Instability Studies of Porcine Somatotropin in Aqueous Solutions and the Possible Reagents for Its Stabilization

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The instability of porcine somatotropin (pST) in various solutions and possible stabilization of the hormone by sugars and mild detergents were studied. Aggregation and decomposition of the hormone molecules in various pH solutions and under presence of sugar or detergent were monitored by gel permeation chromatography (GPC) or ultraviolet spectroscopy (UV). The pST is a very unstable hormone in an aqueous environment. It was found in this project that the peptide hormone underwent aggregation or decomposition quickly in acidic and alkaline solutions but slowly in neutral pH solutions. High losses of pST monomers were seen in concentrated solutions of the hormone. On the other hand, pST monomers were stabilized to a certain degree in glucose solutions and at a low concentration of urea. These results should facilitate the development of efficient controlled-release systems which are essential for commercializing porcine somatotropin.

Keywords: *Porcine somatotropin; aggregation; stabilization; controlled release*

INTRODUCTION

Somatotropin, also known as growth hormone, is a 191 amino acid peptide with a molecular weight of appropriately 22 000 Daltons (slight variation exists among animal species) that is secreted by the anterior pituitary gland in animals and human beings. It is required for normal development during early life and is involved in regulation of a variety of anabolic processes (Chung et al., 1985). Recently, the application of recombinant DNA techniques to clone and express the gene for this hormone has made it possible to obtain an unlimited amount of the hormone, and provides an opportunity to investigate its new applications such as growth stimulation and other physical effects on animals after large-dosage administration (Abdel-Meguid et al., 1987; Pitt, 1993; Seeburg et al., 1983).

It has been known for many years that exogenous administration of porcine somatotropin (pST) stimulates protein synthesis and inhibits lipogenesis, and therefore, can effectively improve growth rate, feed efficiency, and carcass leanness in finishing pigs (Seve et al., 1993; Mikel et al., 1993; Chung et al., 1985; Evock et al., 1988; Verstegen et al., 1991; Jones et al., 1994). Administration of pST to growing pigs could reduce carcass fat content by over 50% and improve production efficiency by 15 to 35% (Etherton et al., 1993). Because meat is a major source of total fat and saturated fatty acids in the diets of human beings, using pST would allow consumers to include leaner, more nutrient-dense pork in their diets and still meet current dietary guidelines. Because of unknown improvements in amino acid utilization and in the energy available for protein synthesis, only marginal increases in dietary protein percentage were required to support 20 to 25% improvements in protein deposition induced by pST administration (Campbell et al., 1991). Also, there were no observed differences in the overall health and immunofunction of the pigs due to pST treatment, on the basis of clinical observations as well as determination of antibody titer to, and isolation of, common swine pathogens (Goff et al., 1991).

Nowadays, daily administration of pST to livestock is impractical for large-scale farm production, which requires a device that, once implanted in the animal, delivers the protein at a steady rate over a period of 4−6 weeks (Hageman et al., 1992; Arnold, 1988). The pursuit of a controlled-release system for pST has therefore been a key component in development of the hormone into commercial products. Unfortunately, the development of such a system for the efficient delivery of somatotropin is complicated by the instability of the protein upon exposure to water, especially at physiological conditions of pH 7.4 and 37 °C. The hormone tends to undergo both aggregation and decomposition in high concentration solutions and physiological temperatures, resulting in irreversible aggregation, destruction of intact protein, and loss of its biological activity (Buckwalter et al., 1992; Pitt, 1993; Ron et al., 1993). Hydration of the release system will also result in hydration of the protein, and subsequent aggregation through formation of disulfide cross-links or isopeptide bonds and through hydrophobic reactions as well as degeneration by unfolding is expected to be rather rapid (Hageman et al., 1992; Bastiras and Wallace, 1992; Bewley and Li, 1972; Brems et al., 1986).

Therefore, a prerequisite for successful development of a pST delivery system is to find a way to stabilize the hormone in solutions and in the controlled release devices. It has been widely reported that pST was much more stable and still in its native structure after being precipitated by a bivalent ion such as zinc or copper (Mitchell, 1995; Azalin et al., 1989). When redissolved, such bivalent ion-precipitated pST could regain its biological activity. Stabilizing pST through this way (bivalent ion precipitation) was found to be useful when

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a biocompatible oil (sometimes in the presence of an antihydration agent) or a reservoir formed with one to several layers of relatively hydrophobic polymers such as paraffin or cellulose acetate (Mitchell, 1995; Azalin et al., 1989; Kim et al., 1996; Raman et al., 1994; Magruder et al., 1991; McCoy and Frost, 1991; Klindt et al., 1995) was used to develop the controlled-release system. The precipitated pST can be dispersed in the oil or put in the reservoir of these systems. However, when a polymer matrix or hydrogel delivery system is preferred, stabilizing pST through bivalent ion precipitation is no longer suitable, because it is impossible to entrap the precipitated pST into a hydrogel or polymer matrix molecularly evenly because of the large size of a precipitate and the limited pore size of the matrix network. Therefore, it is essential to find other ways to stabilize pST for release through these polymer matrix or hydrogel-based delivery systems.

Sugars have been used for decades to preserve the structure and activity of proteins during rehydration and dehydration processes (Capenter et al., 1993). During protein lyophilization, sugars can replace water to form hydrogen bonding to the dried protein. In solutions, sugars are expected to bind to the polar and charged groups and thereby stabilize the protein by preventing unfolding. Another kind of stabilizer is ionic detergent which can bind to proteins through a combination of electrostatic and hydrophobic interactions (Subramanian et al., 1984). Binding of detergent could result in disruption of noncovalent protein−protein interactions which lead to aggregation. Hence, it is essential to recognize those detergents which will maintain the solubility of a protein without significantly altering its native structure and consequently its biological functions. This study is intended to examine the instability properties of pST, and to recognize certain sugars and detergents which can stabilize pST in aqueous solutions.

MATERIALS AND METHODS

Porcine Somatotropin (pST). The hormone used in this experiment was recombinant-derived methionyl-pST (molecular weight 22 000 Dalton) from plasmid pMG93 expressed in *E. coli* (Puri et al., 1991), produced at Bresatec Ltd., Adelaide, Australia. It came as freeze-dried powder, with purity about 60%. It was stored at −40 °C and sealed to prevent re-hydration. The concentration of pST can be determined by ultraviolet (UV) spectroscopy at 278 nm. At this wavelength, the extinction coefficient of the hormone is 15 700 $M^{-1}cm^{-1}$ (e.g., approximately 22 000/1.4), which gives the following formula to determine pST concentration by UV at 278 nm: concentration (mg/mL) = absorbance at 278 nm multiplied by 1.4.

Gel Permeation Chromatography. Concentration of pST was determined by gel permeation chromatography (GPC) with an Asahipak (Tokyo, Japan) GSM-700 column (7.6 mm i.d., 500 mm length, exclusion limit: 10 million Daltons) using a Waters ELC chromatograph coupled with an ultraviolet detector set at 278 nm. The mobile phase (1 mL/min) was 50 mM NaCl buffered at pH 7.2 with 50 mM phosphate. This method was applied to determine various types (monomeric, aggregated, and degraded) of soluble pST in solutions.

Instability and Stabilization of Porcine Somatotropin. For all of the experiments, the initial concentration of pST in the various test solutions was 4 mg/mL except in the experiment examining effect of hormone concentration. Experiments were conducted by incubating at 37 °C. Sodium azide (NaN_3) at a final concentration of 0.02% was used as a preservative. The supernatants, after centrifuging each solution at 4500*g* for 15 min, were periodically analyzed by ultraviolet spectroscopy (UV) and/or by GPC for amount of soluble pST and for monomeric, aggregated, and degraded pST. After the solutions were analyzed, they were re-suspended evenly by shaking and put back into the incubator.

Effects of pH. Solutions of pH 2−9 were prepared from KCl−HCl buffer (pH 2.0), citrate phosphate buffer (pH 3−6), phosphate buffer (pH 7.0), and Tris-HCl buffer (pH 8−9). All solutions had an ionic strength of 0.15 M. The pST was added to start the experiment. The amount of soluble pST was determined by UV (experiment 1) and various types of the hormone (monomeric, aggregated, and degraded) were measured by GPC (experiment 2) of these solution supernatants after they were centrifuged.

Effects of Concentrations of Hormone. Solutions containing 16, 8, 4, 2, 1, and 0.5 mg/mL pST were prepared by diluting the hormone in 0.02 M, pH 7.0 PBS. The pH of each solution was adjusted by 1 N HCl or NaOH. The amount of pST monomers in each solution was detected by the above-described GPC method.

Effects of Sugars and Detergents. Glucose and sucrose were used for the sugar experiment to make 1, 5, and 10% concentrations, dissolved in 0.02 M, pH 7.0 PBS. Sodium dodecyl sulfate (SDS) and urea were used for the detergent experiment, with concentrations of SDS of 0.05, 0.1, and 0.5% and concentrations of urea of 0.1, 0.5, and 1 M. Detergents were dissolved in 0.02 M, pH 7.0 Tris-HCl buffer. The amount of pST monomers was determined by GPC.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) of pST Samples. SDS−PAGE was conducted in polyacrylamide gels (9 cm wide, 6 cm high, and 1 mm thick). The stacking gels contained 4% (w/v) acrylamide and the separating gels contained 10% (w/v) acrylamide. Samples (15 μg/15 μL) in sample buffer [1% (w/v) SDS, 10% (w/v) glycerol, and 50 mM Tris-HCl, pH 6.8] were heated for 5 min at 95 °C prior to loading into gel wells. The gel was run at 150 V for about 1 h with bromophenol blue as the tracking dye. The gel was then stained for protein with 0.12% Coomassie brilliant blue R250, and was calibrated with molecular-mass standards (from Sigma): phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Animal Experiment on the Property of pST in a Controlled Release System. The instability of pST in a slow release system was conducted on hypophysectomized (hypox) female rats (from Charles River Laboratories). The release system was formed through entrapping pST in the hydrogel matrix synthesized by cross-linking poly(gamma-glutamic acid) with dihaloalkane agents (Fan et al., 1996). Release of pST from the subcutaneously injected matrix was monitored by body-weight gain (BWG) measurements (Buckwalter et al., 1992), which were compared with those of the controls. Rats were housed one per cage, at a controlled temperature of 24−26 °C, with lighting regulated on a 14-h light/10-h dark schedule. Feed and a 5% glucose solution for water were made readily available in the feed bins in each cage.

Rats were divided into four groups, with three rats in each group. A total dosage of 3 mg pST for each rat was administered for a 30 day period of experiment (100 μg pST/day). One group with no treatment was for negative control. The second group was for positive control, with each rat receiving subcutaneous injection of 100 μg pST in 0.2 mL of solution every day. The third group was injected with a total dosage of pST (3 mg/0.3 mL) on the first day of the experiment. The fourth group was for evaluation of the slow-release system. For this group, the hydrogel/hormone slow-release preparation with the same total dosage of pST was injected in 0.3 mL volume. Body-weight gains (BWG) of the rats were recorded everyday.

RESULTS

The aggregation and decomposition processes of pST in aqueous solutions were monitored by GPC. A typical GPC plot for determining aggregated, degraded, and

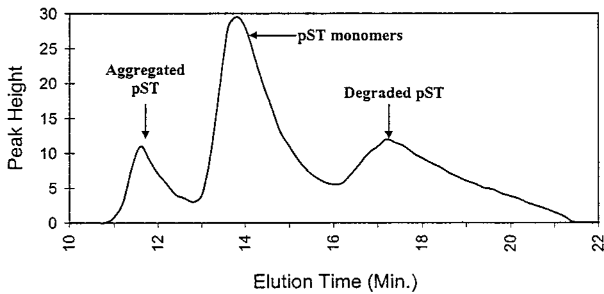

Figure 1. Typical GPC plot for determination of aggregated, degraded, and monomeric pST. The sample for this test was the supernatant of pST in pH 9.0 solution after 15-day incubation at 37 °C. The GPC was run as described in the Materials and Methods section.

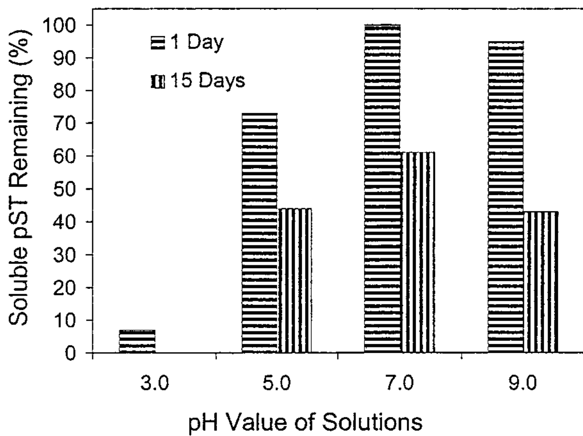

Figure 2. Effects of pH on stability of pST (experiment 1). Hormone solutions of concentration 4 mg/mL were prepared in various pH buffers and incubated at 37 °C for 15 days. The amount of soluble pST was determined by UV method in the supernatant of the solution after centrifugation. The mean value from three experiments was used in this figure.

monomeric pST is shown in Figure 1. The hormone molecules with natural monomeric structure had an elution time of about 14 min, whereas the aggregated and degraded pST appeared before and after the peak for the monomer, with elution times at about 11.8 and 17.2 min, respectively.

The aggregation of pST is partially the result of hydrophobic reactions among molecules. The pH value of the pST solution is very important in this sense. Figures 2 and 3 demonstrate that pST was completely denatured when pH was lower than 4. Because each fraction of pST was determined by the GPC method, the insoluble aggregated and precipitated amounts of pST, which exist heavily at the low pH, were not included in Figure 3. Therefore, this part of pST should be counted when the total pST is considered. The pH at which the hormone was most stable was neutral pH. In alkaline solution, pST underwent both degradation and aggregation fairly quickly, resulting in the loss of monomeric pST (Figure 3). Considering that animal blood is at a pH of 7.2−7.4, the relative stability of pST at neutral pH is an advantage for development of controlled release systems.

The pST concentrations in solutions also affected the rate of hormone aggregation and degradation (Figure 4). It was obvious that pST was more likely to form aggregates when at high concentration. At a concentration of 16 mg/mL, only about forty percent of pST remained in the monomeric form after the 28-day incubation, but nearly 60% of pST remained in this form in a solution of 0.5 mg/mL. In addition, the hormone underwent aggregation rapidly in the initial 15 days, and after that, aggregation occurred slowly.

Two common sugars, glucose and sucrose, were tested for their ability to stabilize the hormone. As indicated in Figure 5, glucose was able to stabilize the hormone fairly well. Even when as little as 1% glucose was added, more than 70% monomeric pST still remained in solution after 30 days. Sucrose, on the other hand, could not stabilize the hormone well except at 5% concentration.

Detergents can, in most cases, prevent aggregation through intermolecular interaction. We studied two relatively mild detergents (SDS and urea) for the purpose of pST stabilization. Results indicated that SDS

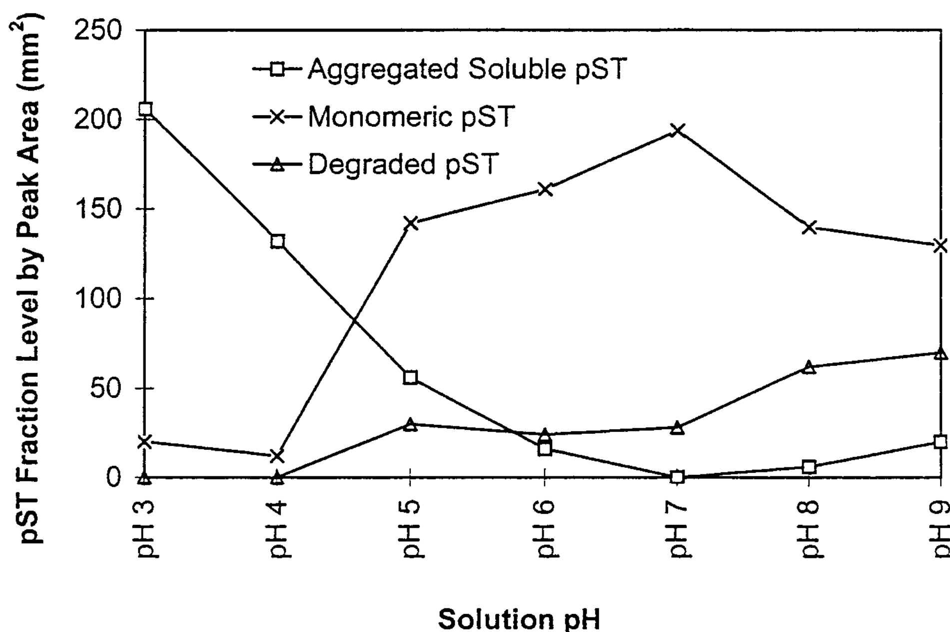

Figure 3. Effects of pH on stability of pST (experiment 2). Hormone solutions with concentration 4 mg/mL were prepared in various pH buffers and incubated at 37 °C for 15 days. The three types of hormone molecules (aggregated, degraded and monomeric) were determined by GPC and their amounts were estimated through the peak area of each types.

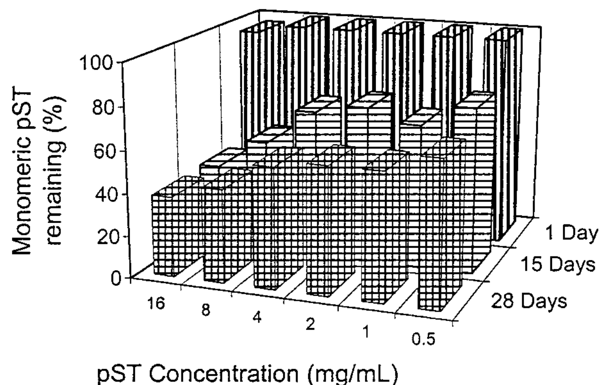

Figure 4. Effects of pST concentrations on its stability. Solutions with various hormone concentrations were incubated at 37 °C. The quantity of hormone monomer was determined by GPC. Data are mean values of three identical tests.

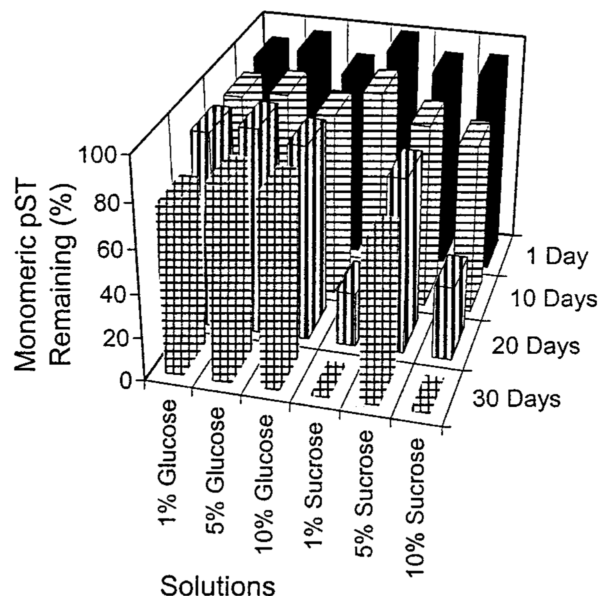

Figure 5. Effects of sugars on pST stability. Hormone solutions with a concentration of 4 mg/mL pST were prepared in the indicated sugar solvents. Experiments were conducted at 37 °C and the amounts of monomers were determined by GPC after centrifugation. Data are the averages of three experiments conducted simultaneously.

could stabilize the hormone only at a concentration of above 0.5%. Urea, however, was effective in all of the three concentrations used in this experiment (Figure 6).

To confirm the existence of aggregated, degraded, and monomeric pST, SDS−PAGE electrophoresis was used to examine various solutions in the above stability experiments. Results (Figure 7) clearly demonstrated that there were several bands besides the band for natural monomeric pST, from bands with very small molecular weights which represented degraded pST fractions, to bands with larger molecular weights which represented aggregated pST molecules. A band with molecular weight about 44 kDa was fairly noticeable. This was likely to be the dimers of pST and certainly indicated that the dimerization reaction occurred during incubation among pST molecules.

The instability of pST inside of hydrogel-based slow-release systems might have been demonstrated by the experiment on hypox female rats. As indicated in Figure 8, the hydrogel system did slowly release the pST for about 20 days as evidenced by the increase or maintenance of rat body-weight gains (BWG) compared to the

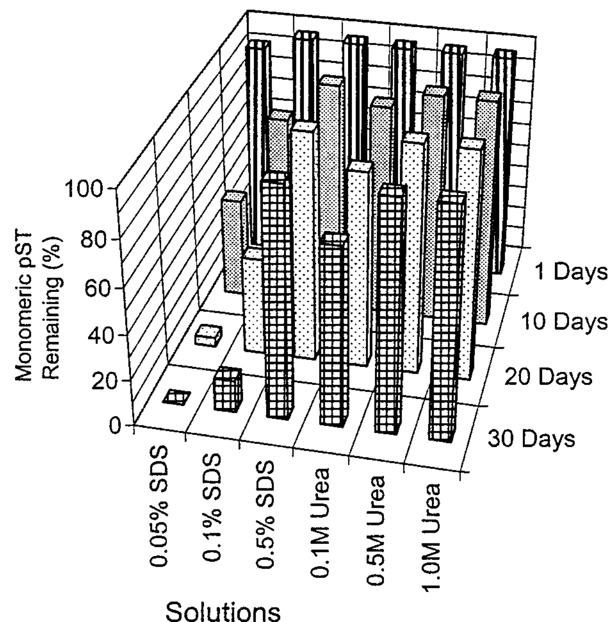

Figure 6. Effects of detergents on stability of pST. Hormone solutions with concentrations of 4 mg/mL pST were prepared in the indicated detergent solutions. The experiment was conducted at 37 °C and the amount of monomers was determined by GPC after centrifugation. Data are the averages of three identical experiments.

negative control group and the group with one-time injection of total dose. However, when compared with the positive control group, which grew substantially while receiving daily injections but lost weight when injections stopped, the rats receiving the pST/hydrogel combinations grew more slowly, and after 15 days, the rats of the latter group failed to make further weight gains.

DISCUSSION

For controlled release of somatotropin, hydrogel delivery system is a frequent choice. However, the instability of the somatotropin in the presence of water has greatly influenced the success of development of a such system. When exposed to an aqueous environment, the hydrogels become hydrated. A concentrated aqueous solution of the somatotropins forms within the network, permitting rapid aggregation of the somatotropin, as reported by Pitt (1993). All of the reactions, like primarily covalent cross-linking, deamidation, and peptide cleavage, seem to be involved in the decomposition of somatotropin. It was reported that the somatotropin release generally reached a plateau at approximately 50% delivery of the total dosage tested when incubated in aqueous media at 37 °C (Hageman et al., 1992). Similarly, our in vitro experiments confirmed the occurrence of the aggregation of porcine somatotropin to form insoluble precipitates (Fan et al., 1996). In the meantime, the likeliness for the aggregation to occur in vivo was also indicated by the experiment on hypox rats, which would not grow unless they received exogenous administration of somatotropin like pST. Referring to Figure 8, the group of rats that received the hydrogel delivery system grew much less than the rats in the positive control group receiving daily injection of fresh pST. Although the two groups received the same total dosage during the experiment, the group with the hydrogel delivery system failed to make further weight

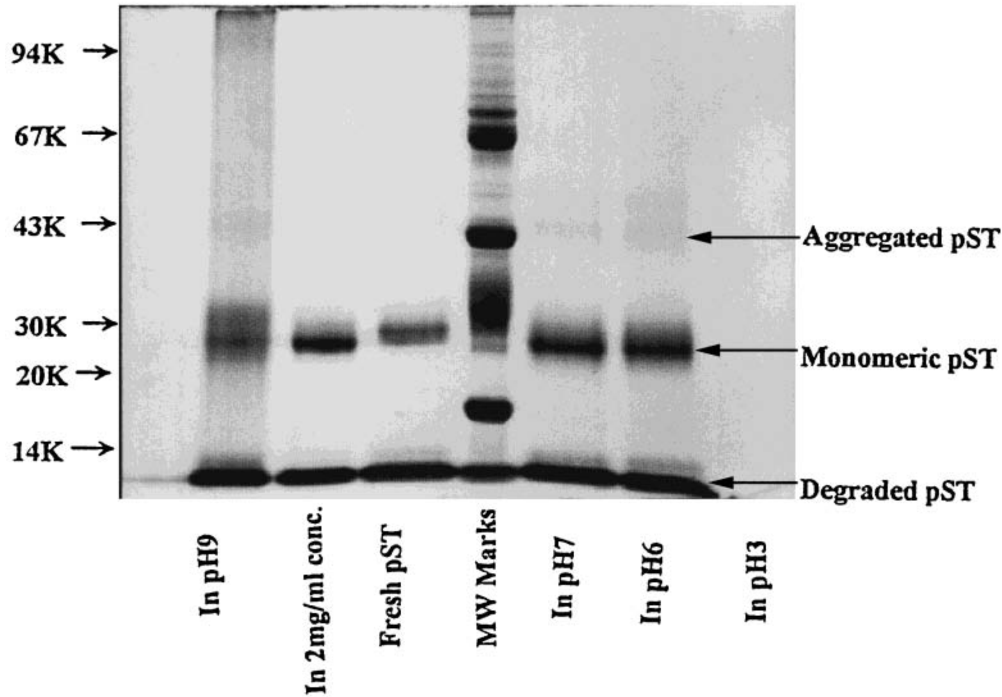

Figure 7. SDS–electrophoresis to determine aggregated, degraded, and monomeric pST. All samples, taken after 15 days of stability studies (except the fresh pST solution, which was prepared immediately before the electrophoresis), were loaded and run on 10% polyacrylamide gels. Bromophenol blue was used as tracking dye. The gel was stained for proteins with Coomassie brilliant blue R250.

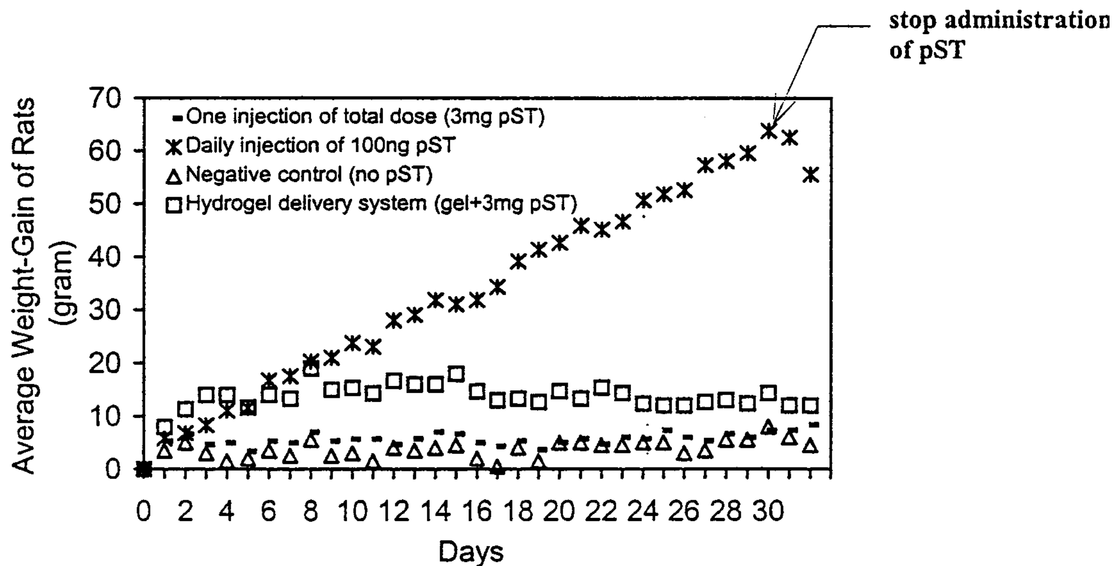

Figure 8. Rat experiments for a pST hydrogel delivery system. Hypophysectomized rats were used with 3 in each group. The experiment was conducted over a 30-day period, and the average body-weight gain in each group was recorded daily.

gains after 15 days. This result suggested that the release of the hormone had greatly decreased by that time. Besides the efficiency of the delivery system, the likelihood of pST aggregation at the high concentration and temperature cannot be excluded from the reasons for the large difference in body-weight gain between the two groups. Consequently, an understanding of protein hydration, as well as the stability of the protein at high levels of hydration and concentration, is especially relevant to developing strategies for delivery devices.

The pH of a solution interacts with the hormone by changing its surface charges. In acidic and alkaline solutions, the hormone molecule is positively and negatively charged, respectively, which increases repulsive forces between the molecules, and thereby hinders the approach of two proteins and limits aggregation. On the other hand, pH at both extremes decreases the hormone's conformational stability and solubility, resulting in a greater tendency for irreversible aggregation or precipitation from solutions (Holzman et al., 1991). As indicated in Figure 2, almost all of the hormone immediately precipitated in a solution of pH 3. Also, in alkaline solution, the decomposition reaction occurred quickly. This may explain that large fraction of degraded pST existing in the pH 9.0 solution as shown in Figure 3.

The irreversible precipitation of somatotropin was concentration-dependent, as shown in Figure 4. At high concentrations, the hormone was highly susceptible to aggregation (Hageman et al., 1992; Buckwalter et al.,

1992). This aggregated protein, which was not redissolved by dilution into PBS, was responsible for a considerable loss of the monomeric protein at the higher concentrations employed. The aggregation reaction was also confirmed by electrophoresis (Figure 7) in which the formation of a covalent dimeric species with a molecular weight of 44 000 Dalton was clearly observed.

For this reason, peptide aggregation could be suppressed by lowering the peptide concentration and by adding stabilizers (Powell, 1993). Sugars and detergents are two possible stabilizers. In our studies, glucose and sucrose for sugars, and SDS and urea for detergents, were tested for stabilization of pST. It was found that glucose was more efficient than sucrose in stabilizing monomers of the hormone (Figure 5). This may be due to the size of the disaccharide. As the size of the sugar increases, the steric hindrance may interfere with the stabilizing binding between the saccharide and the peptide. In addition, different density solutions would be formed at different concentrations of sucrose. Because of the size of sucrose, this phenomenon could be effective enough to let pST molecules have preferential hydration and stabilization at a certain concentration. In this study, the 5% sucrose solution seemingly formed the preferential environment for natural pST molecules (Figure 5).

As to the function of detergents, urea was found to be more effective in stabilizing pST monomers than SDS at the concentration range tested (Figure 6). The latter was effective only at higher concentration. Urea may be a preferable additive because it was reported to be a weaker denaturant at low concentration and be able to leave the protein secondary structure intact in many cases (Subramanian et al., 1984; Cardamone et al., 1994). It may be very beneficial if urea could stabilize the hormone within the delivery matrix without significantly compromising its conformation. As the hormone diffuses out of the matrix and urea molecules are diluted out, it can re-nature and perform its biological activity. Further research to monitor the variation of the dimensional structure of pST by circular dichroism (CD) or other means when it is in urea solutions will help in approaching a conclusion.

Abbreviations Used. pST, porcine somatotropin; SDS, sodium dodecyl sulfate; GPC, gel permeation chromatography; UV, ultraviolet spectroscopy; PBS, phosphate buffer saline.

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