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Release profile of insulin entrapped on mesoporous materials by freeze–thaw method

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ABSTRACT

Adsorption profiles of insulin on porous materials and release profiles of insulin entrapped on folded-sheet mesoporous silica (FSM) were studied. Three types of FSM with different pore sizes (3.0, 6.1, and 9.2 nm) were used as candidates. A simple technique of repeated freezing and thawing resulted in effective adsorption of insulin on mesoporous structures. The amount of adsorbed insulin, estimated by protein assay, increased with an increase in the pore sizes of FSM used. Nitrogen sorption analysis showed that the specific surface area and pore volume decreased according to the insulin adsorption. On the release profile of insulin, the smallest pore size of FSM (3.0) was found to be a suitable material for a fast release of insulin, whereas the medium-pore FSM (6.1) held the insulin inside the pores for a longer time. Consequently, the desired release of insulin could be achieved by selecting the appropriate pore size of FSM.

1. Introduction

The oral administration of insulin represents one of the greatest challenges in pharmaceutical technology. To prepare a composite matrix of insulin and some specific materials may produce advantages for a sustained or delayed insulin release (Jain et al., 2005; Liu et al., 2007; Xiong et al., 2007). Recently, synthetic mesoporous materials in a large range of pore sizes have been produced. There has been growing interest in the use of mesoporous materials as controlled drug delivery matrices (Lin and Mou, 2002). Folded-sheet mesoporous material (FSM) has been prepared by intercalation of quaternary ammonium surfactant as a template in a layered sodium silicate, kanemite, followed by calcination to remove the template (Kresge et al., 1992; Inagaki et al., 1996). FSM showed high specific surface area such as 1000 m^2/g and narrow pore size distribution in the mesopore range of 2–50 nm. FSM has honeycomb one-dimensional straight channels, and it shows excellent heat resistance and pressure resistance (Cassiers et al., 2002; Yanagisawa et al., 1990).

The active pharmaceutical ingredient (API), namely, organic compounds with pharmacological effects, can be incorporated in the pores of mesoporous material, leading to a change in the molecular state of the API from a crystalline to an amorphous state. It was reported that the API could be taken up into the minute holes and the adsorbed substance existed not as a crystal but as a solid dispersion. Moreover, it was reported that the solubility of an API that has been adsorbed to porous powder is higher than that of a drug that exists as single crystals (Tozuka et al., 2003). Therefore, some research in recent years has attempted to improve physical properties by adsorbing drugs into porous powder in order to improve its dissolution and control its delivery (Cavallaro et al., 2004; Doadrio et al., 2004; Mellkaerts et al., 2007; Song et al., 2005).

However, most of the articles published were related to the application of mesoporous material for medicinal compounds having a small molecular weight. There have been few reports of a porous material being used as a carrier for insulin, since the adsorption efficiency may be limited according the steric hindrance of insulin. It was reported that the adsorption profile depended strongly on the pore size of the mesoporous material and chemical structure of medicinal compounds used (Nishiwaki et al., 2009).

In this study, we aimed to incorporate insulin into mesoporous structures of FSMs of different pore sizes by freeze–thaw method. The relationship between the amount of insulin adsorbed and the characteristics of the FSM was investigated, as well as the release profile of insulin from solid dispersions with FSM. The sorption profile or state of insulin was estimated by nitrogen gas sorption analysis and differential scanning calorimetry.

2. Materials and methods

2.1. Materials

Insulin from bovine pancreases (Sigma) was purchased and used as received. Three types of folded-sheet mesoporous materials

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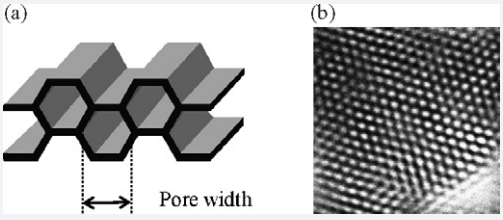
Table 1
Physicochemical properties of porous silicas.

	A schematic representation of FSM structure (a) and a representative transmission electron microscope photograph of FSM (b)		
			
	Pore width (nm)	BET surface area (m^2/g)	Pore volume (cm^3/g)
FSM (3.0)	3.0	1025.8	0.9
FSM (6.1)	6.1	504.4	0.6
FSM (9.2)	9.2	491.3	0.7
SMBSG	11.4	274.0	0.8

(FSM) – FSM (3.0), FSM (6.1), FSM (9.2) – and super micro bead silica gel grade 100-10 (SMBSG) were kindly supplied from Fuji Silysia Chemical Ltd. (Japan). Abbreviations in parentheses beside "FSM" represent the mean pore diameter in nm calculated from nitrogen gas sorption analysis. Those materials were used after drying under reduced pressure at 100 °C for 2 h. Mean pore diameter, specific surface area, and pore volume of porous silicas are listed in Table 1. To prepare a suspension including both silica and insulin, 10 mg of silica and 5 mg of insulin were dispersed into 1 ml of 0.01 M HCl solution, and then sonicated for 5 min.

2.2. Evaluation of drug adsorption on silica

The suspension was mixed by a rotor (RT-50, TAITEC, JAPAN) at 5 °C air temperature for 2 h, and then centrifuged for 10 min at 3500 rpm. The supernatant of insulin solution, without the porous silica, was collected, and the insulin concentration was measured to estimate the amount of drug adsorbed on the silica. The amount of insulin adsorbed on silica was estimated by subtracting the insulin dissolved in solution from the amount of insulin loaded. The drug concentration in the solution was determined by Bio-Rad RC DC™ protein assay kit (Bio-Rad Laboratories, Inc. JAPAN) by measuring the UV absorbance at 750 nm.

2.3. Freeze–thaw method

The suspensions were frozen in a refrigerator at −15 °C for definite intervals and thawed at room temperature (freeze–thawing). All the samples were investigated after undergoing 10 cycles of freezing–thawing. To evaluate the effect of the freezing rate on the drug adsorption amount, freezing operations with two different freezing rates were carried out: rapid freezing using a refrigerated/heating circulator (Julabo Japan Co., Ltd.), and gentle freezing using a Biomedical freezer (SANYO Electric Co., Ltd.). Real-time temperature monitoring was performed to evaluate the temperature change of the sample depending on the freezing treatment, by measuring the temperature of 3 ml of insulin solution with a digital thermometer (Yokogawa Meters & Instruments Corporation, Japan).

2.4. Differential scanning calorimetry (DSC) for the analysis of freezing behavior

The suspension filtered on a filter paper (ADVANTEC, Japan) and the sample immediately after the filtration without further drying treatment was used for DSC analysis. Thermal studies were performed using differential scanning calorimetry (DSC-2000, Seiko Instrument Inc., Japan). Samples were kept in a sealed aluminum pan at a heating and cooling rate of 1 °C /min, over a temperature range from −120 to 20 °C.

2.5. Nitrogen gas adsorption analysis

Nitrogen adsorption–desorption isotherms were recorded at −196 °C on a Micrometrics automatic surface area analyzer (GEMINI 2375, Shimadzu Co. Ltd., Japan). Samples were dried after freeze–thawing at room temperature under vacuum overnight. Specific surface area was calculated according to the Brunauer–Emmett–Teller (BET) equation. BET specific surface areas of the samples were calculated in the range of relative pressures between 0.05 and 0.31. The pore size distribution for the pore diameter range 2–50 nm was estimated by applying the Barrett–Joyner–Halenda (BJH) method to the desorption isotherm of nitrogen.

2.6. Release profile of insulin

The release profile of insulin was carried out in a shaking bath with a shaking speed of 60 strokes/min at 37 °C. The freezed–thawed sample, which was dried at room temperature under vacuum overnight, was put into 10 ml of McIlvaine buffer (pH 7.4), which included 0.02% NaN_3 and 0.1% Tween80®. At definite intervals, 0.3 ml of the solution was pipetted out and filtered through 0.2-μm cellulose acetate membrane filter (ADVANTEC, JAPAN). The amount of insulin released in the medium was determined by a Dc protein assay kit (Bio-Rad Laboratories, Inc., Japan) by measuring the UV absorbance at 750 nm. All measurements were performed in triplicate, and the mean value and standard deviation were calculated.

3. Results and discussion

3.1. Insulin adsorption on silica

Fig. 1 shows differences in the adsorption amount of insulin onto porous silicas of different pore sizes. Open columns show the amount of insulin when each porous silica was simply mixed into the insulin solution. We confirmed that the degradation of insulin was negligible when the freeze–thaw process without adding silica. Without freezing–thawing, no significant difference in insulin adsorption was found among the three types of FSMs. An improvement of adsorption profiles of insulin was observed after freeze–thawing 10 times, as shown in the closed symbol columns of each FSM system. Samples in the presence of SMBSG show little adsorption of insulin regardless of whether or not freezing–thawing was performed. SMBSGs are microbead silica

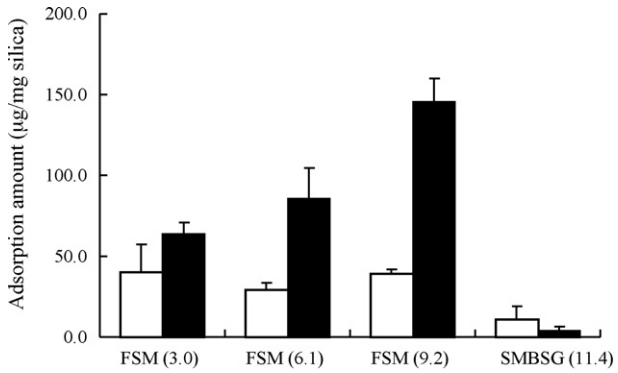


Fig. 1. Calculated amount of insulin adsorbed on silica ($n = 3$). Samples without freeze–thaw process and samples after freezing–thawing 10 times are represented as open columns and closed columns, respectively. Abbreviations in parentheses represent the mean pore diameter in nm.

gels that have a porous structure; however, their structure is not controlled, with wide range pore size distribution. Those findings suggest that one-dimensional straight channels with hexagonal shapes of FSM would be superior environments for insulin adsorption compared with SMBSG having no uniform porous structures.

The adsorbed amount of insulin depends clearly on the pore size of FSM; a large pore tends to hold a larger amount of insulin molecules. In a previous study, the adsorption amount and the adsorption profile of an active pharmaceutical ingredient (API) onto the mesoporous silica depended strongly on the pore size of the material (Nishiwaki et al., 2009). The required space for insulin adsorption is larger than that for the adsorption of the API used in that study, since insulin has a relatively large molecular size and weight. The hydrodynamic diameter of insulin was estimated as 2.69 ± 1.43 nm when the insulin was dissolved as a monomer in an aqueous phase (Oliva et al., 2000). Therefore, large hexagonal mesopores would be superior to small ones in terms of the insulin adsorption onto FSM.

3.2. Adsorption profile of Insulin during the freeze–thaw treatment

Fig. 2 shows the effect of freezing speed on the adsorption amount of insulin during the freeze–thaw process. Closed and open symbols represent rapid freezing under the −15 °C circulation apparatus and gentle freezing in a refrigerator, respectively. Fig. 2A shows the relationship between freezing time and temperature changes detected in the freezing process of insulin solution in the presence of FSM (9.2). The insulin solution with FSM (9.2) was frozen completely within 30 min by the rapid freezing method, whereas more than 3 h was required for freezing by the gentle freezing. In both cases, a sample solution first maintained a liquid state below the freezing point of water; then the supercooled solution crystallized around 0 °C as shown in the plateau region of Fig. 2A. The effect of the cooling rate on the adsorbed amount of insulin on the porous silica is shown in Fig. 2B. In both cases, the amount of adsorbed insulin tended to increase as a function of freeze–thaw repetitions. It was found that the freezing process affected the adsorption profile, since the amount of insulin adsorbed by gentle freezing is higher than that by rapid freezing. Therefore, we selected the gentle freezing condition for the freeze–thaw method in order to achieve more effective adsorption of insulin onto the mesopores.

Thermodynamic profiles of the cooling and heating processes of insulin solution in the presence of porous silicas are shown in Fig. 3. In the cooling process, the bulk water phase in the FSM (9.2) sample froze at around −10 °C, represented as a sharp exothermic peak, but the freezable pore water was present as a liquid state at this temperature (Fig. 3A). Freezable water in the pores of FSM (9.2) froze at around −20 °C as shown by the other, broader exothermic peak. In the heating process, there are at least two distinct endothermic peaks in the heating curves. The two endothermic peaks of lower and higher temperature were attributable to the melting of freezable pore water and bulk water, respectively. By contrast, the sample with SMBSG did not show dual exothermic peaks in the cooling process, as shown in Fig. 3B. It was reported that the melting and freezing temperatures of liquid confirmed as being in pores are lower than those of the bulk liquid (Ishikiriyama and Todoki, 1995a,b; Morishige and Nobuoka, 1997). In the freeze–thaw method performed in this study, insulin solutions were frozen in a refrigerator at −15 °C for definite intervals and thawed at room temperature. The resulting thermodynamic profiles indicated that almost all of the water was frozen at −15 °C for the insulin solution with SMBSG while the freezable pore water existed as a liquid phase during the freeze–thaw process of the insulin solution with FSM (9.2). The existence of supercooled water at −15 °C was also observed for the insulin solution with FSM (3.0) and with FSM (6.1) from the DSC thermograms (data not shown).

Insulin adsorption profiles for the freeze–thaw process may be explained as follows: part of the insulin molecules dissolved near the mesopores of FSMs could move to the unfrozen aqueous phase existing in the mesopores; thereafter, a small amount of insulin still remained after the thawing process. Although the adsorption and desorption of insulin occur simultaneously, the increase of adsorbed insulin suggests that interactions between the insulin and

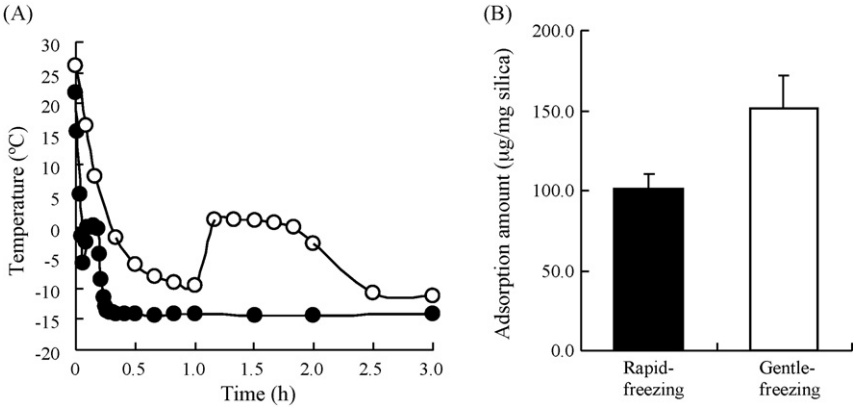


Fig. 2. Effect of freezing condition on the amount of insulin adsorbed during the freeze–thaw process. Change in the sample temperature of insulin solution during freezing as a function of freezing time (A), and differences in the adsorption amount of insulin on FSM (9.2) after freezing–thawing 10 times for each treatment (B): (●) rapid freezing, (○) gentle freezing.

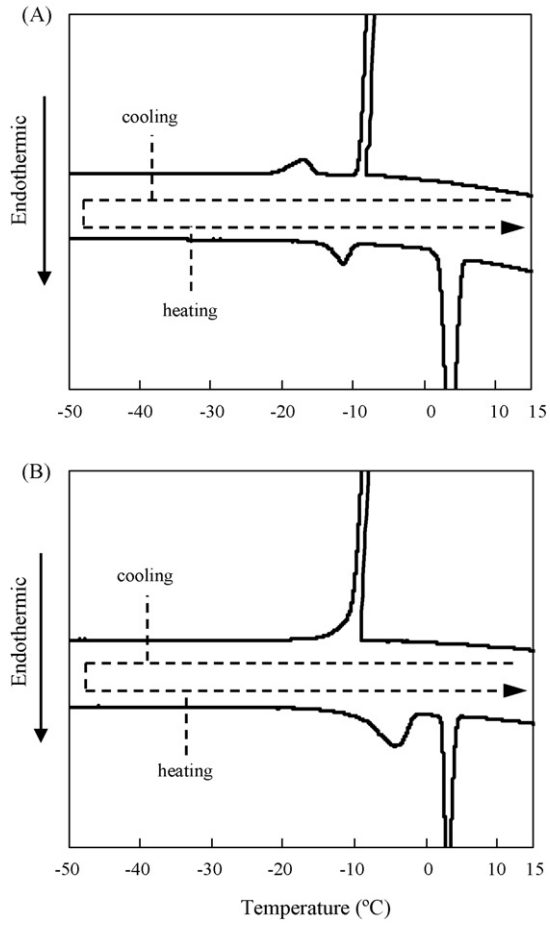


Fig. 3. Thermodynamic profiles of insulin solution in cooling and heating processes: (A) FSM (9.2), (B) SMBSG.

surface of FSM are stronger than that for desorption of insulin from the FSM surface, leading to an increase of insulin concentration into the mesopores during multiple freeze–thaw treatments. The effects of insulin concentration on the adsorption amount in the mesopores of FSM (9.2) are listed in Table 2. The amount of insulin adsorbed increased with an increase in the initial concentration of both untreated and freeze–thawed samples. An increase of insulin concentration may increase the opportunity of insulin molecules to come into contact with supercooled unfrozen water around the mesopores. Therefore, more amount of adsorption was observed for solutions with high insulin concentrations.

3.3. Changes in physicochemical properties of FSMs after the freeze–thaw treatment

Analysis of nitrogen sorption isotherms for a series of FSM before and after the freeze–thaw treatment was carried out to clarify the physicochemical changes of the FSM used. Nitrogen sorption isotherms and pore size distributions of FSM are shown in Fig. 4. Specific surface areas and pore volumes of those samples calculated from the nitrogen sorption isotherms are listed in Table 3. As shown in Fig. 4A, all the FSM samples show a decrease in the amount of nitrogen gas adsorbed after the freeze–thaw treatment with insulin. In all the FSM samples, the peaks of closed symbols in the pore size distribution shifted to smaller pore sizes than those of open symbols (Fig. 4B). Both the specific surface area and pore volume of FSM (3.0), FSM (6.1) and FSM (9.2) decreased after the freeze–thaw treatment with insulin (Table 3).

Table 2
Changes in the amount of insulin adsorbed onto FSM (9.2) surface as a function of insulin concentration.

Insulin concentration (mg/mL)	Adsorbed amount of insulin (μg/mg silica)	
	Untreated	Freezing–thawing (10 times)
1.0	16.4 ± 0.3	76.8 ± 2.9
2.5	16.5 ± 6.1	113.7 ± 0.7
5.0	38.8 ± 2.6	145.8 ± 13.8
10.0	91.4 ± 20.2	205.3 ± 30.4

The nitrogen gas sorption isotherm is commonly used for the characterization of porous materials, especially for the analysis of properties such as specific surface area, pore size, and pore volume. In a previous report, the specific surface area and pore volume of mesoporous silica decreased where flurbiprofen molecules were adsorbed in the mesopores. The decrease in pore volume of mesoporous silica from the nitrogen sorption study corresponded well to the results from the small-angle X-ray diffraction scattering measurement. The decrease in the mean pore width was considered to be a result of the shrinkage of FSM mesopores due to the formation of hydrogen bonds between flurbiprofen and FSM in the solid dispersions. The existence of adsorbed flurbiprofen layers was proposed from the decrease in the specific surface area and pore volume of mesoporous silica (Tozuka et al., 2005). Our findings from nitrogen sorption isotherms suggested that insulin molecules were entrapped in the mesopores of FSMs after the samples were freeze–thawed 10 times.

3.4. Release profile of insulin from silica

Fig. 5 shows the release profiles of insulin from samples after freezing–thawing with FSMs. The release profiles of insulin were clearly different depending on the pore size of FSM used. In the case of FSM (3.0), about 50 percent of the adsorbed insulin was released within 1 h; all was completely released after 48 h. This initial burst of insulin release was not observed in either the FSM (6.1) or FSM (9.2) samples. The FSM (9.2) samples showed a very gradual release of insulin, while the release profile of the FSM (6.1) sample did not change after the first 8 h. Only 20% of insulin was released after 48 h in the case of FSM (6.1), indicating that most of adsorbed insulin still remained in the mesopores of FSM (6.1).

Insulin has been reported to form several association behaviors in solution or suspension, including monomer, dimer and hexamer. The mean hydrodynamic diameters of insulin existing as a monomer, dimer, and hexamer have been estimated as 2.7, 3.8, and 5.5 nm, respectively. It was also reported that the solubility of the insulin hexamer is lower than that of the monomer and dimer (Oliva et al., 2000). According to our assumption as discussed in Figs. 2 and 3, insulin may adsorb on the mesopores via supercooled water in the freeze–thaw treatment. The pore size of FSM (3.0) was a suitable environment for the adsorption of insulin monomers, since the mean pore size of FSM (3.0) is not large enough to accommodate the dimer or hexamer. The fast release of insulin may be attributed to the thermodynamically unstable state of the adsorbed insulin, as well as the fact that monodispersely adsorbed active pharmaceutical ingredients exist in a thermodynamically unstable state (Song et al., 2005; Tozuka et al., 2003). This may be a factor plausibly contributing to the faster dissolution properties of insulin in the FSM (3.0).

For the adsorption step of organic compounds onto porous materials, adsorption occurs in the micropores at very low pressure, followed by adsorption in the mesopores, with capillary condensation accruing at high pressures. When the acetonitrile vapor was adsorbed on the mesoporous silica MCM-41 (pore width: 3.2 nm), the adsorbed state of acetonitrile was changed before and after

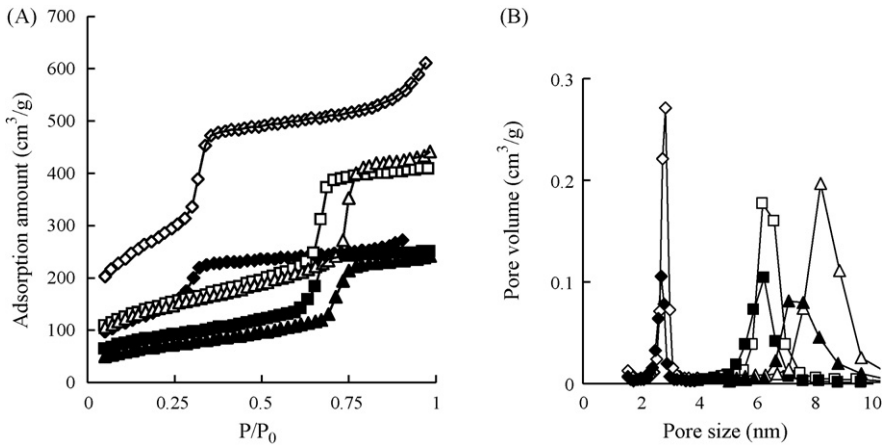


Fig. 4. Nitrogen adsorption isotherms (A) and pore size distributions (B) of FSMs. Open and closed symbols show untreated samples and samples after freeze–thaw treatment, respectively: (♦) FSM (3.0), (■) FSM (6.1), (▲) FSM (9.2).

Table 3
Changes of the physicochemical properties of FSMs after freezing–thawing 10 times with insulin (insulin concentration: 5 mg/ml).

	BET surface area (m^2/g)		Pore volume (cm^3/g)	
	Untreated	Freezing–thawing (10 times)	Untreated	Freezing–thawing (10 times)
FSM (3.0)	1025.8	515.0	0.9	0.5
FSM (6.1)	504.4	301.1	0.6	0.4
FSM (9.2)	491.3	244.3	0.7	0.4

the capillary condensation. It was reported that the relaxation of molecular reorientation depends sensitively on the local nanoenvironment (Tanaka et al., 1998). The adsorption manner of insulin, which has a relatively large molecular weight to organic compound, may be different from that of small organic molecules. Although it is quite difficult to assess the adsorption state of insulin directly, based on size it is possible that insulin forms dimers and/or hexamers in the mesopores of FSM (6.1) and FSM (9.2). Such molecules would have several association behaviors, especially if the associated form of insulin is energetically more stable in the mesopore than the insulin monomer. The reason for the sustained release of insulin from the sample with FSM (6.1) may be explained by the different affinities to the mesopores of insulin in different association forms. In general, it can be said that the strength of interaction between the mesopore wall and insulin complex may differ depending on the pore size of FSM, leading to different release profiles of insulin into an aqueous medium.

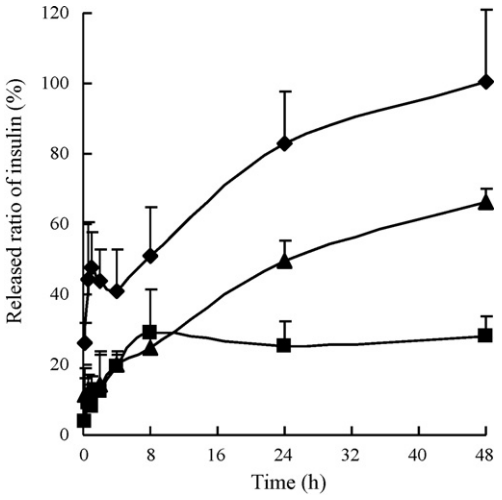


Fig. 5. Release profiles of insulin from the sample after the freeze–thaw treatment with (♦) FSM (3.0), (■) FSM (6.1), (▲) FSM (9.2).

4. Conclusion

Insulin molecules could be entrapped into the mesoporous structure of FSM using the freeze–thaw method. Insulin encapsulated in mesopores of FSM may have the potential to avoid enzymatic degradation. Since freeze–thawing was carried out in the temperature range from −15 °C to room temperature, this method may be a promising way to prepare formulations that was sensitive to temperatures. The release profile of insulin could be controlled by varying the mesopore size of the adsorbent. Consequently, FSMs have potential for allowing controlled release of insulin.

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