0.13 M nitric acid.

Qualitative reaction for chymopsin is also performed. The sample under test should be able to curdle milk. Curdling time for CCC substance should not exceed 50 seconds.

Quantitative assessment is performed by determining protein content by Lowry et. al. and proteolytic activity determination. CCC substance should contain 1.95±0.5 g of protein (chymopsin) per 10 mg of substance.

Proteolytic activity of CCC solution is 2.75 PU/ml. Proteolytic activity of lyophilized CCC subtance at the end of the shelf life should not be less than 0.1 PU/mg.

CONCLUSIONS

Developed novel drug substance can be used either alone or together with antiseptic and anesthetic agents which substantially encreased API performance.

Possible dosage forms for the substance may include ointment, spray, cream, or gel. The latter is preferred over the other ones since it is water-based thus keeping wound moist, decreasing local temperature and providing additional pain relief.

Preferable composition and substance production conditions suggest that API production is low-cost and can be scaled up for commercial manufacturing.

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