ELSEVIER

International Journal of Pharmaceutics 169 (1998) 213–220

international journal of pharmaceutics

# Investigation of pectin as a carrier for oral delivery of proteins using calcium pectinate gel beads

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Received 12 February 1998; received in revised form 9 March 1998; accepted 13 March 1998

**Abstract**

A novel oral delivery of protein to the colon based on the polysaccharide pectin has been investigated using bovine serum albumin (BSA) as a model protein. Calcium pectinate gel (CPG) beads were prepared by extruding BSA-loaded pectin solution into agitated calcium chloride solution. Subsequent drying produced matrix beads in which BSA were embedded. In vitro experiments were conducted on the release of BSA from the CPG beads under conditions pertaining to those in vivo. Monitoring of release gives a sensitive indication of the behavior of pectin under the different conditions. The type of pectin and the presence of enzymes in the medium influence release characteristics. By changing the type of pectin it is possible to protect BSA during conditions of mouth to colon transit and susceptibility to enzymatic attack. Additionally, the release of BSA from CPG beads was strongly affected by cross-linking time but not particularly affected by the amount of protein added. The investigated factors also influenced bead sizes and entrapment efficiency. The results suggest that calcium pectinate gel beads are promising as a carrier for oral delivery of protein by means of colonic delivery. 

*Keywords:* Pectin; Calcium pectinate gel; Bovine serum albumin; Oral protein delivery; Colonic delivery; Beads

## 1. Introduction

Protein drugs are increasingly becoming a very important class of therapeutic agents with the rapid advances in the field of biotechnology. Currently, these drugs are mostly delivered by parenteral administration. Because they are extremely short-acting, repeated injections are often required. To minimize the health hazard by constant injection, there is an urgent need to search for non-parenteral route of administration as well as to develop formulations with controlled-delivery features. Oral delivery, the popular and convenient method for drug delivery, is the alternate route of administration. However, protein drugs are easily degraded in the gastrointestinal tract by the same enzymes that degrade dietary proteins

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0378-5173/98/$19.00 
*PII* S0378-5173(98)00129-X

and are poorly absorbed in the small intestine. In general, the small intestine has the highest amount of peptidase activity (Zhou and Li Wan Po, 1991), whereas the colon has the lowest amount. Hence, delivery of proteins to this region of the gastrointestinal tract where enzyme activity is low offers an attractive means for increasing absorption of proteins when given by the oral route.

Enzymatically controlled delivery systems targeting the oral dosage form to the colon have been reviewed (Friend, 1991; Rubinstein, 1994). A large number of polysaccharides are degraded by colonic bacterial enzymes and may form the basis for a suitable carrier. Pectin is a naturally occurring water-soluble polysaccharide which is found in the cell wall of most plants. Though it is a heterogeneous polysaccharide, pectin contains linear chains of (1→4)-linked α-D-galacturonic acid residues. These uronic acids have carboxyl groups, some of which are naturally presented as methyl esters and others which are reacted with ammonia to produce carboxamide groups. The degree of esterification (DE) and degree of amidation (DA), which are both expressed as a percentage of carboxyl groups (esterified or amidated), are important means to classify pectin. Amidated low methoxy pectin (with DE < 50%) forms more rigid gels by the action of calcium, which cross-links the galacturonic acid chains, than conventional low methoxy pectin. The lower the DE, the more sensitive the pectin is to calcium as a gelling agent. Rolin (1993) has recently provided a good review on pectin, including a comprehensive discussion of the chemistry.

Since pectin can react with calcium ions, calcium pectinate has been investigated as an insoluble hydrophilic coating for sustained-release delivery by an interfacial complexation process (Sriamornsak et al., 1997a,b). Recently, calcium pectinate gel (CPG) beads prepared by the ionotropic gelation method (Aydin and Akbuga, 1996; Sriamornsak and Nunthanid, 1998) were investigated as a sustained-release delivery system for conventional drugs. Combinations of calcium salts and pectin have been used to prepare matrix tablets for colonic delivery of several model drugs (Ashford et al., 1994; Rubinstein et al., 1993). The rationale for this is that calcium pectinate will be degraded by colonic pectinolytic enzymes (Englyst et al., 1987), but will retard drug release in the upper gastrointestinal tract due to its insolubility and because it is not degraded by gastric or intestinal enzymes (Sandberg et al., 1983).

The aim of this research was to investigate the suitability of pectin, in the form of calcium pectinate gel beads, for potential use as an oral delivery system for protein, by means of colonic delivery, using bovine serum albumin (BSA) as a model protein. Several formulation and processing factors can influence the properties and release characteristics of the resultant CPG beads. The effects of three factors, i.e. the different types of pectin, the amount of BSA in the pectin solution and cross-linking time on the bead properties and the release characteristics of model protein (BSA) from BSA-loaded CPG beads were also investigated.

## 2. Materials and methods

### 2.1. Materials

The four types of pectin were obtained from two sources. Amidated low methoxy (LM) pectin with DE of 36% and DA of 14% (GENUpectin type LM-101 AS) and one with DE of 28% and DA of 20% (GENUpectin type LM-104 AS-FS) were the generous gifts of Copenhagen Pectin (Denmark) and are referred to as PG36 and PG28, respectively. The non-amidated LM pectin from citrus fruit with DE of 28% and polygalacturonic acid with DE less than 5% were obtained from Sigma (USA) and referred to as PS28 and PGA, respectively. Bovine serum albumin with average molecular weight of 66400 Da (Fluka AG, Germany) was used as a model protein. Pectinex Ultra SP-L (pectinolytic enzymes) was purchased from Novo Nordisk Ferment (Switzerland). Calcium chloride, Trizma® (Sigma) were used as supplied and where applicable were AR grade. All other chemicals were of reagent grade.

### 2.2. Preparation of BSA-loaded CPG beads

CPG beads were prepared analogously to the main method described earlier (Sriamornsak and Nunthanid, 1998). Pectin was dispersed in distilled water at a concentration of 5% (w/w) unless otherwise noted. BSA was added to aqueous solution of pectin and dispersed thoroughly by stirring. The dispersions were dripped using a nozzle of 0.80 mm inner diameter into a 5% (w/v) solution of calcium chloride with gentle agitation at room temperature. The CPG beads formed were allowed to stand in the solution for 20 min, filtered and washed with distilled water. The beads were then collected by filtration. Diameter of obtained CPG beads was about 2.1–2.2 mm.

### 2.3. Characterization of the BSA-loaded CPG beads

#### 2.3.1. Particle size

After drying at 37°C for 48 h, the mean diameter of 50 dried beads was determined by microscopic method, using an optical microscope (BH-2, Olympus, Japan). The microscope eyepiece was fitted with a micrometer by which the size of the beads can be measured. The mean diameter of beads before and after drying was compared and statistical comparison was carried out using Student's *t*-test.

#### 2.3.2. BSA content and entrapment efficiency

Prior to the determination of BSA content, the CPG beads must be dissolved by phosphate buffer (pH 7.4). The content of BSA was later assayed by UV spectrophotometry (Hitachi U-2000, Japan) in pH 7.4 phosphate buffer at 280 nm. The determinations were made in triplicate. The entrapment efficiency (EE) was calculated according to the relationship:

$$\mathrm{EE} = \frac{\text{Actual BSA content (\%)} \times 100}{\text{Theoretical BSA content (\%)}}$$

### 2.4. BSA release from CPG beads and swelling studies

Release studies of BSA from CPG beads under conditions mimicking mouth to colon transit were evaluated in the absence and presence of pectinolytic enzymes. CPG beads (100 mg) were placed into test tubes containing 10 ml of Tris buffer, pH 6.8 (to simulate the pH conditions of the intestine). Each tube was shaken at 100 rpm at 37°C using a shaking incubator (EYELA MMS-48GR, Japan). At specific times, 5-ml samples were collected and centrifuged for 20 min at 12000 rpm (Biofuge28RS, Haraeus Sepatech, Germany). The supernatant was removed and BSA concentration was determined spectrophotometrically at 280 nm (Hitachi U-2000, Japan). The release study was continued after replacement with 5 ml of fresh buffer. After the 5 h of testing, the release medium was removed and replaced with the medium containing pectinolytic enzymes. The amount of pectinolytic enzymes used were 300 PG (activity 26000 PG/ml at pH 3.5; PG = milliequivalents of reducing groups liberated from pectin per minute per unit of enzyme). Each in vitro release study was performed in triplicate.

The swelling behaviors of the beads was studied by measuring the diameter of the beads under an optical microscope, after exposure to Tris buffer, pH 6.8, using the same conditions as for the release studies. Beads were removed for measurements at specific time intervals. Examination of the bead appearance in the medium, before and after addition of pectinolytic enzymes, was performed after freeze drying of the beads.

## 3. Results and discussion

### 3.1. Preparation and characterization of BSA-loaded CPG beads

The ionic interaction between the negatively charged carboxyl groups of LM pectin and the positively charged counter ion, calcium, was used to prepare CPG beads (Sriamornsak and Nunthanid, 1998). Aqueous solution of LM pectin containing BSA was dropped into calcium chloride solutions and a gelled sphere was formed instantaneously by ionotropic gelation. Intermolecular cross-links were formed between the

Table 1
Effect of the different type of pectin used in the formulation on the shape of wet CPG beads

| | Concentration of pectin (%) | | | | Shape[a] |
|---|---|---|---|---|---|
| | PG36 | PG28 | PS28 | PGA | |
| PG36 | 5 | | | | Spherical |
| PG28 | | 5 | | | Spherical |
| PS28 | | | 5 | | Drop-like |
| PGA # 1 | | | | 5 | Noodle-like |
| PGA # 2 | | | | 10 | Drop-like |
| PG36 + PS28 | 5 | | 2.5 | | Spherical |
| PG36 + PGA | 5 | | | 5 | Spherical |
| PG28 + PS28 | | 5 | 2.5 | | Spherical |
| PG28 + PGA | | 5 | | 5 | Spherical |

[a] Shape of the wet beads was investigated immediately after preparation.

divalent calcium ions and the negatively charged carboxyl groups of the LM pectin molecules, called an 'egg-box' conformation with interstices in which the calcium ions may pack and be coordinated (Grant et al., 1973; Morris, 1986).

As shown in Table 1, the appearance of the beads formed was influenced by the type of pectin used. The non-amidated LM pectins, both DE 28% (PS28) and DE < 5% (PGA), form particles of ellipsoid shape, while the shape of particles of amidated LM pectins (PG28 and PG36) is close to spherical. It is considered that amidated LM pectins can form gels with a higher calcium reactivity than non-amidated ones. However, the lower the DE, the more rigid the pectin gel is to calcium. Moreover, the mixture of amidated and non-amidated pectins provides the spherical beads. So the spherical beads of amidated pectins and the mixtures of amidated and non-amidated pectins were used throughout the experiment.

Upon air-drying, the CPG beads of all preparations shrank significantly ($P < 0.05$). The insufficiently strong beads had a hollow at the middle of the surface. The dried CPG beads containing BSA were translucent. The mean diameter of dried BSA-loaded CPG beads ranged between 1.25 and 1.63 mm (Table 2).

### 3.2. In vitro release of BSA from CPG beads

Release studies were carried out to examine the suitability of the calcium pectinate to use as matrix gel beads for an oral delivery system for proteins, and to check whether CPG beads could retain their drug load for 5 h and degrade in the presence of enzymes afterwards. Release pattern was represented by plotting the amount of BSA release against the time. Fig. 1 demonstrates the

Table 2
Mean diameter and BSA loading capacity of CPG beads

| Variables | Mean diameter (mm ± S.D.[a]) | EE (%, ± S.D.[b]) |
|---|---|---|
| Blank | 1.281 ± 0.11 | — |
| Type of pectin[c] | | |
| PG36 | 1.283 ± 0.13 | 35.0 ± 1.13 |
| PG28 | 1.490 ± 0.09 | 43.8 ± 3.42 |
| PG36 + PS28 | 1.257 ± 0.08 | 27.4 ± 2.43 |
| PG36 + PGA | 1.252 ± 0.10 | 48.3 ± 4.52 |
| PG28 + PS28 | 1.373 ± 0.12 | 52.3 ± 5.59 |
| PG28 + PGA | 1.356 ± 0.11 | 39.1 ± 2.59 |
| Amount of BSA added[d] | | |
| 5 g | 1.505 ± 0.14 | 38.6 ± 6.02 |
| 10 g | 1.594 ± 0.24 | 60.3 ± 2.93 |
| 20 g | 1.611 ± 0.12 | 24.3 ± 1.52 |
| 40 g | 1.629 ± 0.11 | 23.1 ± 0.59 |
| Cross-linking time[e] | | |
| 10 min | 1.621 ± 0.10 | 40.8 ± 2.88 |
| 20 min | 1.594 ± 0.24 | 60.3 ± 2.93 |
| 40 min | 1.382 ± 0.07 | 48.8 ± 2.24 |

[a] S.D. was calculated from 50 measurements.
[b] S.D. was calculated from three repeated measurements.
[c] With 10 g of BSA added and 20 min cross-linked.
[d] Using PG28 + PS28 as polymers, 20 min cross-linked.
[e] Using PG28 + PS28 as polymers, 10 g of BSA added.

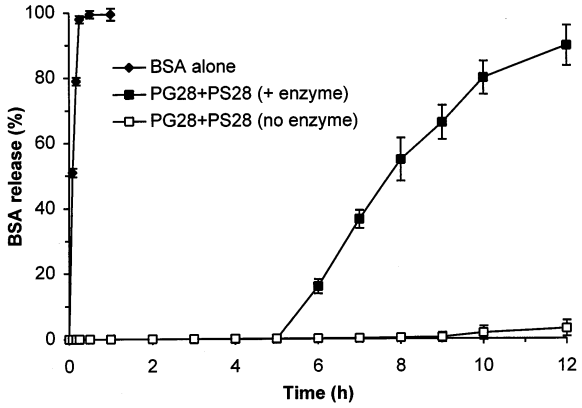


Fig. 1. The effect of pectinolytic enzymes on the release of BSA from CPG beads (bars indicate S.D.; $n = 3$).

release profiles of the pure BSA and BSA-loaded CPG beads (PG28 + PS28). It is seen from the graph that calcium pectinate gel prevents the release of BSA for at least 5 h, while the pure BSA dissolved in 10–15 min. In contrast to low-molecular weight drugs (Sriamornsak and Nunthanid, 1998), BSA is a large molecule which cannot diffuse through the pores of the matrix bead but can be released due to enzymatic degradation of the matrix (Batycky et al., 1997). It was previously reported that gelation of LM pectin droplets in the presence of calcium may provide a valuable approach to the formation of a multiparticulate system for colonic delivery (Munjeri et al., 1997). Therefore, the effect of enzymes on release of BSA from CPG beads is also shown in Fig. 1. The pectinolytic enzymes consist of pectin methylesterase and two types of pectin depolymerases. The pectin methylesterase removes the methoxy groups from the pectin while the polygalacturonase (or hydrolase) hydrolyse the glycosidic linkages next to the free carboxyl groups in the galacturonan chain. The pectate lyases degrade the glycosidic linkages next to the free carboxyl group by $\beta$ elimination, resulting in the formation of double bonds between C4 and C5 monomer. The presence of pectinolytic enzymes considerably accelerated the release of BSA from the CPG beads.

The swelling behavior of the beads was independent of the bead formation (data not shown). When pectinolytic enzymes were added, the beads initially increased in size but were totally degraded within 4–10 h. As shown by electron micrographs in Fig. 2, the bead becomes porous. The pores were formed by enzymatic degradation of the CPG network and by subsequent erosion of the matrix beads. This allows BSA to diffuse through the partially degraded matrix of the calcium pectinate gel.

### 3.2.1. Effect of the different types of pectin

The effect of the amount of protein added and cross-linking time on the mean diameter of the dried beads and entrapment efficiency in CPG beads were given in Table 2. The mean diameter of dried beads with different types of pectin

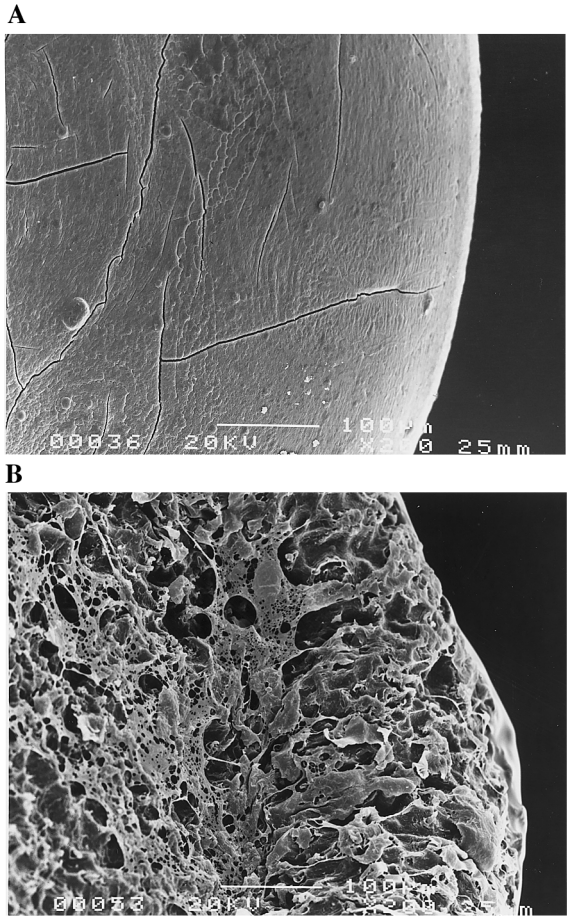


Fig. 2. Scanning electron micrographs of calcium pectinate gel bead (A) before and (B) after addition of pectinolytic enzymes into dissolution medium.

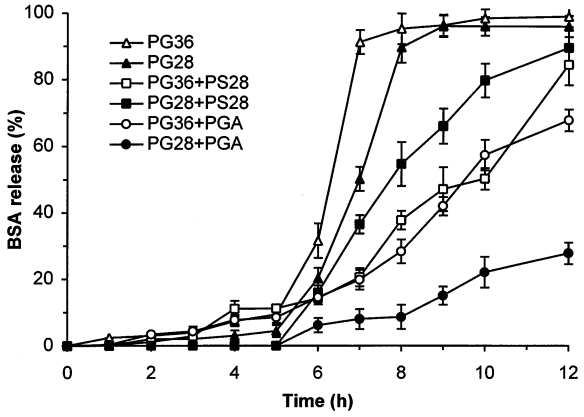


Fig. 3. Release profiles of BSA from CPG beads with the different combination of pectin in the presence of pectinolytic enzymes. Each point represents the mean ( ± S.D.) for at least three determinations.

ranges between 1.25 and 1.49 mm. With different combinations of pectin, the EE ranges from 27.4 to 52.3%. Release characteristics were represented by plotting the cumulative percent BSA release against the time. Fig. 3 gives the release profiles of BSA from CPG beads, with the different combinations of pectin. It was observed that BSA was released from the formulation with PG36, PG36 + PS28, PG36 + PGA and PG28 before 5 h. In these formulations, BSA released from the first hour, and more release was observed after the pectinolytic enzymes were added. It was considered that the calcium ions form the loose linkages with carboxyl groups in the chains of LM pectin (PG36). The formulations with PG36 were, therefore, considered not suitable for using as a carrier for colonic delivery of protein drugs since the proteins can be easily degraded by digestive enzymes in the upper GI tract. In contrast, the formulations with PG28 + PS28 and PG28 + PGA resulted in a lag time of 5 h. Following this, BSA was released at a maximum rate between 6 and 7 h, especially the formulation with PG28 + PS28, which corresponds to the colonic arrival time in the human (Hardy et al., 1985). Hence, this formulation would be considered suitable for further investigation.

Furthermore, the amount of BSA released decreased with a decrease in the DE of the LM pectin. LM pectins with low DE (PG28; DE of 28%) can form more rigid gels with calcium (Rolin, 1993), often known as an 'egg-box' structure, and retard the release of BSA, particularly in the first 5 h. Therefore, no more than 30% of BSA was released over a 12-h period when PGA (DE less than 5%) was mixed in (PG28 + PGA). The formulations in which PGA was incorporated, however, were not suitable for using as a carrier for colinic delivery due to the fact that some BSA was still entrapped in the beads and could not be released from the formulations. So the combination of amidated and non-amidated pectins (PG28 + PS28) was used throughout the experiment.

### 3.2.2. *Effect of the amount of protein added*

The mean diameter of BSA-loaded CPG beads was increased with the increase of amount of BSA added. Also, the EE increased with increased amount of protein added up to 10 g BSA. The EE decreased when the amount of protein added was more than 10 g. This result may be due to the decrease in the polymer/drug ratio in the beads. Fig. 4 demonstrates the effect of amount of BSA added on drug release from CPG beads. It is seen from this graph that calcium pectinate gel prevents the release of protein for 5 h, i.e. until the pectinolytic enzymes are added (conditions mimicking those in the colon). Release of BSA from CPG beads loaded with 5 g BSA was somewhat higher than that from beads loaded with 10 g

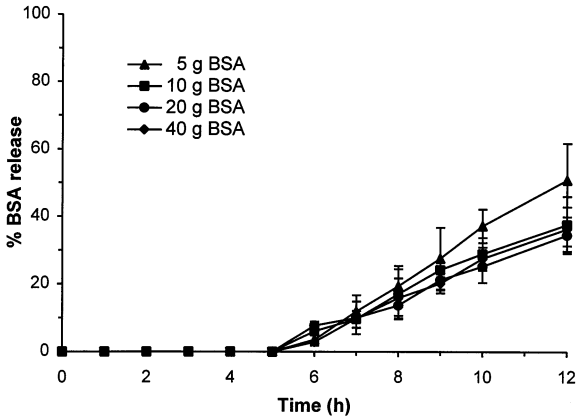


Fig. 4. Effect of the amount of protein added on release of BSA from CPG beads (bars indicate S.D.; $n = 3$).

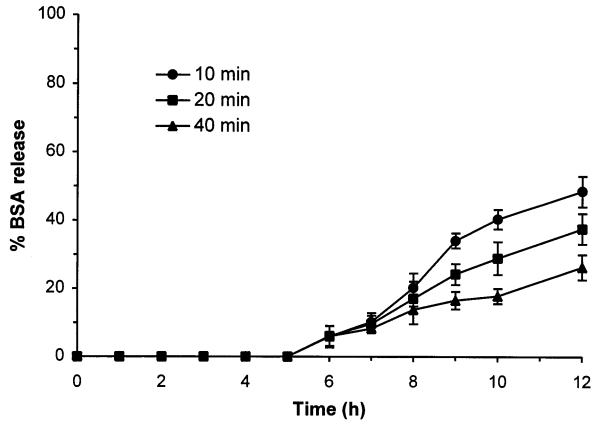


Fig. 5. Effect of the cross-linking time on release of BSA from CPG beads (bars indicate S.D.; $n = 3$).

BSA. However, the patterns of release showed very little variation between CPG bead samples loaded with more than 10 g BSA. Therefore, it appears that the effect of amount of protein added on the release of BSA from CPG beads is minor.

### 3.2.3. *Effect of cross-linking time*

The longer the cross-linking time, the lower the average size of CPG beads. The EE increased with the increased cross-linking time from 10 to 20 min. However, the EE decreased when the cross-linking time was increased to 40 min.

The release profiles of BSA from CPG beads hardened with calcium for various times are shown in Fig. 5. It was observed that the amount of BSA released from CPG beads decreased on increasing the cross-linking time. These properties are probably explained by the promotion of cross-links between pectin chains by the calcium ions. The CPG beads constitute a matrix of enterically intact material, therefore this phenomenon was clearly in evidence and the beads additionally showed a delayed release pattern. BSA is a high-molecular weight protein and its release is strongly influenced by the degradation of the calcium pectinate matrix, which is the result of pectinolytic enzymes presented in the colon.

## 4. Conclusion

In this paper, a novel oral delivery of proteins based on calcium pectinate gel beads was investigated using an ionotropic gelation technique. The delayed release of a model protein (BSA) was achieved by protection of a protein by a dosage form prior to enzymatic attack depending on the type of pectin. No attempt to maximize the benefit of this approach was made, but when using lower DE of pectin, a reduction in release was achieved. Therefore, calcium pectinate gel beads (PG28 + PS28) appear to be suitable for controlled release of protein drugs by mean of colonic delivery since they could retain their drug load for 5 h, and degraded on the presence of enzymes afterwards. Moreover, the release of BSA from CPG beads was considerably affected by cross-linking time, but no significant effect was seen with the amount of protein added. Therefore, CPG beads can be prepared that show reproducible release behavior, which indicates that the development of CPG beads holds promise for manufacturing purposes in the future.

The release of BSA as a model of protein drugs was regulated by the appropriate choice of experimental conditions for the preparation of CPG beads. Hence, CPG beads may be used in oral controlled-release systems for protein drugs.

## Acknowledgements

The author wishes to thank B. Unin, C. Kongsuk, K. Anantawattananon and S. Fangsawud for laboratory assistance. Thanks are also due to Food & Cosmetic Systems Co., Ltd. (Bangkok) who kindly provided the sample of pectin manufactured by Copenhagen Pectin. The financial support from the National Science and Technology Development Agency, Thailand, through the grant of CO-B-06-2E-16-001 is greatly appreciated.

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