Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 303 (2005) 95–103
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Sustained insulin release with biodegradation of chitosan gel beads prepared by copper ions

Kyoko Kofuji *, Yoshifumi Murata, Susumu Kawashima

Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa 920-1181, Japan

Received 24 January 2005; received in revised form 11 June 2005; accepted 9 July 2005
Available online 1 September 2005

Abstract

Chitosan (CS) gel beads were prepared with chelated copper (II) ions as a vehicle for the delivery of peptide and protein drugs. Insulin, which is a model of peptide and protein drugs, was scarcely released from the CS gel beads in vitro, presumably due to the nature of interactions occurring between insulin, CS and the copper (II) ions. The efficacy of insulin released from the CS gel beads was confirmed by implantation into diabetic mice. A consistent reduction in blood glucose level was observed in vivo due to insulin release as the CS gel beads were degraded. Control over insulin release was achieved by altering the properties of the CS. Thus, CS gel beads are promising as a biocompatible and biodegradable vehicle by which peptide and protein drugs can be delivered.

Keywords: Chitosan; Copper (II) ion; Insulin; Biodegradation; Sustained release

1. Introduction

Peptide and protein drugs are potent therapeutic agents. DNA technology has led to an increase in the number of peptide and protein drugs available for pharmaceutical use. However, these drugs have low stability and their absorption is limited. In addition, it is difficult to control the release of peptide and protein drugs from a vehicle due to their high solubility in water. Therefore, numerous attempts have been made to develop delivery vehicles capable of improving the stability and absorption of peptide and protein drugs in addition to enabling their controlled release (Bernkop-Schnürch and Walker, 2001). The development of such peptide and protein drug delivery systems can be expected to improve the efficacy of medical treatments, to minimize side effects and to improve patient compliance.

Chitosan (CS) is an abundant natural polysaccharide that is non-toxic, biocompatible and biodegradable (Yomota et al., 1990; Tomihata and Ikada, 1997; Onishi and Machida, 1999). The use of CS as a material for the development of a drug delivery vehicle that enables sustained drug release has been investigated (Jameela and

* Corresponding author. Tel.: +81 76 229 1165; fax: +81 76 229 2781.
E-mail address: k-kofuji@hokuriku-u.ac.jp (K. Kofuji).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.07.011

Jayakrishnan, 1995; Oungbho and Müller, 1997). In addition, CS is a positively charged polymer. Positively charged polymers are known to enhance the absorption of peptide and protein drugs by mucoadhesion to gastric or nasal mucosa or by opening tight junctions between epithelial cells (Senel et al., 2000; Tengamnuay et al., 2000; Thanou et al., 2001; Lubben et al., 2001). Thus, CS possesses several properties indicating that it might be an ideal vehicle for the delivery of peptide and protein drugs.

The preparation of vehicles for peptide and protein drugs under mild conditions is desirable for preserving the pharmacological activity of the drugs. In our previous work, we prepared CS gel beads in a 10% glycine (pH 9.0) aqueous solution without the use of any toxic cross-linking agent such as glutaraldehyde. The prepared CS gel beads facilitated controlled drug release (Kofuji et al., 2000). We also confirmed the in vitro and in vivo drug release, and the biodegradative properties of the CS gel beads were observed to vary depending on the CS species employed, particularly with respect to the differences in the degree of deacetylation of the CS species. The release of drug from CS gel beads in vivo was controlled by the choice of the CS species used or by modifying the properties of CS (Kofuji et al., 2001; Kofuji et al., 2002). In the previous study, drug release from the CS gel beads prepared in 10% glycine (pH 9.0) aqueous solution was governed by the diffusion of the drug from the matrix. However, limitations on the controlled release of water-soluble drugs, such as insulin, from CS gel beads still exist and mechanisms besides the control of drug diffusion are required to ensure control over drug release (Kofuji et al., 2003).

On the other hand, it has also been demonstrated that CS forms a chelate complex with metal ions. Among the metal ions, copper ions, in particular, strongly interact with CS. (Rhazi et al., 2002). In addition, it is also known that insulin forms polymeric conformations in the presence of divalent metal ions such as zinc. These metal ions have contributed to the stability of insulin (Brange et al., 1987). Furthermore, insulin has been observed to interact with CS (Fernández-Urrusuno et al., 1999; Ma et al., 2002). The use of preparation techniques that utilize the above mentioned properties along with the control of CS biodegradation might optimize the insulin-carrying ability of a CS-based delivery vehicle.

The primary aim of the present study is the development of CS-based drug delivery vehicles that improve the stability and absorption of peptide and protein drugs as well as enable their controlled release. As a model study, we investigated the possibility of achieving a long-term sustained release of insulin from a CS and copper (II) ion-based drug delivery vehicle (i.e., CS gel beads). In addition, the efficacy of insulin released from the CS gel beads was also confirmed by determining the blood glucose levels after the CS gel beads were implanted into subcutaneous air pouches (AP) on the dorsal surfaces of diabetic mice.

2. Materials and methods

2.1. Materials

CS with varying degrees of deacetylation [70% (7B), 82% (8B), 91% (9B) and 100% (10B)], as listed in Table 1 were purchased from Katokichi Co. Ltd. (Japan). Copper (II) sulfate pentahydrate and streptozotocin were purchased from Wako Pure Chemical Industries (Japan). Insulin from bovine pancreas (28 U/mg) was purchased from Nacalai Tesque Inc. (Japan). BCA^{TM} protein assay reagent was purchased from Pierce Chemical Co. (USA). All the other chemicals were of reagent grade.

2.2. Preparation of the CS gel beads

CS gel beads for dissolution testing were prepared as follows: CS (1–4%, w/w) was dissolved in 0.1–0.3 M sodium acetate buffer (pH 4.5). Insulin (2%, w/w) was separately suspended in sodium acetate buffer (pH 4.5) of the same concentration that was used to dissolve CS. Five grams of insulin suspension was dropped into 5 g of CS solution with agitation. Subsequently, the mixture was stirred overnight at room temperature. One gram of the mixture (CS concentration, 0.5–2%, w/w; insulin concentration, 1%, w/w), theoretically containing 10 mg (280 U) of insulin was then dropped slowly into 20 ml of $CuSO_4$ (20–40 mM) aqueous solution by using a pipette and left to stand at room temperature for 25 min. CS gel beads were formed spontaneously under the optimum CS concentration. These gel beads were scooped up with a sieve and used for the experiments.

Table 1
Relationship between CS properties and gel bead formation

CS species	Degree of deacetylation (%)	Mw[a] ($\times 10^4$ Da)	Concentration of acetate buffer (M)	Concentration of CS (%)			
				0.5	1	1.5	2
7B	70	282.6	0.1	–	+	++	++
				(66)	(610)	(8750)	(34940)
8B	82	221.7	0.1	–	+	++	++
				(52)	(400)	(3340)	(33600)
9B	91	98.0	0.1	–	+	+	+
				(24)	(170)	(740)	(1210)[b]
			0.2	–	+	+	+
				(23)	(160)	(660)	(2600)
10B	100	16.2	0.1	–	+	+	+
				(9)	(25)[b]	(21)[b]	(18)[b]
			0.3	–	+	+	+
				(10)	(31)	(109)	(254)

Gel beads formation: – no gelation; + gelation; ++ CS solution could not be dropped into the preparative medium on account of high viscosity. Degree of deacetylation was determined by colloidal titration. Mw: weight-average molecular weight. Values given in parenthesis represents viscosity (mPa s) of CS solution determined at 37 °C using a B-type viscometer (Tokyokeiki).

[a] (Kofuji et al., 2003).

[b] CS was not dissolved completely.

2.3. Determination of the amount of Cu within the CS gel beads

A single bead (weighing approximately 0.06 g) was dissolved in 2 ml of 0.1 M sodium acetate buffer (pH 4.5) with 40 mg of ethylenediaminetetraacetic acid (EDTA) and the volume was made up to 5 ml with 0.1 M sodium acetate buffer (pH 4.5). The absorbance of the solution was determined using a spectrophotometer (Model 200-20, Hitachi Ltd., Japan) at 250 nm. These values were corrected by simultaneously performing a blank test to exclude the factors that may influence the measurement.

2.4. Determination of the amount of insulin within the CS gel beads

The amount of insulin within the CS gel beads was calculated by deducting the amount of insulin detected in the preparative medium after bead formation from that of the initial CS solution to which the insulin was added. Insulin was determined by the BCA^{TM} method using protein assay regents. The absorbance of the BCA^{TM} test solution was determined using a multi-spectrophotometer (Viento, Dainippon Pharmaceutical Co. Ltd., Japan) at 562 nm. These values were corrected by the blank test that was performed simultaneously to exclude the factors that may influence the measurement.

2.5. Dissolution test

The release of insulin from various CS gel beads into 0.1 M sodium phosphate buffer (pH 7.2) or water was determined. CS gel beads (weighing approximately 1 g) were added to 20 ml of dissolution medium in a sample bottle (37 °C). A 25-μl aliquot of the solution was periodically removed for analysis after agitation and replaced with 25 μl of the dissolution medium (pre-warmed to 37 °C) in order to maintain a constant volume. Insulin concentration in each sample was determined by the BCA^{TM} method. All the dissolution tests were performed in triplicate.

2.6. Preparation of diabetic mice

Diabetic mice were prepared as follows: Streptozotocin (10 mg/ml) was dissolved in 0.1 M sodium citrate buffer (pH 4.5). Streptozotocin (0.1 mg/g body weight) was then injected intraperitonealy into the mice (ddy strain, 6-week-old male). Blood was collected from the tail vein and the blood glucose levels were determined periodically using a blood glucose determination system (Medisafereader, Terumo Co., Japan). When the

blood glucose level did not increase after one injection of streptozotocin, mice were injected a second time. These mice were utilized as diabetic mice when blood glucose levels were greater than 300 mg/dl for 4 consecutive days. At the same time, air (3 ml) was subcutaneously injected into the dorsal surface to form AP. An oval AP was formed after an additional 1 ml of air was injected at days 1 and 4. All research protocols were approved by the committee for animal research at Hokuriku University.

2.7. Evaluation of the efficacy of insulin released from the CS gel beads

CS7B (1%) and CS9B (2%) gel beads with 0.1 M sodium acetate buffer (pH 4.5) for implantation were prepared as described above; however, the amount of insulin was altered. A single bead retained approximately 20.0 U of insulin, which was selected in consideration of a sustained release. A single bead or only insulin suspension (0.1 U) was implanted into the AP of mice under ether anesthesia 7 days after the first air injection. Blood was collected from the tail vein and glucose levels were periodically determined. The biodegradation of the CS gel beads implanted into the AP was assessed by direct observation inside the AP with naked eyes.

2.8. Pharmacodynamic analysis

The areas under the reduction of blood glucose concentration–time curve (AUC) and the area under the first moment curve (AUMC) after injection of the insulin suspension or CS gel beads retaining insulin were calculated from 0 to 360 h by using the linear trapezoidal rule. The area under the baseline (blood glucose level: 600 mg/dl)-time from 0 to 360 h (AUC_{base}) was calculated. The extent of reduction in blood glucose levels (D) was assessed from the ratio of AUC/AUC_{base}. The mean residence times (MRT) were obtained from the ratio of AUMC/AUC. The minimum blood glucose concentration times (T_{min}) were obtained from experimental values.

2.9. Statistical analysis

Data were represented as the mean ± S.D. and were analyzed statistically using Tukey–Kramer multiple comparison procedure after F-test or Smirnov–Grubbs' outlier test ($\alpha = 0.05$).

3. Results and discussion

3.1. Preparation of the CS gel beads

The spontaneous formation of spherical CS gel beads with diameters of approximately 5.0 mm was observed when CS solution was dropped into $\geqq$20 mM $CuSO_4$ aqueous solution. In particular, $\geqq$40 mM of $CuSO_4$ concentration resulted in the formation of tightly packed gel beads. All the CS gelated without any leakage. This gelation is due, in large part, to chelation between CS and copper (II) ions. It has been proposed that CS binds to copper ions via four nitrogen ligands in a square-planar geometry (Schlick, 1986). The ease of preparation of CS gel beads was dependent on the properties of the particular CS species employed. As shown in Table 1, before the addition of CS into the $CuSO_4$ aqueous solution for the formation of spherical gel beads, the optimum CS concentration was 1% for both CS7B and CS8B and 1–2% for CS9B and CS10B, irrespective of the presence of insulin. Lower concentrations of CS of low viscosity did not result in the formation of spherical gel beads on account of the rapid diffusion rate of CS solution in the preparative medium. Higher concentrations could not be dropped into the preparative medium by using a pipette due to the high viscosity of the resultant solution. The dissolution of CS was aided by acetic acid, which forms a salt with some amino groups of CS. When the CS solution containing acetic acid was dropped into $CuSO_4$ aqueous solution, CS-acetic acid salts dissociated rapidly in the solution and copper ions chelated with CS. It is important that bead formation occur rapidly after CS is dropped into the preparative medium because this enables a more efficient drug encapsulation by inhibiting the escape of the drug into the medium. On the other hand, 2% CS9B and 1–2% CS10B did not dissolve completely in 0.1 M sodium acetate buffer (pH 4.5) (Table 1). In order to achieve the dissolution of CS at these concentrations, a greater amount of acetic acid was required. A concurrent increase in viscosity was observed with dissolution as greater amounts of acetic acid were added to these concentrations of CS. However, the addition of acetic acid did not increase

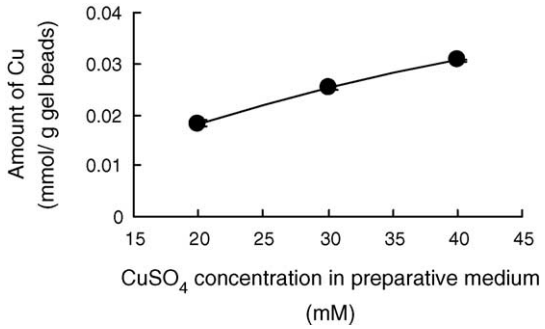

Fig. 1. Effect of $CuSO_4$ concentration in preparative medium on retention of Cu within CS gel beads. CS gel beads preparative condition: CS species, CS7B; CS concentration, 1%; sodium acetate buffer concentration, 0.1 M. Data represent the mean ± S.D. ($n = 3$).

the viscosity when soluble concentrations of CS that did not require additional acetic acid for dissolution were used. An optimum acetic acid concentration of 0.1 M was required for the formation of spherical gel beads in the case of both CS7B and CS8B, 0.1–0.2 M for CS9B and 0.1–0.3 M for CS10B. Under the conditions of excess acetic acid (i.e., when the concentration of acetic acid was above the optimal level), CS did not form spherical gel beads even at high concentrations of $CuSO_4$.

3.2. Retention of Cu within the CS gel beads

Copper (II) ions in the preparative medium were retained within the CS gel beads. Fig. 1 shows the amount of Cu present in 1% CS7B gel beads. Retention within the CS gel beads was increased when greater concentrations of $CuSO_4$ were added in the preparative medium. In this preparative condition, the retention of Cu within the CS gel beads was not affected by the temperature, within the range of room temperature. To form tightly packed gel beads and minimize the influence of copper when the CS gel beads were implanted into the body, 40 mM of $CuSO_4$ was used in the preparative medium in future experiments. As shown in Fig. 2, the amount of Cu retained within the CS gel beads (CS concentration, 1%; sodium acetate buffer concentration, 0.1 M; $CuSO_4$ concentration, 40 mM) increased with the increase in the degree of deacetylation of CS. The amount of Cu retained within the CS gel beads also increased with increased initial CS concentrations of CS9B and CS10B (data not shown). In both the cases described above, the observed increase in Cu within the CS gel beads can probably be attributed to the increase in the number of amino groups of CS, since Cu ions are incorporated into the CS gel beads on chelation with the amino groups. On the other hand, altering the concentration of sodium acetate buffer required to dissolve CS had no effect on the Cu content within the CS gel beads.

3.3. Retention of insulin within the CS gel beads

The amount of insulin within the CS gel beads was calculated by deducting the amount of insulin detected in the preparative medium after bead formation from that of the initial CS solution to which insulin was added. When the CS solution containing insulin was dropped into the preparative medium, the total amount of insulin in the CS solution was always retained within the CS gel beads, irrespective of the CS species, CS concentration, or acetic acid concentration required to dissolve CS. Variations in $CuSO_4$ concentration also did not reduce the retention of insulin below the maximal values. The values obtained by this method were also confirmed to be identical to those determined directly as a result of dissolution of the CS gel beads in 0.1 M sodium acetate buffer (pH 4.5) with EDTA. CS is a positively charged polymer with a pK_a of approximately 6.2–6.5. After the addition of CS dissolved in 0.1 M acetate buffer (pH 4.5), the pH of the $CuSO_4$ aqueous solution in which the beads were prepared was approximately 4.7. Therefore, peptide or protein drugs with carboxyl groups might form electrostatic complexes with the amino groups of CS. Furthermore, the

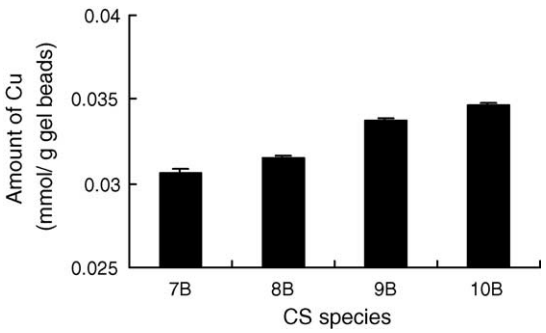

Fig. 2. Effect of CS species on retention of Cu within CS gel beads. CS gel beads preparative condition: CS concentration, 1%; sodium acetate buffer concentration, 0.1 M; $CuSO_4$ concentration in preparative medium, 40 mM. Data represent the mean ± S.D. ($n = 3$).

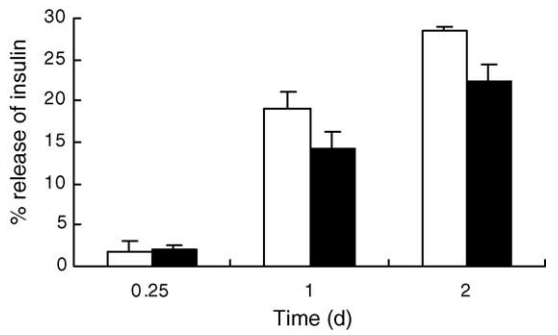

Fig. 3. Effect of $CuSO_4$ concentration in preparative medium on release of insulin from CS gel beads. $CuSO_4$ concentration in preparative medium: (□) 20 mM; (■) 40 mM. CS gel beads preparative condition: CS species, CS7B; CS concentration, 1%; sodium acetate buffer concentration, 0.1 M. Dissolution medium: 0.1 M phosphate buffer (pH 7.2). Data represent the mean ± S.D. ($n = 3$).

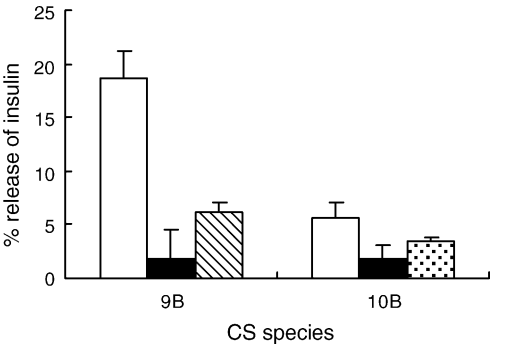

Fig. 5. Effect of CS concentration and acetate buffer concentration on the release of insulin from CS gel beads. CS gel beads preparative condition: (□) (CS concentration, 1%; sodium acetate buffer concentration, 0.1 M); (■) (2%, 0.1 M); (▨) (2%, 0.2 M); (⊡) (2%, 0.3 M); $CuSO_4$ concentration in preparative medium, 40 mM. Dissolution medium: 0.1 M phosphate buffer (pH 7.2). Time of dissolution test: after 2 days. Data represent the mean ± S.D. ($n = 3$).

polymeric conformation of insulin induced by the copper (II) ions might also play a role in insulin retention (Brange et al., 1987).

3.4. Release of insulin from the CS gel beads

The dissolution of the insulin powder in the dissolution medium was immediate. In contrast, only a small amount of insulin was released from the CS gel beads even after incubation for 6 h in the dissolution medium (Figs. 3 and 4). Further release of insulin was not observed after 2 days. It was conjectured that insulin, which did not interact with chitosan and copper ions was diffused from the CS gel beads into the dissolution medium and insulin, which interacted with them was remained in the CS gel beads. This phenomenon was observed, regardless of the dissolution medium employed (0.1 M phosphate buffer with pH 7.2 or water); however, swelling of the CS gel beads was observed in water possibly due to the absence of an interaction between the amino groups of CS and the anions in the dissolution medium. In addition, the CS gel beads in both the dissolution mediums did not disintegrate over time. Furthermore, the percentage of insulin released from the CS gel beads was not influenced by the amount of insulin retained within the CS gel beads. Fig. 3 shows the percentage of insulin released after 0.25, 1 and 2 days from 1% CS7B gel beads prepared in 20 or 40 mM $CuSO_4$ aqueous solution. The release of insulin was reduced on increasing the concentration of $CuSO_4$ in the preparative medium. As shown in Fig. 4, insulin release was also reduced by the use of CS species with greater degrees of deacetylation. Fig. 5 shows the percentage of insulin released from 1 or 2% CS9B and CS10B gel beads after 2 days, indicating that insulin release was also inhibited by the increased initial CS concentration. In contrast, insulin release was accelerated by increasing the concentration of sodium acetate buffer required to dissolve CS (Fig. 5). Fig. 6 shows the relationship between the

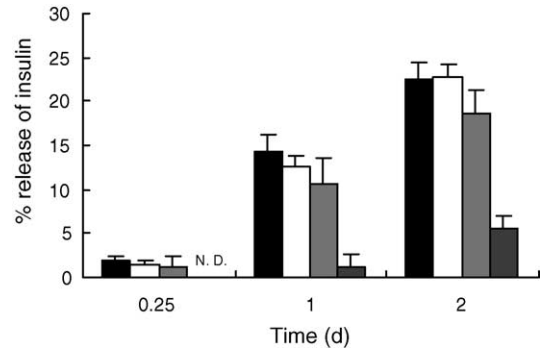

Fig. 4. Effect of various CS species on release of insulin from CS gel beads. CS gel beads preprative condition: CS species; (■) 7B; (□) 8B; (▩) 9B; (■) 10B; CS concentration, 1%; sodium acetate buffer concentration, 0.1 M; $CuSO_4$ concentration in preparative medium, 40 mM. Dissolution medium: 0.1 M phosphate buffer (pH 7.2). Data represent the mean ± S.D. ($n = 3$).

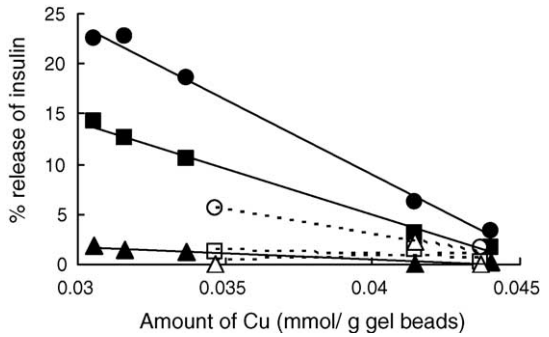

Fig. 6. Relationship between the percentages of insulin released after 0.25, 1 and 2 days from CS gel beads and the amount of Cu retained within CS gel beads. CS was dissolved completely prior to being added to the preparative medium: (●) 2 days (r^2 = 0.9934); (■) 1 day (r^2 = 0.9934); (▲) 0.25 day (r^2 = 0.9303). CS did not dissolve completely prior to being added to the preparative medium: (○) 2 days (r^2 = 0.9531); (□) 1 day (r^2 = 0.3662); (△) 0.25 day (r^2 = 0.0768). Dissolution medium: 0.1 M phosphate buffer (pH 7.2). r^2: correlation coefficients (subjected to least-squares fitting).

percentage of insulin released after 0.25, 1 and 2 days from CS gel beads and the amount of Cu retained within the CS gel matrix. The data points were subjected to least-squares fitting. When the CS was dissolved completely prior to being added to the preparative medium, an almost linear relationship was observed between the percentage of insulin release and Cu retention within the CS gel beads. Insulin release from CS gel beads was inhibited by increased retention of Cu within the CS gel beads. This indicates that the period of sustained insulin release from CS gel beads correlated closely with the extent of Cu retention within a CS gel bead. It may be deduced that polymeric conformations of insulin were formed on account of the copper (II) ions (Brange et al., 1987) and the gelation of CS induced by copper (II) ions was strengthened with the increase in Cu retention within the CS gel beads. When CS was not dissolved completely prior to being added to the preparative medium, insulin release was inhibited to an even greater extent (Fig. 6). It is conjectured that in the case when CS is not completely dissolved, greater number of free amino groups of CS chelated with copper ions (Rhazi et al., 2002).

These results suggest that a diverse range of factors, such as the improvement in stability by formation of polymeric conformations of insulin with metal ion and the enhanced interaction between insulin and CS by metal ions, would play a role in the high retention and sustained release of insulin from Cu-retaining CS gel beads.

3.5. Evaluation of insulin activity

Blood glucose levels rose gradually and usually exceeded 300 mg/dl 2 days after injection of streptozotocin. Mice with blood glucose levels greater than 300 mg/dl for 4 consecutive days were utilized as diabetic mice. The average blood glucose level of the diabetic mice just prior to injection of insulin or CS gel beads was 593 ± 16 mg/dl. In our previous study, the biodegradation of 100% deacetylated CS10B was not observed (Kofuji et al., 1999). Therefore, to investigate the efficacy of insulin released from the CS gel beads, 1% CS7B and 2% CS9B gel beads retaining insulin were implanted into subcutaneous AP on the dorsal surfaces of diabetic mice. The gel strengths determined by the rheometer (RHEO TEX, Sun Scientific Co. Ltd., Japan) were 4000 ± 500 g/cm^2 for 1% CS7B gel beads and 8000 ± 1100 g/cm^2 for 2% CS9B gel beads. Cu retentions within the CS gel beads were approximately 30 μmol/g gel beads for 1% CS7B gel beads to approximately 40 μmol/g gel beads for 2% CS9B gel beads. Both the types of CS gel beads did not swell in the AP. An insulin suspension (0.1 U) was injected into the AP of mice under ether anesthesia. As shown in Fig. 7 blood glucose levels were immediately reduced by the injection of the insulin suspension. However, the blood glucose levels of all mice rose above 300 mg/dl within 8 h. In addition, injection of 0.15 U of insulin

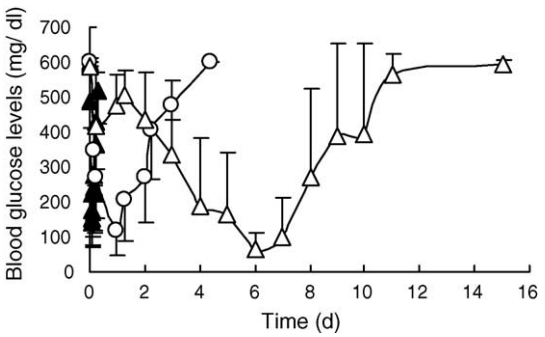

Fig. 7. Profiles of blood glucose levels after injection of insulin suspension or implantation of CS gel beads retaining insulin. (▲) Insulin suspension; (○) 1% CS7B; (△) 2% CS9B. CS gel beads preparative condition: sodium acetate buffer concentration, 0.1 M; $CuSO_4$ concentration in preparative medium, 40 mM. Data represent the mean ± S.D. (n = 4–8).

Table 2
Pharmacodynamic parameters (blood glucose reducing effect) after injection of insulin suspension or implantation of CS gel beads retaining insulin

	$AUC_{(0–360\,h)}$ (mg h/dl)	D (%)	MRT (h)	T_{min} (h)
Insulin suspension	2070 ± 750	1.0 ± 0.4	3.2 ± 0.6	2.3 ± 1.0
1%CS7B	24300 ± 4800[a]	11.2 ± 2.2[a]	74.3 ± 1.9[a]	24.0 ± 0.0
2%CS9B	97200 ± 20800[a,b]	45.0 ± 9.6[a,b]	163 ± 24[a,b]	140 ± 32[a,b]

CS gel beads preparative condition: sodium acetate buffer concentration, 0.1 M; $CuSO_4$ concentration in preparative medium, 40 mM. Data represent the mean ± S.D. (n = 4–8).
[a] $p < 0.05$ significantly different from insulin suspension.
[b] $p < 0.05$ significantly different from CS7B gel beads.

into the AP induced hypoglycemia. In contrast, a gradual decline in blood glucose levels was observed after implantation of a single bead retaining insulin (approximately 20 U). This finding strongly suggests that there was a release of insulin from the CS gel beads and the activity of insulin was preserved. In addition, tolerance to implantation of CS gel beads containing such a high dose of insulin indicates sustained release from the CS gel beads. Initially, insulin was gradually released after implantation of 1% CS7B gel beads; however, a time-specific acceleration in biodegradation of the 1% CS7B gel beads also occurred. Some of the 1% CS7B gel beads within the AP started degrading within 1 day and all of them had degraded within 2 days after implantation. In addition, 2% CS9B gel beads were also observed to cause a gradual reduction in blood glucose levels despite the fact that insulin was scarcely released from the CS gel beads in the in vitro condition (Fig. 5: (■) (2%, 0.1 M)), in which the enzymatic degradation of the CS gel beads was not observed on account of the absence of enzymes. The 2% CS9B gel beads displayed a more gradual degradation as compared to the 1% CS7B gel beads. The biodegradation of 2% CS9B gel beads continued for 2 weeks. Thus, the biodegradation of CS gel beads was inhibited with the increase in their degree of deacetylation, as observed in our previous study (Kofuji et al., 2002). In general, the in vivo drug release from a vehicle is governed by the diffusion of a drug from a vehicle and the biodegradation of the vehicle. In a previous study (Kofuji et al., 2002), it was demonstrated that the in vivo release rate of a drug from CS gel beads is very slow compared with the in vitro diffusion rate because the amount of dissolution medium in implantation site is little. Then, the finding obtained in the in vitro experiment indicated that insulin was hardly diffused from the CS gel beads in vivo. Therefore, with regard to the CS gel beads examined in this experiment, insulin release was strongly governed by the biodegradation of the CS gel beads.

The pharmacodynamic parameters are summarized in Table 2. The respective mean values of AUC of 1% CS7B and 2% CS9B from 0 to 360 h were 12 and 47 times greater than that obtained after injection of insulin suspension. Approximately 11 and 45% of D values were obtained after implantation of 1% CS7B and 2% CS9B gel beads, respectively. Furthermore, the respective values of MRT of 1% CS7B and 2% CS9B were increased to 23 and 51 times that obtained after the injection of insulin suspension. The increase in the values of T_{min} was also observed after implantation of both types of CS gel beads. These calculations indicate that the use of the CS gel beads resulted in a more prolonged continuous reduction in blood glucose level than that obtained by the injection of insulin suspension. Further, 2% CS9B beads, which exhibited a slower CS biodegradation, resulted in a more prolonged reduction in blood glucose levels than that obtained by the use of 1% CS7B gel beads. Thus, the use of the CS gel beads, which showed a biodegradation-dependent drug release, resulted in a more prolonged continuous release of insulin.

4. Conclusion

CS gel beads prepared by chelation with copper ions were able to retain insulin and preserve its activity. The use of metal ions to assist in the formation of CS gel beads enabled the long-term sustained release of insulin, since the release of insulin was controlled by the biodegradation of the CS gel beads. More prolonged control of blood glucose levels could be observed when CS gel beads were prepared using a CS species that exhibited slower CS biodegradation. The ability to

control drug release will allow the supply of required doses of a drug and may prolong the duration of drug activity as well as reduce the side effects of the drug. Therefore, CS gel beads are a promising, biocompatible and biodegradable vehicle for the delivery of peptide and protein drugs capable of improving the stability and their controlled release.

Acknowledgment

This work was supported by the specific research fund of Hokuriku University.

References

Bernkop-Schnürch, A., Walker, G., 2001. Multifunctional matrices for oral peptide delivery. Crit. Rev. Ther. Drug Carrier Syst. 18, 459–501.

Brange, J., Skelbaek-Pedersen, B., Langkjaer, L., Damgaard, U., Ege, H., Havelund, S., Heding, L.G., Jørgensen, K.H., Lykkeberg, J., Markussen, J., Pingel, M., Rasmussen, E., 1987. Galenics of insulin: The Physico-Chemical and Pharmaceutical Aspects of Insulin and Insulin Preparations. Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo.

Fernández-Urrusuno, R., Romani, D., Calvo, P., Vila-Jato, J.L., Alonso, M.J., 1999. Development of a freeze-dried formulation of insulin-loaded chitosan nanoparticles intended for nasal administration. S.T.P. Pharm. Sci. 9, 429–436.

Jameela, S.R., Jayakrishnan, A., 1995. Glutaraldehyde cross-linked chitosan microspheres as a long acting biodegradable drug delivery vehicle: studies on the in vitro release of mitoxantrone and in vivo degradation of microspheres in rat muscle. Biomaterials 16, 769–775.

Kofuji, K., Shibata, K., Murata, Y., Miyamoto, E., Kawashima, S., 1999. Preparation and drug retention of biodegradable chitosan gel beads. Chem. Pharm. Bull. 47, 1494–1496.

Kofuji, K., Ito, T., Murata, Y., Kawashima, S., 2000. The controlled release of a drug from biodegradable chitosan gel beads. Chem. Pharm. Bull. 48, 579–581.

Kofuji, K., Ito, T., Murata, Y., Kawashima, S., 2001. Biodegradation and drug release of chitosan gel beads in subcutaneous air pouches of mice. Biol. Pharm. Bull 24, 205–208.

Kofuji, K., Ito, T., Murata, Y., Kawashima, S., 2002. Effect of chondroitin sulfate on the biodegradation and drug release of chitosan gel beads in subcutaneous air pouches of mice. Biol. Pharm. Bull. 25, 268–271.

Kofuji, K., Akamine, H., Oshirabe, H., Maeda, Y., Murata, Y., Kawashima, S., 2003. Retention and release behavior of insulin in chitosan gel beads. J. Biomater. Sci. Polym. Edn. 14, 1243–1253.

Lubben, I.M., Verhoef, J.C., Borchard, G., Junginger, H.E., 2001. Chitosan and its derivatives in mucosal drug and vaccine delivery. Eur. J. Pharm. Sci. 14, 201–207.

Ma, Z., Yeoh, H.H., Lim, L.Y., 2002. Formulation pH modulates the interaction of insulin with chitosan nanoparticles. J. Pharm. Sci. 91, 1396–1404.

Onishi, H., Machida, Y., 1999. Biodegradation and distribution of water-soluble chitosan in mice. Biomaterials 20, 175–182.

Oungbho, K., Müller, B.W., 1997. Chitosan sponges as sustained release drug carriers. Int. J. Pharm. 156, 229–237.

Rhazi, M., Desbrières, J., Tolaimate, A., Rinaudo, M., Vottero, P., Alagui, A., Meray, M.E., 2002. Influence of the nature of the metal ions on the complexation with chitosan. Application to the treatment of liquid waste. Eur. Polym. J. 38, 1523–1530.

Schlick, S., 1986. Binding sites of Cu^{2+} in chitin chitosan. An electron spin resonance study. Macromolecules 19, 192–195.

Senel, S., Kremer, M.J., Kas, S., Wertz, P.W., Hincal, A.A., Squier, C.A., 2000. Enhancing effect of chitosan on peptide drug delivery across buccal mucosa. Biomaterials 21, 2067–2071.

Tengamnuay, P., Sahamethapat, A., Sailasuta, A., Mitra, A.K., 2000. Chitosans as nasal absorption enhancers of peptides: comparison between free amine chitosans and soluble salts. Int. J. Pharm. 197, 53–67.

Thanou, M., Verhoef, J.C., Junginger, H.E., 2001. Oral drug absorption enhancement by chitosan and its derivatives. Adv. Drug Deliv. Rev. 52, 117–126.

Tomihata, K., Ikada, Y., 1997. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. Biomaterials 18, 567–575.

Yomota, C., Komuro, T., Kimura, T., 1990. Studies on the degradation of chitosan films by lysozyme and release of loaded chemicals. Yakugaku Zasshi 110, 442–448.