J. MICROENCAPSULATION, 2001, VOL. 18, NO. 3, 285–297

Release characteristics of microspheres prepared by co-spray drying *Actinobacillus pleuropneumoniae* antigens and aqueous ethyl-cellulose dispersion

C. W. LIAO, I. C. CHENG, K. S. YEH, F. Y. LIN and C. N. WENG*

Department of Pathobiology, Pig Research Institute Taiwan, Chu-Nan, Miaoli, Taiwan, Republic of China

(*Received 18 May 2000; revised 31 July 2000; accepted 9 August 2000*)

Using formalin inactivated *Actinobacillus pleuropneumoniae* antigens and aqueous ethylcellulose dispersions, microspheres of oral vaccines were developed by a co-spray drying process. The present study attempted to determine whether the dosage formulations of microspheres could form enteric matrices. To assess the enteric characteristics, an *in vitro* dissolution test was performed with the AQ6-AP microspheres; 95% of the *A. pleuropneumoniae* protein was released within 3 h at pH 7, but there was no release at pH 1.5. The scanning microscopy revealed that the surface structure of AQ6-AP microspheres became porous at neutral pH. The SDS-PAGE analysis showed that the release rate of proteins from the microspheres was pH dependent not only for the AQ6-AP formulation but also when antigens of *A. pleuropneumoniae* were replaced with porcine serum. The results suggest that the *A. pleuropneumoniae* antigens were entrapped in the AQ6 microspheres under the acidic conditions. In a mouse model, oral immunization with AQ6-AP microspheres containing *A. pleuropneumoniae* evoked systemic IgG and mucosal IgA responses against *A. pleuropneumoniae* antigens. Thus, the present method may further provide an opportunity to develop oral vaccines and mucosal immunity.

Keywords: Spray drying, ethyl cellulose antigen, vaccine, oral.

Introduction

Actinobacillus pleuropneumoniae causes a highly contagious disease characterized by pleuropneumonia with necrotic, haemorrhagic, and fibrinous lesions in pigs of all ages, but primarily affects growing pigs aged 2–6 months old. Recently, because of intensified pig production practices in Taiwan, the disease has occurred frequently. Although some antibiotics are being used to treat the infected pigs, the emergence of resistant strains against those antibiotics has made the treatment relatively difficult. The most practical and economic way to prevent the occurrence of the disease would be a vaccination (Chang and Lai 1996). Among the vaccines of *A. pleuropneumoniae*, the formalin-killed vaccine has been the most commonly used. Nevertheless, it has long been known that protection against pathogenic bacteria correlates better with the presence of IgA antibody in local secretions than with serum antibody only (Bosse *et al.* 1992). The high induction of IgA responses

* To whom correspondence should be addressed. e-mail: CWL02@mail.prit.org.tw

Journal of Microencapsulation ISSN 0265–2048 print/ISSN 1464–5246 online © 2001 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/02652040010019442

can often be achieved through direct immunization via gut-associated lymphoid tissue (GALT), of which the largest compartment is represented by Peyer's patches of the gastrointestinal (GI) tract (Chao *et al.* 1998).

In the past years, based on formalin inactivated *A. pleuropneumoniae* antigens and aqueous ethylcellulose dispersions, microspheres of oral vaccines were developed using a novel spray drying process in the laboratory. This spray drying process, either with an organic solvent or an aqueous system, has been applied extensively in pharmaceutical systems. The impact of various external and internal forces against solvent systems, such as the risk of explosive hazard and environmental pollution, has revitalized an interest in aqueous coating alternatives (Chang *et al.* 1987). However, using water as a solvent, more time or energy is required for evaporation. Recently, ethylcellulose in aqueous polymeric dispersion has been formulated by FMC corporation (Philadelphia, USA). This product can be applied in a film coating process according to a new mechanism of film formation—one that is distinctly different from that of water soluble polymers or the organic solvent solutions of polymers. The mechanism of film formation involves 'coalescence' of the individual latex particles (Zdanowski and Brown 1958). Not only is less energy required for removing water in the evaporation process, but the film is less porous, and water vapour transmission through the film lowers after film formation. It is, therefore, a suitable protective coating for water sensitive drugs.

Recently, co-spray-dried microspheres for drugs have been prepared with an ethylcellulose aqueous polymeric dispersion (Kulvanich and Leesawat 1996). In this process, encapsulation and film formation occur simultaneously in a spray drying process. It has also been proposed that a hydrophilic agent, such as lactose, sugars, or water soluble polysaccharides, may be selected as a channelling agent to modify the release characteristic of a matrix product (Fites *et al.* 1970, Kulvanich and Leesawat 1996). Therefore, the objectives of the study were to introduce a new application of the process for an oral vaccine delivery in the gastrointestinal tract, and to demonstrate the potential use of microspheres, prepared by a co-spray drying process, as an oral vaccine in the gastrointestinal tract.

Materials and method

Materials

Ethylcellulose aqueous polymeric dispersion (Aquacoat) was purchased from FMC Corporation (Philadelphia, USA); hydroxypropyl methylcellulose acetate succinate (HPMCAS, AS-MF) from Shiu-Etsu Chemical Co., Ltd. (Tokyo, Japan); NAD (β-nicotinamide adenine dinucleotide), $CaCl_2.2H_2O$, Na.EDTA, soybean trypsin inhibitor, phenyl methyl sulphonyl fluoride (PMSF) from Sigma Chemical Co. (St. Louis, MO, USA); gelatin, NaN_3, NaCl, KCl, Na_2HPO_4, sodium citrate, isopropanol from Merck (Darmstadt, Germany); Coomassie protein assay reagent, p-nitrophenyl phosphate from Pierce (IL, USA); Tryptic soy broth (TSB) and brain heart infusion (BHI) from Becton Dickinson (Sparks, MD, USA); and mouse immunoglobulin reference serum, alkaline phosphatase conjugated goat anti-mouse IgA alpha chain specific, alkaline phosphatase conjugated goat anti-mouse IgG from Bethyl Lab. Inc. (Montgomery, AL, USA).

Preparation of the antigens of Antinobacillus pleuropneumoniae

A. pleuropneumoniae used in this study was a field isolate (P-99-158 strain) and identified as serotype 1. The bacterial stock was maintained at −70 °C in BHI-NAD broth (brain heart infusion broth with 0.1% NAD) containing 50% glycerol. For preparation of the formalin-killed bacterial antigens, 1 ml inocula was incubated in 10 ml BHI-NAD broth at 37 °C overnight, and transferred into 200 ml of BHI-N culture broth in a 500 ml Hinton flask and cultured at 37 °C for 15 h. After the culture was lightly homogenized by a homogenizer (polytron pt-300, Kinematica AG), 0.5% formalin was added into the culture broth and mixed at 37 °C for 1 h and then incubated at 4 °C for 15 h. To assure that there were no viable bacteria in the broth, 5 ml of the inactivated broth was aspirated into 200 ml of BHI-N broth in a 500 ml Hinton flask and cultured at 37 °C, until there was no bacterial growth after 1-week of cultivation. The formalin inactivated broth was then used as an antigen for vaccine formulation.

Preparation of vaccines for subcutaneous injection

The solution of formalin-killed bacterial antigens was lightly homogenized using a homogenizer (polytron pt-300, Kinematica AG), an equal volume of aluminium gel was then added, and mixed at 37 °C for 1 h. Several batches of aliquot were stored at 4 °C.

Preparation of microspheres

Microspheres were produced by co-spray drying using a Model L-8 (Ohkawara Kakohki Co., Ltd., Yokohama, Japan) as described by Kulvanich and Leesawat (1996). The microsphere formulations were prepared according to table 1. The antigen with or without HPMC-AS was constantly stirred while Aquacoat solution was added dropwise. Stirring procedure was kept for another hour followed by the addition of magnesium stearate. The mixing was continued for 3 h under ambient conditions. The optimal condition of co-spray drying was conducted on several manufacturing factors, including atomizer speed, feeding rate, hot air inlet and outlet temperatures, and the pressure of cyclone.

Table 1. Formulations of microspheres.

Formulation I.D.	Formalin inactive broth (g)	HPMC-AS (g)	Ethylcellulose aqueous polymeric dispersion (Aquacoat) (g)	Magnesium Stearate (g)	Double deionized water (g)
A0-AP	100	0	0	1	99
A1-AP	100	0	10	1	89
A2-AP	100	0	20	1	79
A4-AP	100	0	40	1	59
A6-AP	100	0	60	1	39
AQ6	0	2	60	1	137
AQ6-AP	100	2	60	1	37
AQ6-Ser	25% Serum 100	2	60	1	37

For further observation of microsphere formation, the size and surface structure of the particles coated with gold were examined by a scanning electron microscope (Liao *et al.* 2000). For a pilot experiment, antigen of *A. pleuropneumoniae* was replaced with 25% porcine serum (table 1, AQ6-Ser formulation) to determine whether or not the protein molecules released from microspheres were pH dependent. Proteins released from microspheres were analysed by SDS-PAGE or Coomassie protein assay (Pierce and Suelter 1997).

Protein content of microspheres

The protein content was determined according to the extraction procedure of Delgado *et al.* (1999). Briefly, 100 mg of microspheres was suspended in 1 ml of 5% SDS (in PBS, pH 7.5) and shaken for 1 h. A mixture of dichlomethane : acetone (1 ml, 50 : 50 ratio) was added to dissolve the polymer, followed by a vortex for a few seconds and shaken for 12 h at ambient temperature to allow the organic solvent to evaporate. The extracted samples were centrifuged, and the protein concentration in the supernatant was determined. Protein concentration was determined by a Coomassie protein assay using BSA standards.

In vitro *protein release study*

The dissolution test of AP enteric-coated microspheres was conducted according to the pH changing method of USP-XXII A (United States Pharmacopoeia XXII/NF-XII 1990). In general, 3 g microspheres were placed into a jacketed beaker containing 500 ml of 0.02 N HCl solution (pH 1.5) at 37 °C and 100 rpm. Five ml of 0.2 M tri-sodium phosphate buffer was added to adjust the pH to 7 for further dissolution experiment. The dissolution test was also carried out at various pH (2 mM phosphate buffer) at 37 °C for 6 h. Each test was performed in triplicate.

During the dissolution test, 1.5 ml of samples were taken out at 30 min intervals and 1.5 ml of buffer solution was added to maintain the volume of the dissolution vessel. Samples were centrifuged at 15 000 g for 5 min, and the supernatants were aspirated and stored at −20 °C. The amount of protein released was determined by a Coomassie protein assay (Pierce and Suelter 1997) and characterized by SDS-PAGE.

Protein assay using a microtiter plate

A set of protein standards (bovine serum albumin; BSA) was prepared using phosphate-buffered saline (PBS) as a diluent. A 100 µl volume of each standard, along with the blank and samples, was pipetted into the wells of a microtiter plate to which 100 µl of Coomassie protein reagent was added. The microtiter plate was covered with a lid and incubated at 37 °C for 2 h. Using a microplate reader (DYNEX Technologies, Inc. MRX type. USA), the absorbance of the reaction product was measured at 595 nm for Coomassie protein reagent.

Immunization in mice model

Six-week old female Balb/c mice were obtained, quarantined for 1 week prior to study, and maintained throughout the study on libitum with pelleted food and water throughout the experiment. The animals were randomly assigned to groups of six which received combinations of oral or subcutaneous dosing. Sixty mg of

AQ6-AP in 0.4 ml of acetic buffer (0.5% acetic acid, pH 3) was used for oral immunization. Mice were orally administered with this microsphere solution via a blunt-tipped feeding needle inserted into stomach. The positive control group was immunized by subcutaneous injection with 0.5 ml vaccine containing 0.25 ml of formalin inactive broth at intervals of 2 weeks for a total of three immunizations. Two weeks after the final immunization, mice were exsanguinated, and blood was collected from the puncture of retroorbital plexuses. Serum was obtained from coagulation at 4 °C for 12 h and centrifugation. Intestine larvage samples were collected by instilling 1 ml of washing buffer (PBS containing 100 μg/ml soybean trypsin inhibitor, 50 mM EDTA, 1 mM PMSF, 0.5% gelatin, and 0.05% NaN_3) into the intestine. The larvage was collected and stored at −20 °C.

Measurement of antibody responses by an Enzyme-linked Immunosorbent Assay (ELISA) (Voller et al. *1976, Tzan* et al. *1989)*

Microtiter plate (Costar) was coated with whole cell lysate of *A. pleuropneumoniae* in this study. To obtan the lysate, formalin-inactivated *A. pleuropneumoniae* with 1% triton X-100 was diluted to 500 μg/ml of protein concentration by PBS buffer. Each well of a 96-well plate was coated with 100 μl of antigen at a concentration of 50 μg/ml protein in carbonate-bicarbonate buffer, pH 9.6 for overnight adsorption at 4 °C. To determine the titers of antibodies, a standard curve with dilutions of a mice immunoglobulin was constructed (0.06, 0.2, 0.6, 2, 6 μg/ml for IgG or 0.048, 0.14, 0.48, 1.4 μg/ml for IgA) (Liao *et al.* 2000). One unit/ml of antibodies was defined as the absorbance value from 1 μg/ml of immunoglobulin. In general, a dilution of 1:100–1:500 was prepared for serum and 1:10–1:30 for intestinal larvage to yield an absorbance value within the sensitive ranges of the assay.

Results and discussion

Production and characteristics of Aquacoat microspheres

A novel method for preparing microsphere vaccine was developed by a co-spray drying method. Various parameters for optimizing the processes were investigated. Several conditions that could directly affect the particle size and morphology were shown in figure 1. In AQ6 formulation (without antigens), the microspheres were prepared at the disc speeds of 20 000–30 000 rpm and with an 80 °C hot inlet of air. However, AQ6-AP microspheres did not form a sphere-shape in the presence of *A. pleuropneumoniae* antigen. The structure change should be affected by the bacterial material. To study the temperature effect, various temperature settings of the hot inlet air were tested. It was found that temperature setting was very important for AQ6-AP formulations. As shown in figure 2, the shape of microspheres remained spherical at temperatures below 60 °C, but was altered to irregular particle shapes above 60 °C.

Ethylcellulose in aqueous polymeric dispersion was used for film coating whose mechanism is involved in 'coalescence' of individual latex particles. The percentage of Aquacoat is a critical factor in encapsulating and assembling antigen or protein in aqueous solution. It may also be essential to the microsphere shell wall formation. Microspheres prepared by various formulation are listed in table 1. The amount of Aquacoat used in formulation appeared to be important. In table 2, 40%

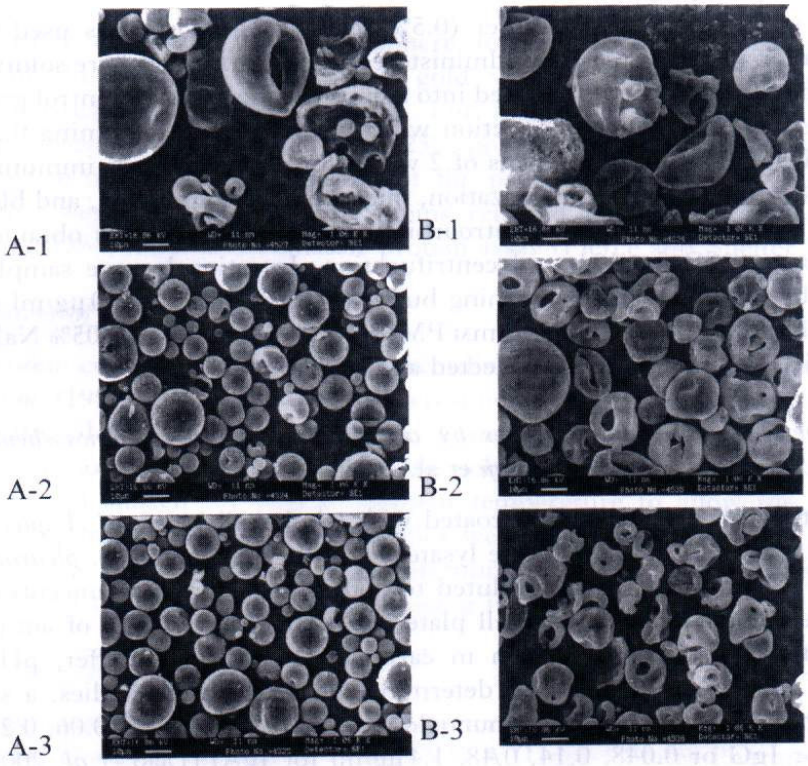

Figure 1. Microsphere formation at various speeds of rotary disc sprayer in co-spray drying. AQ 6 (A-1,2,3) and AQ6-AP formulation (B-1,2,3) as the feed liquid, respectively. The speed of rotary disc at 10 000 rpm centrifugal force in A-1, B-1, 20 000 rpm in A-2, B-2, and 30 000 rpm in A-3, B-3, respectively.

Table 2. Yields of microspheres prepared by various formulations.*

Compositions and production efficiency	Formulation I.D.							
	A0-AP	A1-AP	A2-AP	A3-AP	A4-AP	A6-AP	AQ6	AQ6-AP
Solid content of 100 ml bacterial broth (g)	3	3	3	3	3	3	0	3
Solid content of Aquacoat (g)	0	3	6	9	12	18	18	18
HPMC-AS (g)	0	0	0	0	0	0	2	2
Magnesium stearate (g)	1	1	1	1	1	1	1	1
Average yields of microspheres (g)**	0	1.4 ± 0.4	6.3 ± 0.6	9.2 ± 1.2	12.3 ± 2.5	16.9 ± 3.5	17.7 ± 1.5	20.6 ± 1.7
Average weight loss after production (%)***	100	80	37	29	23	23	15.7	14

* Formulations as shown in table 1.
** Microsphere particles were observed and confirmed by light microscope.
*** Average weight loss: [1-(weight of microsphere/solid content of bacterial broth + Aquacoat)] × 100. Each formulation was prepared in triplicate.

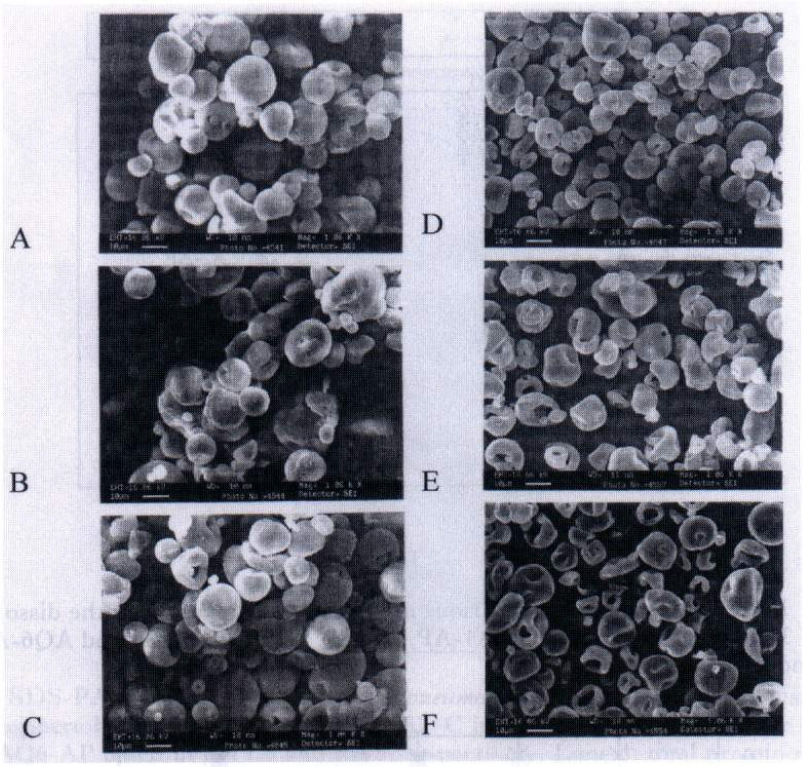

Figure 2. Microsphere morphologies of co-spray dried samples at various inlet air temperatures. The temperature of inlet air were 40 °C (*a*), 45 °C (*b*), 50 °C (*c*), 60 °C (*d*), 80 °C (*e*), and 100 °C (*f*), respectively. The speed of rotary disc was 30 000 rpm.

of Aquacoat (12% solid content of Aquacoat) was sufficient to encapsulate the antigen, either AQ6 or AQ6-AP. A content of Aquacoat greater than 60% did not improve the yield (table 2). The microspheres became sticky and often adhered to the wall of the drying chamber (data not shown). The production yield of AQ6-AP was significantly higher than that of the formulation without HPMC-AS, as shown in table 2. Interestingly, HPMC-AS may serve as a material to assist microspheres recovery from tank during the drying process.

In vitro *release of protein from Aquacoat microspheres*

In addition to the yield of microspheres, the release characteristics varied with the formulations containing various contents of Aquacoat. The protein release profiles of A6-AP and AQ6-AP microspheres are shown in figure 3. Ninety-five per cent of *A. pleuropneumoniae* protein was released within 3 h at pH 7, but less than 5% was released at pH 1.5. The release rates antigens from Aquacoat microspheres at pH 7 buffer solution were noticeably faster than those of antigens coated with the poly(lactide) and/or poly(lactide-co-glycolide) systems reported previously (Coombes *et al.* 1999). The Aquacoat microspheres were, therefore, used as a short-term or prompt release system.

According to the USP-XXII, the enteric product can be characterized and approved whenever the release of its active ingredient is no more than 10% in 2 h at pH 1.5. Thus, either A6-AP or AQ6-AP would be appropriate for an enteric

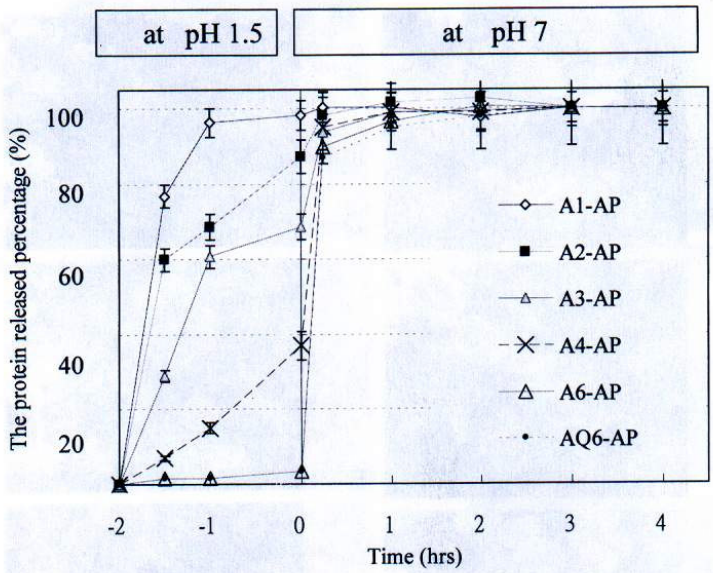

Figure 3. Protein release profile of various microshere formulations in the dissolution test ($n = 3$). For the formulation of A1-AP, A2-AP, A3-AP, A6-AP, and AQ6-AP see the legend for table 1.

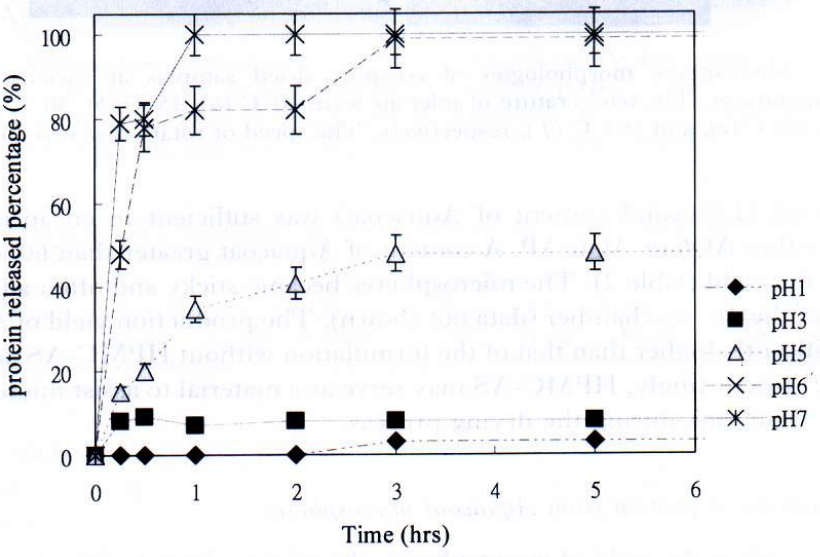

Figure 4. Protein release profile of AQ6-AP microspheres at various pH in the dissolution test ($n = 3$). For the formulation of AQ6-AP see the legend for table 1.

formulation. In addition to the yield of microspheres prepared by AQ6-AP formulation (table 2), AQ6-AP should be a promising formulation for an enteric dosage form. To investigate this enteric characteristic, the dissolution behaviour of AQ6-AP was studied at various pH at 37 °C for 6 h. A trace amount of protein was released at pH between 1.5 and 3 (figure 4) and the release was pH dependent. The SDS-PAGE (figure 5) showed that the protein release from AQ6-AP was pH-dependent. The surface structure of AQ6-AP microsphere looked smooth at pH 1.5, but became porous at pH 7 under the scanning microscope (in figure 6).

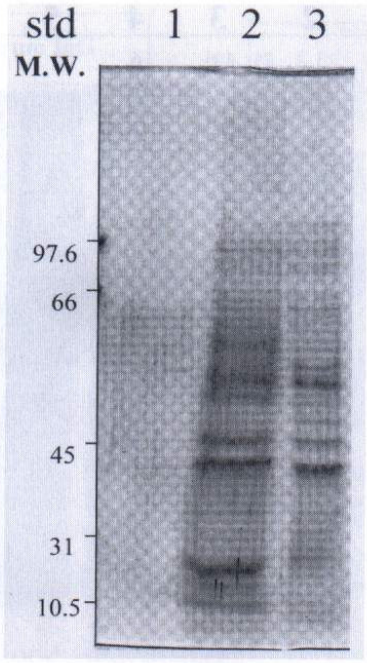

Figure 5. SDS-PAGE analysis of *A. pleuropneumoniae* bacterial protein released from microsphere batches after incubation at 37 °C in pH 1.5 or pH 7 buffer for 4 h. Lane 1,2: AQ6-AP batch in pH 1.5 and pH 7, respectively. Lane 3: total protein pattern of bacterial lysate.

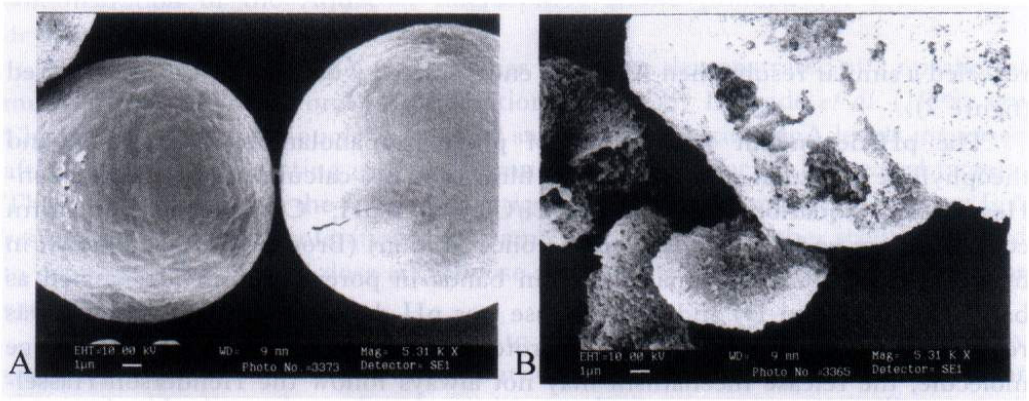

Figure 6. AQ6 microsphere morphology after incubation at 37 °C, pH 1.5 (*a*) or pH 7 (*b*) for 1 h.

Hydroxypropyl methylcellulose acetate succinate (HPMCAS) is cellulose ether derivatives that has been demonstrated to be useful in control release for oral delivery (Hogan 1994). In the next experiment, the effect was studied of HPMC-AS on protein release from the formulation. The dissolution test of Aquacoat microspheres showed no significant difference in the protein release between formulation with or without 1% HPMCAS added. Thus, it provided one with another piece of evidence to support that Aquacoat material could be used not only for antigen encapsulation but also for a pH-dependent release system. The SDS-PAGE analysis of the protein released from the microspheres at various pH

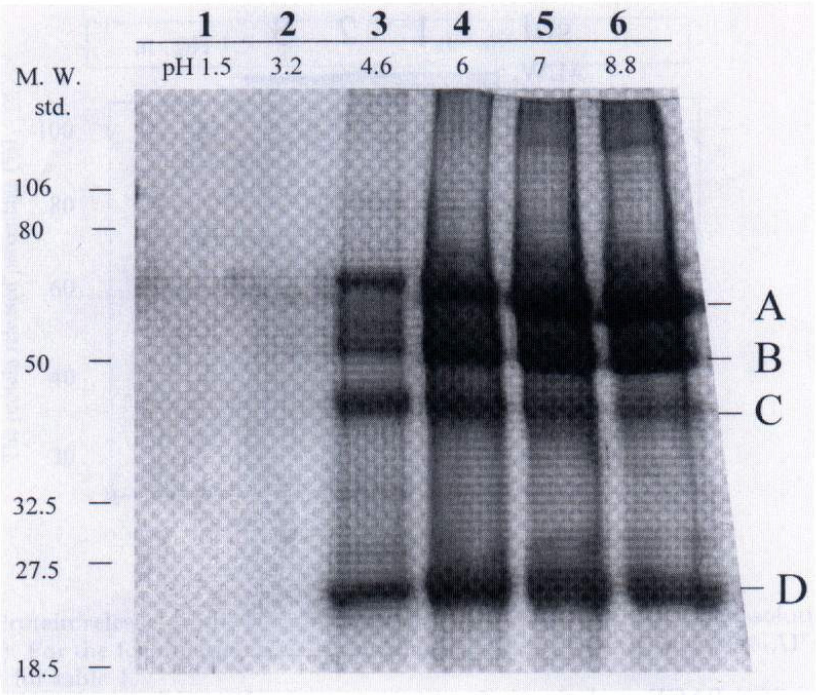

Figure 7. SDS-PAGE analysis of the serum protein released from AQ6-Ser microspheres after incubation at 37 °C in various pH buffer solutions for 2 h. Lane 1, at pH 1.5; lane 2, at pH 3.2; lane 3, at pH 4.6; lane 4, at pH 6; lane 5, at pH 7; lane 6, at pH 8.8. Four major bands (A, B, C, D) were detected.

revealed a similar result when AQ6-Ser encapsulated with porcine serum was used (figure 7).

The pH-dependent release rate of phenylpropanolamine HCl (PPA) and theophylline through the Aquacoat film can be calculated by Henderson-Hasselbalch equations, where ($\log Ci/Cu = pKa\text{-}pH$; Cu = non-ionized form concentration; and Ci = ionized form concentration) (Brodie 1964). As shown in figure 7, there were four major protein bands in porcine serum (designated as bands A, B, C, and D) and their release was pH dependent, where band B was released at a higher pH than other proteins. Due to the protein being a large molecule, the release mechanism may not always follow the Henderson-Hasselbalch equation and it needs to be further investigated.

Immune response of mice

The levels of mice antiserum to whole cell antigens of *A. pleuropneumoniae* were very low after the first oral immunization (data not shown). Following the booster immunization on the 14th day after the third oral immunization, the systemic IgG and mucosal IgA responses were brought about against *A. pleuropneumoniae* antigens. Higher levels of antiserum to whole cell antigens could be detected after booster immunization, but the oral administration with AQ6-AP microspheres still demonstrated low activity compared with the group of subcutaneous administration (shown in figure 8(*a*)). The underlying reason for eliciting a lower amount of specific serum IgG needs to

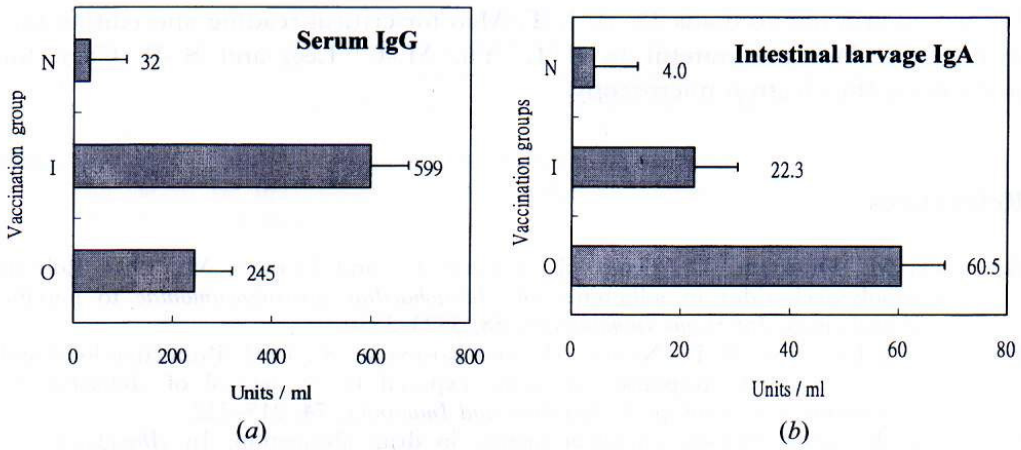

Figure 8. Titers of IgG and IgA following peroral immunization with AQ6-AP microsphere or subcutaneous injection with aluminium vaccine of *A. pleuropneumoniae* (A. p.). (*a*) Titers of serum IgG against whole antigens of A. p.; vaccination group of N is negative control; group I is subcutaneous injection, and group O is peroral immunization. (*b*) Titers of intestinal larvage IgA against A. p. ($n = 6$).

be further investigated in oral administration. Nevertheless, by oral administration, a good mucosal IgA response was brought about in small intestine, as shown in figure 8(*b*). It demonstrated that a mucosal immune response was easily induced through oral administration on *A. pleuropneumoniae* antigen. From these studies, the elicitation of systemic and mucosal immune responses through oral administration of the Aquacoat microsphere shows promise for oral vaccine development.

Induction of specific IgA antibody has also been demonstrated at a number of mucosal sites following oral administration in mice by Delgado *et al.* (1999) and Chao *et al.* (1998). Although they found salivary or intestinal IgA levels increased after boosting, long-lasting mucosal immune could be affected by several factors. There is a concern that the feeding of antigen inducing a strong local secretary IgA response may produce tolerance at the same time. Thus, a further study is needed to determine the optimal dose and choose appropriate encapsulation polymers for oral vaccine.

The nature of protective immunity to pleuropneumonia has been studied by several groups. They showed that neutralizing antibodies against the capsular polysaccharide, lipopolysaccharide, and haemolysin were protective (Bosse *et al.* 1992, Loftager *et al.* 1993). The major adhesion factor of *A. pleuropneumoniae* has been identified as the LPSs (Belanger *et al.* 1990). If the antibody against LPS appears in the secretions of the respiratory tract, the antibody may easily protect the animal against pleuropneumonia. In the mouse model, it was demonstrated that a good mucosal antibody of IgA can be evoked, but a further investigation is needed to find out whether the IgA of LPSs is elicited in intestine or salivary gland.

Acknowledgements

The authors would like to thank the National Science Council of the Republic of China for the financial support under Contract No. NSC-89-2317-B-059-001.

We would also like to thank Dr S. J. T. Mao for critical reading and editing this manuscript. We are grateful to B.-L. Yu, M.-C. Lee, and S.-P. Chen for performing the electron microscopy.

References

Belanger, M., Dubreuil, D., Harel, J., Girard, C., and Jacques, M., 1990, Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. *Infectious Immunology*, **58,** 3523–3530.

Bosse, J. T., Johnoson, R. P., Nemec, M., and Rosendal, S., 1992, Protective local and systemic antibody responses of swine exposed to an aerosol of *Actinobacillus pleuropneumoniae* serotype 1. *Infection and Immunity*, **74,** 215–222.

Brodie, B. B., 1964, Physico-chemical factors in drug absorption. In *Absorption and distribution of drugs*, edited by T. B. Binns (London: E&S Livingston, Ltd.), pp. 16–24.

Chang, R. K., Hsiao, C. H., and Robinson, J. R., 1987, A review of aqueous coating techniques and preliminary data on release from a theophylline product. *Pharmaceutical Technology*, **11,** 56–68.

Chang, W. M., and Lai, S. S., 1996, Characterization of *Actinobacillus pleuropneumoniae* RTX-toxin (Apx-I). *Journal of the Chinese Society of Veterinary Science*, **22,** 394–401.

Chao, N.-H., Seong, S.-Y., Chun, K.-H., and Kim, Y.-H., 1998, Novel mucosal immunization with polysaccharide-protein conjugates entrapped in alginate microspheres. *Journal of Controlled Release*, **53,** 215–224.

Coombes, A. G. A., Lavelle, E. C., and Davis, S. S., 1999, Biodegradable lamellar particles of poly(lactide) induce sustained immune responses to a single dose of adsorbed protein. *Vaccine*, **17,** 2410–2422.

Delgado, A., Lavelle, E. C., Hartshorne, M., and Davis, S. S., 1994, PLG microparticles stabilized using enteric coating polymers as oral vaccine delivery systems. *Vaccine*, **17,** 2927–2938.

Fites, A. L., Banker, G. S., and Smolen, V. F., 1970, Controlled drug release through polymeric films. *Journal of Pharmaceutical Science*, **59,** 610–613.

Hogan, J. E., 1994, Some formulation factors involved in hydroxypropyl methycellulose controlled release matrices. In *Excipients and delivery systems for pharmaceutical formulations*, edited by D. R. Karsa and R. A. Stephenson (Cambridge: Royal Society of Chemistry), pp. 186–190.

Kulvanich, P., and Leesawat, P., 1996, Release characteristics of the matrices prepared from co-spray dried theophylline and ethylcellulose with/without channeling agents. *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, **23,** 143–144.

Liao, C.-W., Hsu, M.-I., Yu, B.-L., Lee, M.-C., Chen, S.-P., Cheng, I. C., and Wen, C.-N., 2000, *Mycoplasma hyopneumoniae* antigens entrapped in alginate microspheres for oral administration. *Taiwanese Journal of Agricultural Chemistry and Food Society*, **38**, 310–320.

Loftager, M. K., Eriksen, L., and Nielsen, R., 1993, Antibodies against *Actinobacillus pleuropneumoniae* serotype 2 in mucosal secretions and sera of infected pigs as demonstrated by an enzyme-linked immunosorbent assay. *Research in Veterinary Science*, **54,** 57–62.

Pierce, J., and Suelter, C. H., 1997, An evaluation of the Coomassie® Brilliant Blue G-250 dye-binding method for quantitative protein determination. *Analytical Biochemistry*, **81,** 478–480.

Tzan, Y. L., Weng, C. N., Lee, C. J., and Lin, S. Y., 1989, Determination of antigenic characteristics of *Mycoplasma Hyopneumoniae* by enzyme-linked immunosorbent assay. I. The establishment and performance of ELISA. *Journal of the Chinese Society of Veterinary Science*, **15,** 193–198.