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Preparation of Alginate/Chitosan Microcapsules and Enteric Coated Granules of Mistletoe Lectin

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The aqueous extract of European mistletoe (*Viscum album,* L.) has been used in cancer therapy. The purified mistletoe lectins, main components of mistletoe, have demonstrated cytotoxic and immune-system-stimulating activities. Korean mistletoe (*Viscum album* L. *coloratum*), a subspecies of European mistletoe, has also been reported to possess anticancer and immunological activities. A galactose- and *N*-acetyl-D-galactosamine-specific lectin (*Viscum album* L. *coloratum* agglutinin, VCA) with Mr 60 kDa was isolated from Korean mistletoe. Mistletoe preparations have been given subcutaneously due to the low stability of lectin in the gastrointestinal (GI) tract. In the present study, we investigated the possibility of alginate/chitosan microcapsules as a tool for oral delivery of mistletoe lectin. In addition, our strategy has been to develop a system composed of stabilizing cores (granules), which contain mistletoe lectin, extract or powder, coated by a biodegradable polymer wall. Our results indicated that successful incorporation of VCA into alginate/chitosan microcapsules has been achieved and that the alginate/chitosan microcapsule protected the VCA from degradation at acidic pH values. And coating the VCA with polyacrylic polymers, Eudragit, produced outstanding results with ideal release profiles and only minimal losses of cytotoxicity after manufacturing step. The granules prepared with extract or whole plant produced the best results due to the stability in the extract or whole plant during manufacturing process.

Key words: Mistletoe, Lectin, Microcapsules, Enteric coating, Oral administration

INTRODUCTION

The aqueous extract of European mistletoe (*Viscum album,* L.) has been used in conventional cancer therapy for decades, mainly in Europe (Jung *et al.,* 1990; Hajto *et al.*, 1990; Büssing *et al.*, 1996). Recent several studies have shown that mistletoe induces apoptotic killing of cultured tumor cells and lymphocytes, and stimulates the immune system (Bantel *et al.,* 1999; Büssing, 2000; Lyu *et al.*, 2001; Lyu *et al.*, 2002). Among the mistletoe components the cytotoxic and immunological properties of mistletoe preparation are considered to be clearly linked to lectins which are the focus of modern biomedical research (Büssing, 2000; Lyu *et al.*, 2002). The European mistletoe lectins (*Viscum album* agglutinins, VAAs) are D-galactose- and/or *N*-acetyl-D-galactosamine-specific and have molecular masses between 55 and 63 kDa (Büssing, 2000; Lyu *et al.*, 2002). Korean mistletoe (*Viscum album* L. *coloratum*), a subspecies of European mistletoe, has also been reported to possess anticancer and immunological activities (Park *et al.*, 1998; Yoon *et al.*, 1999; lyu *et al.*, 2000). A galactose- and *N*-acetyl-D-galactosamine-specific lectin (*Viscum album* L. *coloratum* agglutinin, VCA) with Mr 60 kDa was isolated from Korean mistletoe.

The mistletoe lectins are type-2 ribosome-inactivating proteins (RIPs) composed of two different subunits, A- and B-chain linked by a disulfide bridge. The A-chain is capable of inactivating the 60S ribosomal subunit of eukaryotic cells resulting in inhibition of protein synthesis. The B-chain is capable of binding to cell-surface glycoconjugates and thereby permits entry into the cell of the toxic subunit (Peumanns *et al.,* 1996). Cell surface carbohydrate chains provide potential binding sites to endogeneous carohydrate binding proteins (Schumacher *et al.,* 1994). The glycosylation of tumor cells can be different

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from that of the normal cells, since the changes in carbohydrate expression on tumor cells can alter cell adhesion. Thus, the cell-surface glycoconjugates can be suitable targets for therapeutic exploitation because the toxicity of mistletoe lectins can be specific for cancer cells (Taylor-Papadimitriou *et al.,* 1994; Schumacher *et al.,* 1995).

Oral applicability is crucial for anticancer agents applied chronically (Schellens *et al.*, 2000). However, mistletoe preparations have been given subcutaneously because most proteins are known to be ineffective by oral administration due to the low stability in the gastrointestinal (GI) tract and poor absorption (Büssing, 2000). The epithelial layer lining the GI tract is generally known to be a barrier to oral protein absorption. However, mistletoe lectin selectively labelled the surface of epithelial cells called intestinal membranous/microfold (M) cells in the dome region of the Peyers patch. Moreover, the lectin induced powerful anti-cancer effects when provided by the oral route showing limited endocytosis of the lectin (Sharma *et al.,* 1996). However, compared to parenteral administration, oral administration required large doses due to degradation of lectin structure in the GI tract (Pusztai *et al.*, 1998).

Many different strategies such as encapsulation of protein or/and enteric coating have been attempted to protect protein from degradation in the GI tract. An ideal oral delivery system would be characterized as having a high efficiency of encapsulation, maximal stability in acidic pH and provide rapid release at neutral pH. One of the problems for the drug delivery system is, however, the structural or conformational change, or integrity loss, of proteins during preparation because of the harsh preparation or formulation conditions (Singh and O'Hagan, 1998).

Alginates are anionic polysaccharides derived from brown algae and comprise D-manuronic and L-guluronic acid residues joined linearly by 1,4-glucosidic linkages (Polk *et al.,* 1994a). Addition of a polycation (chitosan) to the alginate gelation medium induces formation of polyanionicpolycationic complexes, which stabilize the ionic gel network and reduce alginate permeability (Polk *et al.,* 1994). Recently, alginate/chitosan microcapsules have been suggested as candidates to orally deliver a wide range of compounds including drugs (Takka *et al.*, 1998), vaccines (Polk *et al.,* 1994b; Bowersock *et al.*, 1999), DNA (Quong and Neufeld, 1998), and a host of therapeutic proteins and peptides (Hari *et al.*, 1996) past the stomach to intestinal sites of absorption.

Enteric coatings have been studied extensively in attempts to deliver protein orally and used in the pharmaceutical industry. These can be engineered to remain stable at low pH in the stomach and then release drugs at the higher pH of the intestine. Polyacrylic polymers which have pH-dependent solubility can be used to encapsulate proteins and protect them from degradation conditions (Singh and O'Hagan, 1998). Eudragit® is an aqueous dispersion of an anionic copolymer based on methacrylic acid and acrylic acid ethyl ester. The polymer dissolves above pH 5.5 by forming salts with alkalis, thus affording coatings that are insoluble in a gastric media, but soluble in the small intestine conditions (Singh and O'Hagan, 1998).

In this study, we investigated the possibility of alginate/chitosan microcapsules as a tool for oral delivery of mistletoe lectin. In addition, our strategy has been to develop a system composed of stabilizing cores (granules), which contain mistletoe lectin, extract or powder, coated by a biodegradable polymer (Eudragit®) wall. Loss of activity was estimated during incorporation into the delivery systems such as alginate microcapsules, granulation and surface-coating of granules.

MATERIALS AND METHODS

Preparation of VCA, mistletoe extract or powder of mistletoe

Korean mistletoe (*Viscum album* L. var. *coloratum*) growing on oak tree was collected in winter in Kangwon province, South Korea. Leaves, berries and 1- to 4-year old stems of the plants were sorted and chopped in slices. The VCA was purified from the Korean mistletoe by affinity chromatography on asialofetuin-Sepharose 4B as described previously (Lyu *et al.*, 2000). The purity check and the molecular weight were determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described previously (Park *et al*, 1998; Lyu *et al.*, 2000). The concentration of total protein was determined by BCA assay (Pierce, Rockford, IL). To prepare mistletoe extract, the leaves, berries and 1- to 4-year old stems of the plants sorted and chopped in slices were crushed with 10 vols of saline between two rollers going in opposite directions in a vegetable juice miller (Angel Life Co., Korea). The mixture was separated by filtration through a cheese cloth, and centrifuged at 12,000 rpm for 30 min. Thereafter, the supernatant was filtered in stages with 60, 20, 7.2 and 0.45 μm pore sizes and lyophilized. To prepare powder of mistletoe, the leaves, berries and 1- to 4- year old stems of the plants were lyophilized and ground into powder.

Determination of VCA by ELLA

The concentration of VCA was determined by ELLA (enzyme-linked lectin assay) as described previously (Lyu *et al.*, 2000). For ELLA (enzyme linked lectin assay), asialofetuin-1 (100 μL/well of 0.1 mg asialofetuin-1/mL in PBS) in a Nunclon microtiter plate (Nunc Immuno Plate Maxisorb, Nunc. Rosklide, Denmark) was incubated overnight, and washed with PBS-T. Blocking solution (200

μL/well of 1% BSA in PBS) was added, incubated, and washed. Then lectins or extracts at varied concentrations (100 μL/well of antigen in PBS) were added and incubated. The plate was washed with PBS-Tween 20. Primary antibody (a rabbit polyclonal anti-VAA antibody) solution was added, incubated, and washed. Secondary antibody (rabbit-antimouse-IgG-conjugated peroxidase) solution (100 μL/well) was added, incubated, and washed. Each washing process above repeated three times. Hydroperoxidase substrate ABTS (2,2'-azinobis [3-ethylbenzotiazoline-6-sulfonic acid]-diammonium salt (100 μL/well) solution was added and incubated for 20 min at RT in dark. The development was stopped with 100 μL/well of 1% sodium dodecyl sulfate. Absorbance was measured at 405 nm.

Preparation of alginate/chitosan microcapsules

Alginate/chitosan microcapsules were prepared by the emulsification/gelation method (Esquisabel *et al.,* 1997) with slight modification. Two percent (w/v) of alginate (BDH Co. Montreal, QC, Canada) containing 0.2% (w/v) $CaCl_2$ was dissolved in water and the VCA was added to a final concentration of 100 μg/mL. The well-mixed suspension was used as water phase. Five milliiter of water phase was added to 50 mL of mixed oil phase composed of soybean oil containing Tween 80 (0.2%) under gentle magnetic agitation. After 5-min emulsification, a water-in-oil emulsion was formed. The pH of the oil phase was adjusted to be 5.0 with acetic acid, and agitated for 15 min to complete the gelation reaction between calcium ion and sodium alginate. Then, distilled water (80 mL) and *n*-hexane (50 mL) were added with gentle mixing, allowing the microcapsules to partition to the aqueous phase. Chitosan (Pronova Biopolymers, Washington, OR, USA) was dissolved in acetic acid solution of 1.0% (w/v). The microcapsules aqueous suspension was separated and added to chitosan solution to react for 30 min. The microcapsules were filtered off, washed with distilled water, dried using acetone as dehydration agent and collected.

Encapsulation efficiency and retention of VCA in microcapsules

The amount of VCA entrapment was measured by placing whole microcapsules in 0.2 M phosphate buffer (pH 7.2) and by shaking at 100 rpm at 37±0.5°C for 12 h. And the VCA retention during acid incubation was measured by placing microcapsules in 0.1 M HCl (pH 1.5) and by shaking at 100 rpm at 37±0.5°C for 12 h. The VCA content of the extraction solution was determined using BCA assay (Pierce, Rockford, IL), compared with a standard curve of data obtained by assaying known concentrations of BSA (bovine serum albumin) solutions. The encapsulation efficiency (E.E.) of the process indicates the percentage of total amount of VCA encapsulated with respect to the total amount used for the preparation of microcapsules. And the VCA retention in microcapsules during acid incubation indicates the percentage of total amount of VCA retained in microcapsules after acid incubation with respect to the total amount used for the preparation of microcapsules. The tests of encapsulation efficiency and retention of VCA were carried out in triplicate.

Preparation of granules and enteric coating

VCA-containing granules were prepared by forming an excipient blend of cellulose, starch, sucrose and gelatin at a ratio of 30:30:30:10 in a Waring blender. One batch of granules was prepared by adding 1 mL of solution containing 1 mg of VCA to 10 g of excipient blend. The wet mass was dried under vacuum at 4°C and then ground with a small mill to form granules. These were passed through a series of sieves (0.3-3 mm), and granules of 1-3 mm were collected and stored at 4°C under vacuum with desiccant.

Granules were prepared by floating mixture of powder (75%) of freeze-dried mistletoe extract or powder (75%) of freeze-dried mistletoe (whole plant), and starch (15.0% w/w) as an excipient and spraying a binder (10.0%) using a Centrifugal Fluidizing Granulator (CF-Granulator, CF-360, Freund Industrial Co., Ltd, Japan). HPMC (hydroxypropylmethycellulose) ethanol (95.0 v/v%) solution (1.5 w/w%) was used as a binder. Operating conditions were as follows: rotating speed, 250 rpm, inlet air temperature, 35°C, inlet air volume, 150 L/min, spray air volume, 10 L/min and binder flow rate, 15.3 g/min.

A commercially available aqueous methacrylate coating dispersion (Eudragit® L 30 D-55, Roehm GmbH, Darmstadt, Germany) was used for enteric coating. One batch of granules (3 kg) was enterically coated with Eudragit L30D, using a Wurster spray coating method in a laboratory scale fluid bed, STREA-1 (Aeromatic Inc. Columbia, MD). About 7% weight of granules increased by enteric coating process. Accordingly enteric coated granules contained 70% of mistletoe extract or powder.

VCA release *in vitro*

To study the VCA release from alginate/chitosan microcapsules or enteric coated granules the following pH values were chosen- pH 1.5 and pH 7.5 that simulates the physiological microenvironment in the stomach and intestinal tract, respectively. Microcapsules or enteric coated granules were incubated at pH 1.5 (0.1 M HCl) for 12 h in a thermostated shaker bath set at 37±0.5°C and agitated at 50 rpm agitator. Alternatively microcapsules or enteric coated granules incubated at pH 1.5 for 2 h were subsequently transferred and incubated at pH 7.5 (0.2 M Tris, 2.5% sodium deoxycholate) for an additional 10 h in a rotating. Same release test at pH 7.5 was performed without

preincubation at acidic conditions. Samples were removed at appropriate time intervals and centrifuged for 10 min at 15000 rpm. The concentration of VCA was determined by ELLA (Lyu *et al.*, 2000). The profile of VCA-release indicates the percentage of VCA released with respect to the total amount of VCA to prepare microcapsules or granules. Release tests were carried out in triplicate.

Cytotoxicity against cell lines *in vitro*

Microcapsules or enteric coated granules incubated at pH 1.5 for 2 h were subsequently transferred and incubated at pH 7.5 (0.2 M Tris, 2.5% sodium deoxycholate) for an additional 4 h in a rotating agitator with sampling at indicated time intervals. The samples were centrifuged and the concentration of protein (VCA) was measured by BCA assay (Pierce, Rockford, IL).

SK-Hep-1 human hepatocarcinoma cell lines were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea) and maintained in RPMI 1640 medium (GIBCO BRL, Grand Island, MD, USA) containing 10% fetal bovine serum (GIBCO BRL), 100 U/mL penicillin (GIBCO BRL), and 100 μg/mL streptomycin (GIBCO BRL) at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2. Cells (1×10^4 cells/mL) were plated, maintained and treated with varied concentrations of VCA protein released after incubation.

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay colorimetric dye reduction method. The cytotoxicity was measured as IC_{50} values (inhibitory concentration values, i.e. drug concentration required to inhibit viability by 50%) and each assay was done in triplicate.

Statistical analyses

All data are expressed as mean±SD of the three independent experiments. A one-way ANOVA was used for multiple comparisons (SPSS program, ver 10.0).

RESULTS

Encapsulation efficiency (EE%) and VCA retention during acid incubation

Alginate/chitosan microcapsules were prepared through electrostatic interaction using emulsification/gelation method (Polk *et al.*, 1994a). Fig. 1A shows the effects of alginate concentration on the encapsulation efficiency and VCA retention during acid incubation. Encapsulation efficiency increased with alginate concentration and was maximized at 2% alginate whereas VCA retention during acid incubation was maximized at 2.5% alginate. Fig. 1B shows the effects of $CaCl_2$ concentration on the encapsulation

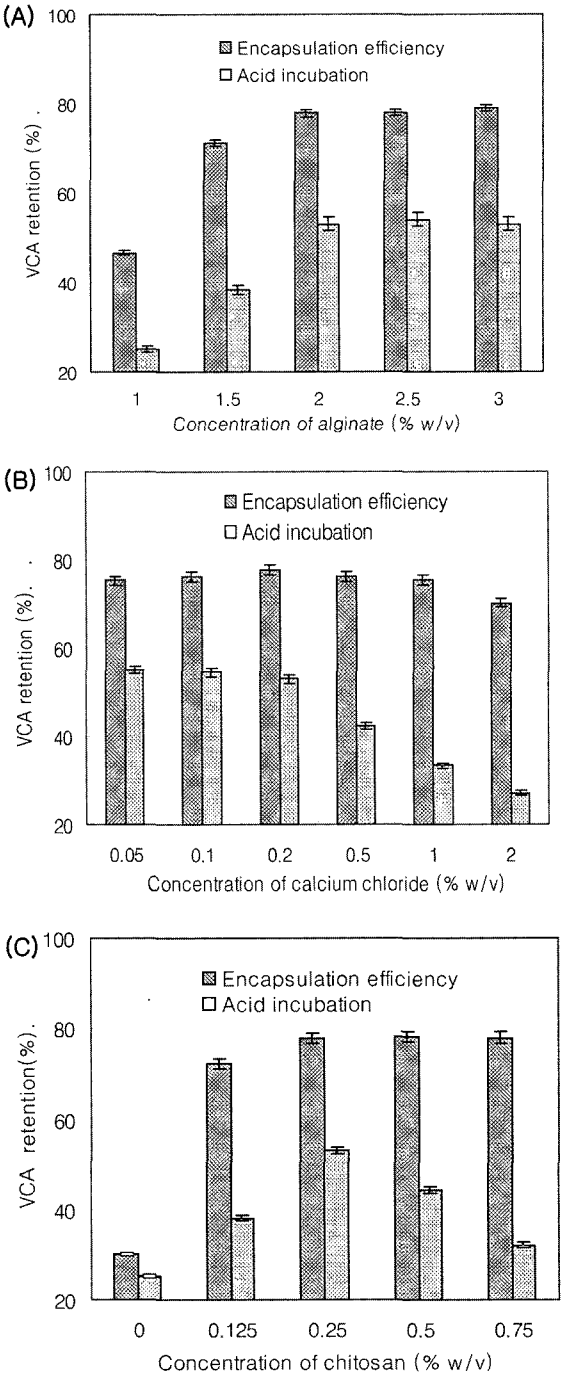

Fig. 1. *Effects of alginate (A), $CaCl_2$ (B) and chitosan (C) concentration* on the encapsulation efficiency of VCA into alginate/chitosan microcapsules and on the VCA retention in microcapsules during acid incubation. The basal concentration of alginate, chitosan and $CaCl_2$ for the preparation of microcapsules was 2% (w/V), 0.25% (w/v) and 0.2% (w/v), respectively. The encapsulation efficiency was measured by placing whole microcapsules in 0.2 M phosphate buffer (pH 7.2) and the VCA retention during acid incubation was measured by placing microcapsules in 0.1 M HCl (pH 1.5) and by shaking at 100 rpm at 37±0.5°C for 12 h, respectively. The concentration of the VCA was measured by BCA assay. Each point represents the mean±SD of the three independent experiments.

efficiency and VCA retention during acid incubation. The encapsulation efficiency in response to increasing $CaCl_2$ in the medium showed similar tendencies, while protein retention during acid incubation was maximal at lower calcium levels, and decreased significantly above 0.5% $CaCl_2$. The addition of chitosan to the encapsulation medium has a considerable effect on the encapsulation efficiency and VCA retention during acid incubation (Fig. 1C). The addition of lowest concentration of 0.125% chitosan significantly increased encapsulation efficiency from 30% without chitosan to 72%. Encapsulation efficiency increased with increasing chitosan concentration, reaching a maximum at 0.5%. The influence of increasing chitosan concentration on VCA retention during acid incubation reveals a different pattern of response. VCA retention is maximized with 0.25% chitosan in acid incubation, above which retention decreases significantly. Taken together, the microcapsules prepared with 2% alginate, 0.2% $CaCl_2$ and 0.25% chitosan were chosen for further studies.

VCA release *in vitro*

To study the VCA release from alginate/chitosan microcapsules or enteric coated granules containing VCA the following pH values were chosen- pH 1.5 and pH 7.5 that simulates the physiological environment in the stomach and intestinal tract, respectively. Hence the suspensions of microcapsules or granules were incubated for 2 h in acidic medium, followed by incubation in a phosphate buffer at neutral pH. The VCA released was estimated by ELLA that only determines sugar binding lectin (Lyu *et al.*, 2000).

Fig. 2A shows that 8% of VCA was released from the alginate/chitosan microcapsules within 2 h and that 12% of VCA was released within 6 h from microcapsules when incubated at pH 1.5. On the other hand, 78% of VCA was released within 6 h and practically all of VCA (93%) was released in 12 h when incubated at pH 1.5, followed by incubation at pH 7.5. And practically the similar release profile at pH 7.5 was obtained without preincubation at acidic conditions. Fig. 2B shows the release profiles of VCA-containing granules coated with Eudragit®. Practically no release of granules was observed in acidic medium but after transferring to pH 7.5 buffer the VCA content in solution increased abruptly. When the granules were incubated at pH 7.5, 95% VCA was released within 1 h. There were no significant differences in the release profiles from granules prepared with VCA, powder of mistletoe extract or powder of whole plant (data are not shown).

Effect of manufacturing processes of microcapsules and granules on the activity of VCA

Fig. 3A shows the influence of microcapsulation processes on the cytotoxic activity of purified VCA in micro-

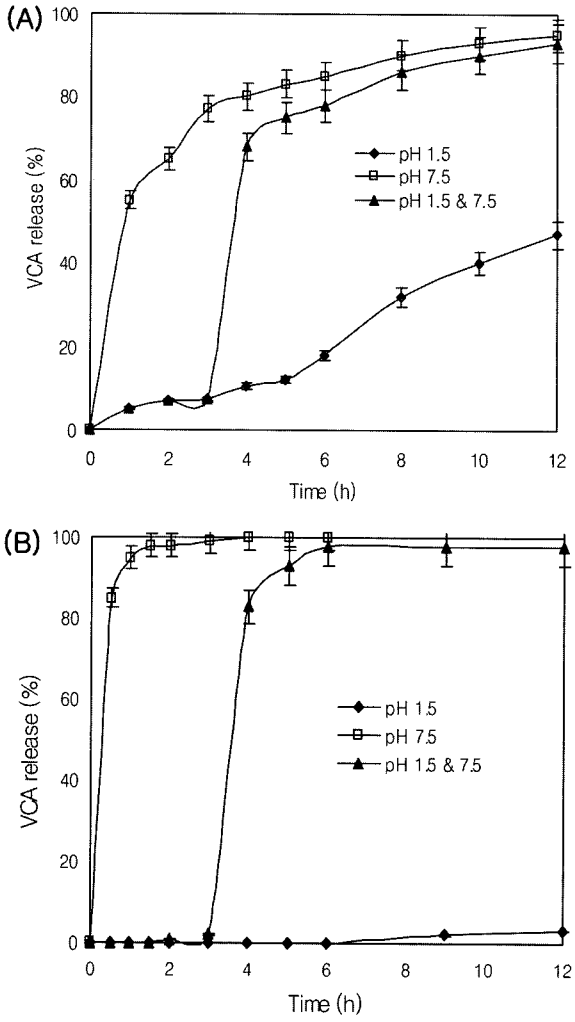

Fig. 2. Release profiles of VCA from alginate/chitosan microcapsules (A) and from VCA-containing granules coated with Eudragit L 30 (B) as a function of time. The concentration of VCA was measured during incubation in an acid medium (pH 1.5) and incubation in a neutral medium (pH 7.5). In order to simulate conditions through the GI tract, the enteric coated granules containing VCA were incubated for 2 h in acidic medium, followed by incubation in a phosphate buffer at neutral pH (pH 1.5 & 7.5). The VCA released was estimated by ELLA. The profile of VCA-release indicates the percentage of VCA released with respect to the total amount of VCA to prepare microcapsules or granules. Each point represents the mean± SD of the three independent experiments.

capsules or enteric coated granules. The VCA inhibited strongly the growth of the SK-Hep1 cells (IC_{50} = 8 ng/mL), but the VCA incubated at pH 1.5 for 2 h did not show any cytotoxicity even at the highest concentration (100 ng/mL) (data are not shown). The activity of purified VCA decreased more significantly by the microcapsulation process (IC_{50} = 32 ng/mL) than the process of granulation and enteric coating (IC_{50} = 14 ng/mL). On the other hand, the activity of VCA in the extract or whole plant maintained after granulation and enteric coating (Fig. 3B).

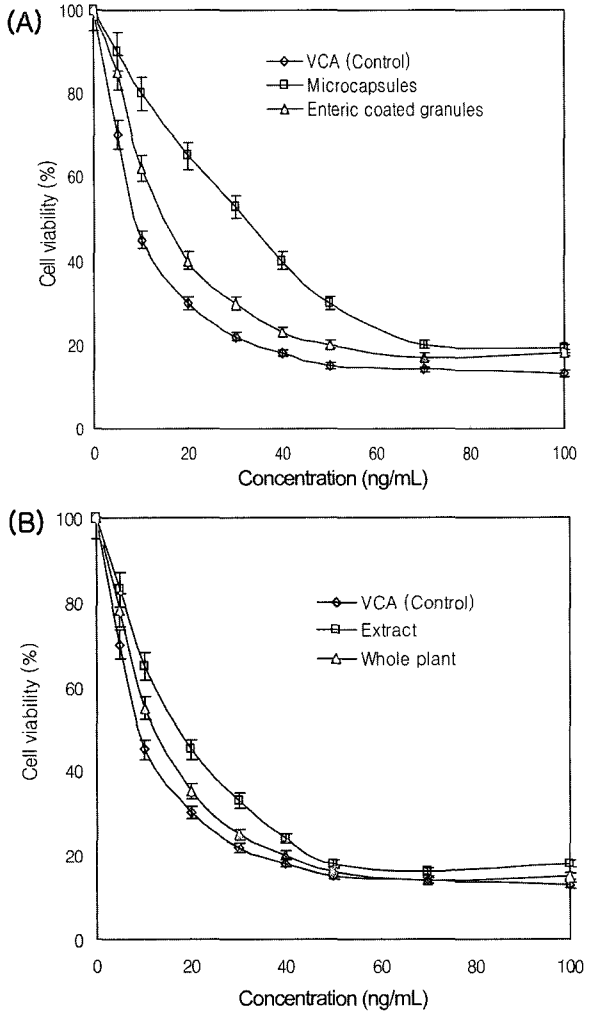

Fig. 3. Effect of manufacturing process on the cytotoxicity of VCA in microcapsules or in enteric coated granules. Dose-dependent viability was measured by treating SK-Hep-1 cells with VCA protein released from microcapsules or from enteric coated granules. The concentration of VCA protein was measured by BCA assay. (A) Cells were treated VCA released from microcapsules or from enteric coated granules prepared with VCA for 48 h. (B) Cells were treated with VCA protein released from enteric coated granules prepared with extract or whole plant of mistletoe. Cell viability was determined by the MTT assay. Values from each treatment were expressed as a percent relative to the control. Each point represents the mean± SD of the three independent experiments.

DISCUSSION

Mistletoe, a semiparasitic plant, holds interest as a potential anticancer agent. The purified mistletoe lectins, main components of mistletoe, have demonstrated cytotoxic and immune-system-stimulating activities. Mistletoe extract has been given by subcutaneous injection because protein is known to be unstable and absorbed poorly in the GI tract. Dietary proteins do not normally cross the intestinal epithelium intact, but must first be broken down to their constituent free amino acids which are then absorbed. Of course, this route of protein absorption destroys all physiological activity of the protein and explains why typical oral bioavailabilities of proteins are usually less than 12% (Schellens *et al.*, 2000). Overcoming these barriers is the focus of efforts to develop oral protein delivery systems.

The epithelial layer lining the GI tract is a barrier to oral protein absorption. However, specialized epithelial cells lie in the dome region of the Peyers patch. The cells are called follicle associated epithelial or intestinal membranous/ microfold (M) cells. Lectin binding studies have revealed that the M cell surface glycocalyx differs in carbohydrate composition from that of enterocytes (Giannasca *et al.*, 1994; Jepson *et al.*, 1996; Gebert and Posselt, 1997; Clark *et al.*, 2000) The lectin *Ulex europaeus* agglutinin 1 selectively binds to mouse Peyer's patch M cells both in vitro and in vivo showing the potential for lectin-mediated targeting of synthetic delivery vehicles to M cells (Florence, 1997; McClean *et al.*, 1998). Moreover mistletoe lectin selectively labelled the surface of human Peyer's patch M cells (Sharma *et al.*, 1996). Morphological studies of the small bowel indicated that the mistletoe lectin binds avidly to lymphoid tissue of Peyer's patches and the plasma level of TNF-α and IL-1β were significantly increased in rats that were given high oral doses of mistletoe lectin (Pusztai *et al.*, 1998). More importantly it has been reported that mistletoe lectins induced powerful anti-cancer effects when provided by the oral route showing limited endocytosis of the lectin. The growth of a murine non-Hodgkin lymphoma (NHL) tumor has been shown to be reduced by incorporating mistletoe lectin into the diet (Pryme *et al.*, 2002). An experiment where mistletoe lectin was added to the diet of mice three days after inoculation of tumor cells showed that the lectin was able to slow down further growth of tumor. However, compared to parenteral administration (up to 1 mg kg^{-1} BW of rats) of mistletoe lectin, oral administration required large doses (up to 200 mg kg^{-1} BW of rats) possibly due to the instability of lectin in the environment of the GI tract and the poor permeability of the active components *via* the epithelial layer lining the GI tract (Pusztai *et al.*, 1998).

Oral drugs should be stable at the harsh conditions of the gastrointestinal tract and have a good pharmaceutical dissolution profile (Zhou *et al.*, 2001). Permeation enhancers (Nakane *et al.*, 1996), protease inhibitors (Marschutz and Bernkop-Schnurch, 2000), enteric coatings (Reddy *et al.*, 1999) and polymer microsphere formulations (Delgado *et al.*, 1999) have all been applied towards the development of oral protein formulations. One of the problems for these polymeric drug delivery systems is the structural or conformational change of proteins during preparation (Reddy *et al.*, 1999). One reason for the protein structural or

conformational change, or integrity loss, may be the harsh preparation or formulation conditions.

Sodium alginate contains a negative charge, while chitosan contains a positive one. With regard to the properties, they are able to form microcapsules through electrostatic attraction. The problems destroying the structure of protein would not appear when the encapsulation materials form a microcapsule by electrostatic attraction (Polk *et al.*, 1994). Hence, we used sodium alginate and chitosan to prepare alginate/chitosan microcapsules using emulsification/gelation method. Encapsulation efficiency was maximized at 2% alginate and protein retention was maximal at lower calcium levels and decreased significantly above 0.5% $CaCl_2$ (Fig. 1A, B). It has been reported that contact between the alginate and the calcium in solution induces immediate interfacial ionic polymerization of the alginate *via* binding of calcium within the cavities of the guluronic residues, thus forming a polyanionic microcapsule (Wang *et al.,* 2002). The addition of chitosan to the encapsulation medium has a considerable effect on the encapsulation efficiency and VCA retention during acid incubation (Fig. 1C). Encapsulation efficiency increased with increasing chitosan concentration, reaching a maximum at 0.5%. It has been reported that a polyelectrolyte complex membrane formed on the microcapsule surface when alginate microcapsules were treated with chitosan (Sezer and Akbuga, 1999). The membrane became thick and tight with the increase in chitosan concentration so that the encapsulation efficiency of VCA could be elevated.

Next, we investigated the VCA release profiles of microcapsules or enteric coated granules incubated in the simulated microenvironment in the stomach and intestinal tract, respectively. It is well known that mean gastric emptying time in human is about 2 h (Davis *et al.*, 1986). Hence the suspensions of microcapsules or granules were incubated for 2 h in acidic medium (pH 1.5), followed by incubation in a phosphate buffer at pH 7.5. Eight percent of VCA was released from the microcapsules within 2 h when incubated at pH 1.5 while 78% of VCA was released within 6 h when incubated at pH 1.5, followed by incubation at pH 7.5 (Fig. 2A). The similar data were obtained with the alginate beads loaded with chymotrypsin, which was encapsulated inside the beads at pH 1.5 during 1 day and released at pH 7.5 during 2 h (Tiourina *et al.,* 2002). Apparently, in our case the alginate/chitosan interaction stabilized the gel structure and protected the alginate from degradation at acidic pH values.

The acidic groups of granules coated with poly methacrylic acid-co-methylmethacrylate (Eudragit®) are protonated at acidic pH, which leads to insolubility of the material. When the pH is increased the solubility increases due to deprotonation of the polymer. In the present study, a system composed of stabilizing cores (granules), which contain mistletoe lectin, coated by a biodegradable polymer wall to stabilize the protein in the core and to be released following the erosion of the polymer coat. No dissolution of granules was observed in acidic medium but after transferring to pH 7.5 the VCA content in solution increased abruptly. When the granules were incubated at pH 7.5, 95% VCA was released within 1 h (Fig. 2B).

It has been reported that mistletoe lectin (VAA and VCA) had a strong inhibitory effect on the growth of the SK-Hep1 cells (IC_{50} = 8±2.5 ng/mL). Hence the influence of manufacturing process on the VCA activity was estimated by measuring anticancer activity of VCA in the microcapsules or enteric coated granules. The activity of the purified VCA decreased more significantly by the microcapsulation process than the process of granulation and enteric coating. And the activity of VCA in the extract or whole plant maintained after granulation and enteric coating (Fig. 3A, B). In the previous studies the purified VCA has been observed to be unstable, but the VCA in the water extract or in the whole plant was considerably stable (Lyu *et al.*, 2000). It is quite possible that the preservation of activity in the sample contributed to the stabilization of the VCA by some other components in the extract or whole plant. It has been similarly reported that alkaline phosphatase that was freeze-dried with serum albumin had 70% activity left after the process, while the enzyme that was freeze-dried without albumin only had 5% activity remaining (Ford and Allahiary, 1993).

In conclusion, our results indicated that successful incorporation of VCA into alginate/chitosan microcapsules is dependent on the concentration of alginate, $CaCl_2$ and chitosan and that the alginate/chitosan interaction stabilized the gel structure and protected the alginate from degradation at acidic pH values. And coating the VCA with polyacrylic polymers, Eudragit, produced outstanding results with ideal release profiles and only minimal losses of cytotoxicity after manufacturing step. The granules prepared with extract or whole plant produced the best results due to the stability in the extract or whole plant during manufacturing process. The successful delivery of this study opens the possibility to deliver other therapeutically interesting lectins in the natural products to the intestine. However, the activity of microcapsules and enteric coated granules should be confirmed by investigating *in vivo* studies.

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