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Characterisation of salmon calcitonin in spray-dried powder for inhalation Effect of chitosan

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Abstract

Salmon calcitonin (sCT) powders suitable for inhalation, containing chitosan and mannitol as absorption enhancer and protection agent, respectively, were prepared using a spray-drying process. The effect of chitosan on physicochemical stability of sCT in the dry powder was investigated by different analytical techniques. High-performance liquid chromatography (HPLC) analysis indicated that sCT was chemically stable upon spray-drying. With the proportion of chitosan in spray-drying formulation being increased, dissolution of sCT from the dry powders was decreased both in phosphate buffer and acetate buffer. The thioflavine T fluorescence assay showed that no fibrils were present in the spray-dried powder. However, sCT partly fibrillated in the phosphate buffer, but not in acetate buffer. Fourier transform infrared (FTIR) spectra showed that the secondary structure of sCT was slightly changed in the dry powder, yet no aggregate signal was observed. Circular dichroism analysis indicated that the structure of sCT in an aqueous formulation was slightly altered by addition of chitosan. Nevertheless, recovery of sCT was not influenced by chitosan in the aqueous formulation as indicated by HPLC analysis. This study suggested that sCT, in absence of any additives, was stable during the spray-drying process under certain conditions. Addition of chitosan affects recovery of sCT from spray-dried powders, which may be due to formation of a partially irreversible complex between the protein and chitosan during the spray-drying process.

Keywords: Salmon calcitonin; Spray-drying; Chitosan; Physicochemical characterization

1. Introduction

Proteins and peptides drugs have been the most rapidly growing class of drugs in the last two decades (van de Weert et al., 2005; Walsh, 2005). Due to their inherent physicochemical instability and limited transport through biomembranes, proteins and peptides are mainly delivered parenterally, which causes very poor patient compliance. Therefore, safe and effective noninvasive administration routes are required as an alternative to injection. It is generally realized that pulmonary administration is superior to other noninvasive routes such as nasal, dermal, ocular or oral, owing to its relatively high bioavailability, limited side effects, rapid and sustained benefits, good long-term compliance, and ease of use (Byron and Patton, 1994; Service, 1997; Patton, 1998).

As protein and peptide drugs are more stable in the solid state than in the liquid state, a dry powder formulation may be more feasible than a liquid or suspension one for delivering proteins and peptides to the lung. Spray-drying is one of the most popular techniques to produce a dry protein powder suitable for inhalation in one single step (Maa and Prestrelski, 2000). Since thermal and shearing stresses are present in the spray-drying process, chemical and physical degradation of proteins and peptides may take place and thereby result in safety and efficacy problems. Thus, it is important to characterize proteins and peptides both in the spray-dried product and after release as well as understanding the basic principle for degradation during the manufacturing and application.

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Salmon calcitonin is a polypeptide of 32 amino acids with a 1–7 disulphide bond. It is currently formulated as a sterile solution for intramuscular or subcutaneous injection in the management of several bone-related diseases including Paget's disease, hypercalcemia and osteoporosis (Patton, 2000). A needle-free formulation with good patient compliance would be desirable to extend its application. In this study, sCT was spray-dried into powder suitable for inhalation, with mannitol and chitosan as excipients. Mannitol was used as a stabilizer, and chitosan, a biodegradable mucoadhesive polysaccharide was employed as absorption enhancer. Since chitosan exhibits interesting properties in the controlled-release and absorption promotion for active proteins and peptides, it has been formulated with many biomacromolecular drugs including vaccines and genes, and applied to different administration routes, such as the gastrointestinal tract, nasal cavity and lungs (Alpar et al., 2005; Yamamoto et al., 2005; Grenha et al., 2005). Yet, few studies have reported on the interaction between the chitosan and the biomacromolecular drugs in the solid formulation, which might affects release and physicochemical stability of these active pharmaceutical ingredients. This paper will focus on understanding the effect of chitosan on the physicochemical properties of sCT during spray-drying.

2. Material and experiments

2.1. Materials

Salmon calcitonin was kindly supplied by Polypeptide Laboratories A/S (Hillerod, Denmark). Mannitol was purchased from Sigma (Seelzee, Germany). Chitosan with a M_W of 150 kDa and greater than 85% deacetylated was purchased from SeeLab (Wesselburenerkoog, Germany). Other reagents and chemicals were of analytical grade or chromatography grade.

2.2. Preparation of spray-dried powder

For this study, six aqueous formulations containing 1:0:0, 1:0:18, 1:1:18, 1:2:17, 1:3:16 and 1:4:15 (w/w) of sCT:chitosan:mannitol were spray-dried into powder using a B-290 Büchi Mini Spray-Drier (Flawil, Switzerland). The processing conditions are listed in Table 1. The formulation of 1:1:18 of sCT:chitosan:mannitol was used as a reference throughout this paper unless otherwise indicated.

Table 1
Spray-drying process condition of B-290 Büchi Mini Spray Drier

Condition	Parameter
Atomizing air volumetric flow rate	357 L/h
Feeding rate	5 mL/min
Aspirator rate	80%
Inlet (outlet) temperature	140 (74) °C
Solid concentration	1%
pH of feeding solution	4.4–4.8

2.3. High-performance liquid chromatography (HPLC) analysis of protein chemical stability

About 4.0 mg of spray-dried powders was weighed into 1.5 mL Eppendorf Protein LoBind Tubes (Hamburg, Germany) and mixed with 1 mL of phosphate buffer (pH 7.4, 50 mM) or acetate buffer (pH 4.4, 50 mM). The resultant suspension or solution was rotated at a speed of 19 rpm for 120 min. Centrifugation was then performed at a speed of 15,000 rpm for 10 min. The supernatant was analyzed by the HPLC system under conditions described below.

The HPLC system consisted of two pumps (Programmable Solvent Module 126, Beckman, Fullerton, California, USA), a gradient controller (Programmable Solvent Module 126, Beckman, Fullerton, California, USA), an autosampler injector (717 plus, Waters, Milford, Massachusetts USA), an ODS C-18 column (5 μm, 250 mm × 4.6 mm, LiChrospher®, Darmstadt, Germany), a UV detector set to 220 nm (Programmable Detector Module 166, Beckman, Fullerton, California, USA), and a computer system with Gold Nouveat 1.72 (Beckman Instrument, Inc., Fullerton, California, USA). A linear gradient was used from 30 to 55% A in 25 min. Mobile phase A was 0.1% trifluoroacetic acid (TFA) in Milli-Q water and mobile phase B was 0.1% TFA in acetonitrile. The injection volume was 40 μL and the flow rate was 1.5 mL/min.

2.4. Fourier transform infrared (FTIR)

FTIR-spectra were collected on a Bomem IR-spectrometer (Bomem, Quebec, Canada). KBr pellets were prepared by admixing ca. 4 mg spray-dried powders containing approximately 0.2 mg of sCT with ca. 300 mg of spectroscopy-grade KBr. The mixture was then pressed into a 13 mm disk at 4-tons pressure with a die press. For each spectrum, a 256-scan interferogram was collected in single-beam with 4 cm^{-1} resolution at room temperature. The spectra of the excipients and water vapor were subtracted from the protein spectra separately. The second derivative spectra were obtained with a nine-point Savitsky-Golay derivative function and the baseline was corrected using a two-point adjustment. In addition, spectra were area-normalized in the amide I region from 1600 to1700 cm^{-1} using the Bomem-Grams software (Galactic Industries, Salem, NH). The spectra obtained for the different formulations were compared using the area overlap method described by Kendrick et al. (1996), where identical spectra give a value of 1.0.

2.5. Thioflavin T (ThT) fluorescence assays for fibrillation

A stock solution of ThT was prepared at a concentration of 1 mM in milli-Q water and stored at 4 °C protected from light until use to prevent photobleaching.

The fibrillation of sCT in liquid or solid samples was studied in 1.5 mL Eppendorf Protein LoBind Tubes by dispersing spray-dried powders or neat sCT in 0.5 mL of either phosphate buffer or acetate buffer. After 2-h incubation, the samples were centrifuged to separate supernatant and pellet. Fifty microlitres of the supernatant was directly added to the fluorescence cuvette

(1 cm path length semi-micro quartz cuvette) containing 1 mL of a ThT mixture (20 μM ThT). The pellet was re-dispersed in 1 mL ThT solution for fluorescence determination.

The fluorescence measurements were performed using a Spex Fluorolog 3-22 (Jobin Yvon Horiba, Longjumeau, France) with a 450-W Xenon lamp. Emission spectra were recorded immediately after addition of the aliquots to the ThT solution from 470 to 560 nm (excitation at 450 nm, 1 nm step size, 1 s integration time, and slits of 5 nm for both excitation and emission). For each sample, the signal was obtained as the ThT intensity at 482 nm from which was subtracted a blank measurement recorded prior to addition of sample to the ThT solution. Data were processed using DataMax/GRAMS software.

2.6. Circular dichroism

To study the effect of chitosan on sCT in liquid state, the neat sCT solution was freshly prepared and mixed with different concentration of chitosan in acetate buffer (50 mM). The circular dichroism (CD) spectra of sCT were collected at ambient temperature in square quartz cells (path length 0.1 cm) with a dual-beam DSM 10 circular dichroism spectrophotometer (On-Line Instrument systems, Bogart, GA, USA). The subtractive double-grating monochromator was equipped with a fixed disk with holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24 mm slits. Far-UV spectra were taken from 260 to 195 nm. Each measurement was the average of five repeated scans (step resolution 2 nm, 6 s each step) from which the corresponding solvent spectrum was subtracted. The measured CD signals were converted to molar extinction coefficient ($\Delta\varepsilon$) based on a mean residual weight of sCT of 107.2 g/residue. The sCT concentration in the liquid samples was 0.20 mg/mL. All CD measurements were carried out on freshly prepared protein solutions.

3. Results and discussion

3.1. Chemical integrity of sCT in spray-dried powder

The chemical integrity of sCT in spray-dried powder was studied by RP-HPLC. To maximize the extraction of sCT from spray-dried powders, the powders were incubated in both phosphate buffer and acetate buffer for 2 h.

The results (Fig. 1) show that in the absence of excipients sCT could be recovered completely from spray-dried powder in acetate buffer. In contrast, in phosphate buffer, the recovery of sCT was less than 90%.

In the presence of excipient, the recovery of sCT from the dried powder was decreased with an increase in proportion of chitosan in the formulation both in acetate buffer and in phosphate buffer. Even though no significant difference could be found between any adjacent two formulations, statistical differences could be found when sCT:chitosan:mannitol was equal to 1:4:15, and 1:3:16 formulation were compared to 1:1:18 formulation ($p < 0.05$). Further, an apparent reduced recovery of sCT was observed when the proportion of chitosan was increased.

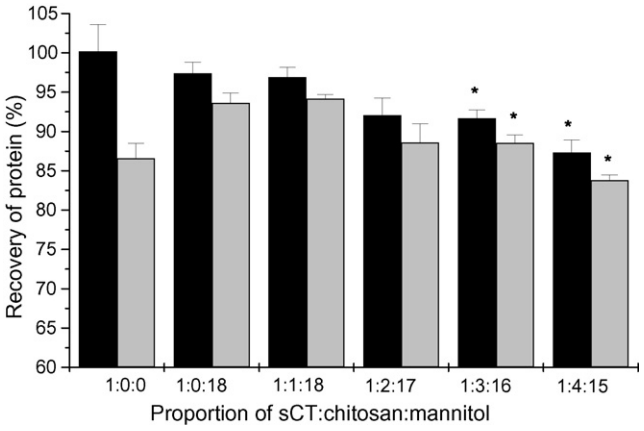

Fig. 1. Recovery of sCT from spray-dried powder (SDP) in different buffers after 2 h: black columns are data from acetate buffer (pH 4.4), and gray column are data from phosphate buffer (pH 7.4). $^{*}p < 0.05$, compared to sCT:chitosan:mannitol = 1:1:18 formulation.

The reduced recovery in phosphate buffer can possible be explained by the fact that chitosan does not dissolve at pH 7.4, which impeded release of sCT by forming gel-like pellets. However, sCT is expected to be recovered completely in the acetate buffer (pH 4.4) as long as it is stable enough during the spray-drying process, as chitosan completely dissolves at this acidic condition. In another control formulation, when mannitol was the only additive in the solution, a relative high recovery of protein was achieved. Therefore, the result from HPLC analysis suggested that chitosan negatively affects the recovery of sCT from spray-dried powder to some extent.

Several degradation pathways might be involved to compromise sCT integrity during the spray-drying in the presence of chitosan:

1. chitosan might influence chemical stability of protein causing degradation of sCT;
2. chitosan might be detrimental to physical stability of sCT, leading to irreversible aggregation or fibrillation of sCT;
3. or some irreversible complex might be formed by the interaction between sCT and chitosan, which negatively affects reconstitution of sCT in aqueous solution.

As no obvious degradation peaks were found in the HPLC chromatograms (data not shown), this suggests that some physical degradation takes place.

3.2. Thioflavin T fluorescence assays for fibrillation

The histological dye ThT has been widely used to detect amyloid fibrils of proteins (LeVine, 1993, 1995). A 450 nm fluorescence excitation maximum will appear when ThT binds to amyloid fibrils, and an enhanced emission can be determined at 482 nm, whereas unbound ThT is essentially nonfluorescent at these wavelengths. This assay has been employed to detect the presence of amyloid fibrils of human and salmon calcitonin previously (Khurana et al., 2005). In this study, as no significant enhanced emission fluorescence was observed in terms of

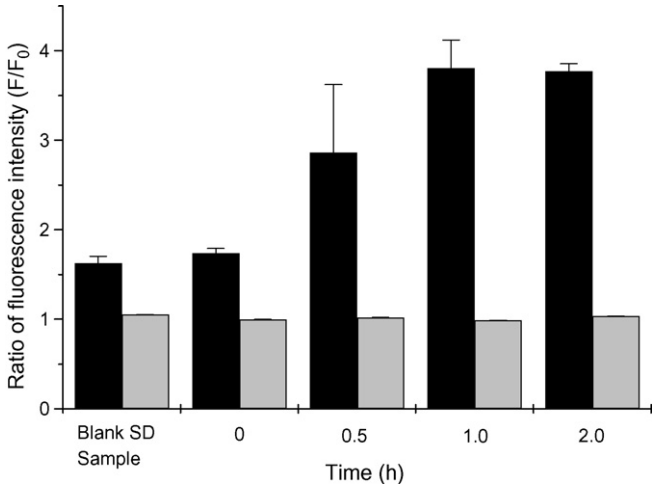

Fig. 2. ThT fluorescence assay of SDP (sCT:chitosan:mannitol = 1:1:18 formulation) in different buffers: black column are data from phosphate buffer, and gray column are data from acetate buffer. F: fluorescence intensity of ThT solution after adding fibril suspension; F_0: fluorescence intensity of ThT solution before adding fibril suspension.

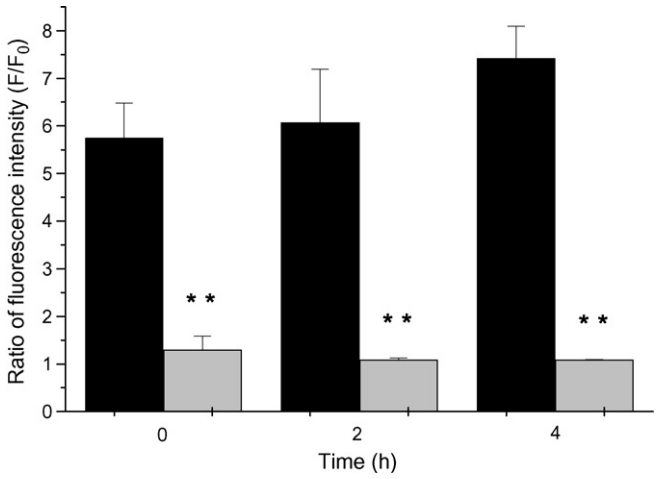

Fig. 4. ThT fluorescence assay on sCT fibrils incubated in different buffers: black column are data from phosphate buffer, and gray column are data from acetate buffer. $^{**}p < 0.01$, compared to black column. F: fluorescence intensity of ThT solution after adding fibril suspension; F_0: fluorescence intensity of ThT solution before adding fibril suspension.

supernatant samples, the results reported here were derived from redispersion of pellet in ThT solution.

As indicated in Fig. 2, compared with blank spray-dried powder, no enhanced ThT emission fluorescence intensity was observed when spray-dried powders containing sCT were incubated in acetate buffer for up to 2 h. In contrast, a pronounced enhancement of ThT fluorescence intensity was observed in phosphate buffer already after 30 min. However, ThT fluorescence intensity did not show any increase when the spray-dried powder was reconstituted in phosphate buffer and directly measured, as compared with the blank spray-dried powder. This indicated that no detectable fibrils were found in spray-dried products.

As shown in Fig. 3, sCT readily fibrillates when incubated in phosphate buffer, both in the presence and absence of a blank

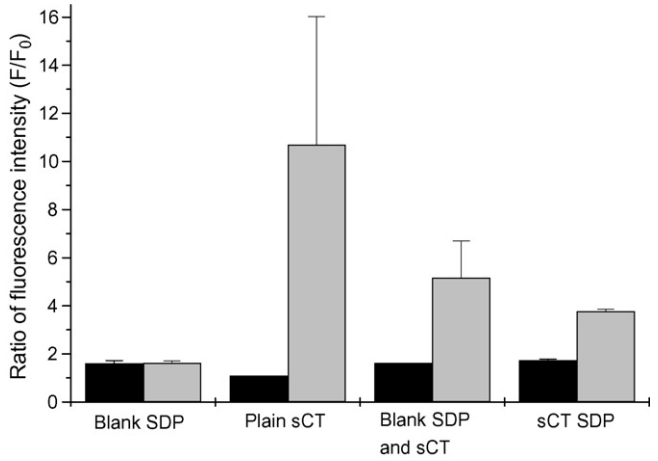

Fig. 3. ThT fluorescence assay of different samples incubated in phosphate buffers for 2 h: black column are data before incubation, and gray column are data after 2 h incubation. F: fluorescence intensity of ThT solution after adding fibril suspension; F_0: fluorescence intensity of ThT solution before adding fibril suspension.

spray-dried powder containing mannitol and chitosan. The final intensity is lower in the presence of the blank spray-dried powder, which may suggest that the addition of excipients such as mannitol and/or chitosan might reduce the fibrillation rate of sCT in phosphate buffer. The effect of these excipients on fibrillation of sCT remains to be investigated further.

A ThT fluorescence assay was further carried out to investigate sCT fibrils in different buffers. sCT fibril suspension was prepared by incubation of 10 mg/mL sCT aqueous solution in 1.5 mL Eppendorf Protein LoBind Tube overnight. After a same amount of fibril suspension was withdrawn and transferred to either phosphate buffer or acetate buffer for incubation, the ThT fluorescence assay was performed to detect fibrils by sampling at different time points. As shown in Fig. 4, after 2 h, the ThT fluorescence intensity had decreased in acetate buffer, while the intensity gradually increased in phosphate buffer. This suggests that sCT fibrils formation is reversible in acetate buffer. This also explains why sCT amyloid fibrils were only found during the dissolution test in phosphate buffer, and not in acetate buffer. These findings are consistent with previous reports that sCT is more stable at acidic condition than at neutral conditions (Lee et al., 1992; Stevenson and Tan, 2000).

To summarize, the results in the ThT fluorescence assays demonstrate that the reduced dissolution of sCT from spray-dried powder in phosphate buffer may be due to partial fibrillation of sCT during incubation. However, the decrease in recovery of sCT from spray-dried powder in acetate buffer still remains unclear, since the protein fibrils are reversible in this buffer. Hence, FTIR and CD were subsequently employed to investigate the structural integrity of the protein in different chitosan formulations.

3.3. FTIR analysis

The secondary structure of sCT in spray-dried powders was studied by FTIR. FTIR has been extensively used to study struc-

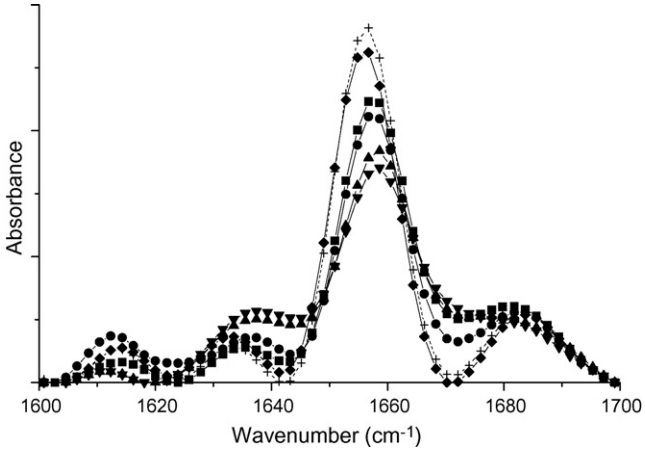

Fig. 5. FTIR spectra of sCT and its spray-dried products: (♦) sCT as received; (■) sCT:chitosan:mannitol = 1:0:18; (●) sCT:chitosan:mannitol = 1:1:18; (▲) sCT:chitosan:mannitol = 1:3:16; (▼) sCT:chitosan:mannitol = 1:4:15; (+) sCT:chitosan:mannitol = 1:0:0.

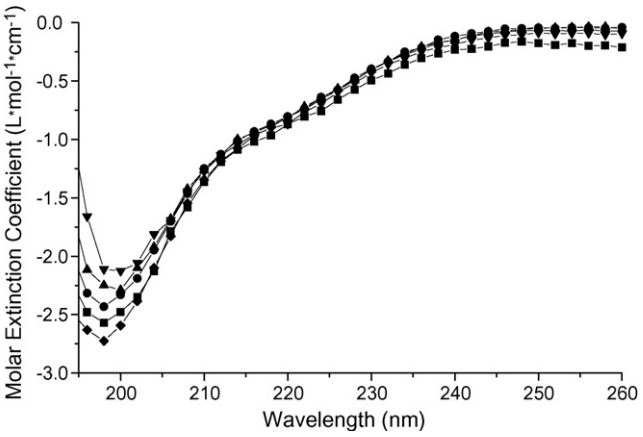

Fig. 6. Effect of chitosan on the sCT circular dichroism spectra in acetate buffer (pH 4.4): (♦) sCT 200 ug/mL; (■) sCT 200 μg/mL and chitosan 500 μg/mL; (●) sCT 200 μg/mL and chitosan 1000 μg/mL; (▲) sCT 200 μg/mL and chitosan 1500 μg/mL; (▼) sCT 200 μg/mL and chitosan 2000 μg/mL.

tural changes of protein in solid formulations (Dong et al., 1995; Carpenter et al., 1998). The structural changes can be monitored conveniently in the amide I, II or III region, where amide I (1600–1700 cm^{-1}) is the most commonly exploited region. As illustrated in Fig. 5, no obvious structural changes of sCT were found when it was spray-dried solely, as compared with the FTIR spectra of sCT as received from the supplier. Only a slight increase in α-helical content (1656–1658 cm^{-1}) was observed in the spectra of plain sCT spray-dried powder, which is probably due to the fact that the solid-state structure of sCT became more compact upon spray-drying. However, a relatively pronounced decrease in α-helical content of sCT was observed in other spray-dried powders with an increase in proportion of chitosan. Compared with sCT as received, the overlap area of the FTIR spectra of the spray-dried powders with that of the sCT as received was reduced with the proportion of chitosan in spray-dried formulation being increased. This reduction correlates with the reduced recovery of sCT from the dry powder in acetate buffer ($r^2 = 0.92$). In addition, no aggregate signal of protein was observed in any of the FTIR spectra, which would be expected around 1625–1630 cm^{-1} (REF). These findings, in parallel with the results from HPLC analysis and ThT fluorescence assays, suggested that spray-drying affected the structural integrity of sCT in the presence of chitosan.

3.4. Circular dichroism spectroscopy

Circular dichroism spectroscopy was used to study the effect of chitosan on the secondary structure of sCT in aqueous formulation. As shown in Fig. 6, sCT has little ordered secondary structure in aqueous solution, which is consistent with other reports (Arvinte and Drake, 1993).

A clear decrease in molar ellipticity at ca. 198 nm was observed in the presence of chitosan. Also, a shift of the minimum was observed with the concentration of chitosan in acetic buffer being increased. These results suggest that a change in secondary structure of sCT occurred upon the addition of chitosan. Subsequently, HPLC was employed to study recovery of sCT from chitosan solution. Interestingly, unlike the spray-dried powder, sCT could be recovered completely from chitosan solution. These findings suggested that chitosan apparently influences the structure of sCT in aqueous formulation, yet the interaction between chitosan and sCT does not compromise the protein solubility.

The p*I* value of sCT is around 10.4. Thus, it is expected to be positively charged, just like chitosan, at acetic and neutral conditions. Repulsive electronic interactions between sCT and chitosan may possibly influence the secondary structure of sCT. However, chitosan may possess various conformations and different charge distribution on its polymer chain under different conditions. Therefore, the interaction between sCT and polymer may be more complicated and unpredictable. The negative effect of chitosan on recovery of sCT from spray-dried powder may thus be due to the interaction between chitosan and sCT being strengthened by dehydration during the spray-drying process, and some irreversible complex may have been formed in the spray-dried powder.

4. Conclusion

Salmon calcitonin, in absence of any excipients, can be spray-dried into powder with fine structural and chemical integrity under certain conditions. Addition of chitosan in the formulation decreased the recovery of sCT from the spray-dried powders, which is probably due to interaction between chitosan and sCT being strengthened by dehydration during the spray-drying, and some irreversible complex may form in the dry powder. Chitosan has slight effect on secondary structure of sCT in an aqueous formulation, but it is not detrimental to chemical integrity of sCT under the conditions studied. The presence of mannitol and/or chitosan decreases the fibrillation rate of sCT in phosphate buffer. The effect of these excipients on fibrillation of sCT is subject to further investigation.

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References

Alpar, H.O., Somavarapu, S., Atuah, K.N., Bramwell, V.W., 2005. Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. Adv. Drug Deliv. Rev. 57, 411–430.

Arvinte, T., Drake, A.F., 1993. Comparative study of human and salmon calcitonin secondary structure in solutions with low dielectric constants. J. Biol. Chem. 268, 6408–6414.

Byron, P.R., Patton, J.S., 1994. Drug delivery via the respiratory tract. J. Aerosol Med. 7, 49–75.

Carpenter, J.F., Prestrelski, S.J., Dong, A., 1998. Application of infrared spectroscopy to development of stable lyophilized protein formulations. Eur. J. Pharm. Biopharm. 45, 231–238.

Dong, A., Prestrelski, S.J., Allison, S.D., Carpenter, J.F., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–423.

Grenha, A., Seijo, B., Remuñán-López, C., 2005. Microencapsulated chitosan nanoparticles for lung protein delivery. Eur. J. Pharm. Sci. 25, 427–437.

Kendrick, B.S., Dong, A., Allison, S.D., Manning, M.C., Carpenter, J.F., 1996. Quantitation of the area of overlap between second-derivative amide I infrared spectra to determine the structural similarity of a protein in different states. J. Pharm. Sci. 85, 155–158.

Khurana, R., Agarwal, A., Bajpai, V.K., Verma, N., Sharma, A.K., Gupta, R.P., Madhusudan, K.P., 2005. Unraveling the amyloid associated with human medullary thyroid carcinoma. Endocrinology 145, 5465–5470.

Lee, K.C., Lee, Y.J., Song, H.M., Chun, C.J., DeLuca, P.P., 1992. Degradation of synthetic salmon calcitonin in aqueous solution. Pharm. Res. 9, 1521–1523.

LeVine III, H., 1993. Thioflavine T interaction with synthetic alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution. Protein Sci. 2, 404–410.

LeVine III, H., 1995. Thioflavine T interaction with amyloid β-sheet structures. Amyloid: Int. J. Exp. Clin. Invest. 2, 1–6.

Maa, Y.F., Prestrelski, S.J., 2000. Biopharmaceutical powders: particle formulation and formulation considerations. Curr. Pharm. Biotechnol. 1, 283–302.

Patton, J.S., 1998. Breathing life into protein drugs. Nat. Biotechnol. 16, 141–143.

Patton, J.S., 2000. Pulmonary delivery of drugs for bone disorders. Adv. Drug Deliv. Rev. 42, 239–248.

Service, R.F., 1997. Drug delivery takes a deep breath. Science 277, 1199–1200.

Stevenson, C.L., Tan, M.M., 2000. Solution stability of salmon calcitonin at high concentration for delivery in an implantable system. J. Pept. Res. 55, 129–139.

van de Weert, M., Jorgensen, L., Moeller, E.H., Frokjaer, S., 2005. Factors of importance for a successful delivery system for proteins. Expert Opin. Drug Deliv. 6, 1029–1037.

Walsh, G., 2005. Biopharmaceuticals: recent approvals and likely directions. Trends Biotechnol. 23, 553–558.

Yamamoto, H., Kuno, Y., Sugimoto, S., Takeuchi, H., Kawashima, Y., 2005. Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. J. Control. Release 102, 373–381.