(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 June 2006 (08.06.2006)

PCT

(10) International Publication Number
WO 2006/059846 A1

(51) International Patent Classification:
A61K 9/16 (2006.01) *A61K 38/04* (2006.01)

(21) International Application Number:
PCT/KR2005/003871

(22) International Filing Date:
16 November 2005 (16.11.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10-2004-0099624
1 December 2004 (01.12.2004) KR

(71) Applicant *(for all designated States except US)*: **LG LIFE SCIENCES, LTD.** [KR/KR]; LG Twin Tower, 20, Yoido-dong, Youngdungpo-gu, Seoul 150-721 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KANG, Myung-Soo** [KR/KR]; 203, 210-49, Sinseong-dong, Yuseong-gu, Daejeon 305-805 (KR). **CHANG, Byoung Sun** [KR/KR]; 501, Hongik Villa, 458-7, Jeonmin-dong, Yuseong-gu, Daejeon 305-810 (KR). **LEE, Jin Hee** [KR/KR]; 108-303, Woosung Apt., Jeongrim-dong, Seo-gu, Daejeon 302-795 (KR). **YOON, Ki-young** [KR/KR]; 201-1102, Hyundai Apt., Ssangmun 4-dong, Dobong-gu, Seoul 132-779 (KR). **KIM, Yun-Sik** [KR/KR]; 201-402, Taesan 2-Cha Apt., 887-56, Gosaek-dong, Gwonseon-gu, Suwon-si, Gyeonggi-do 441-729 (KR).

(74) Agent: **SOHN, Chang Kyu**; 1403, Seongji Heights 2-cha Bldg., 642-16, Yoksam 1-dong, Kangnam-gu, Seoul 135-910 (KR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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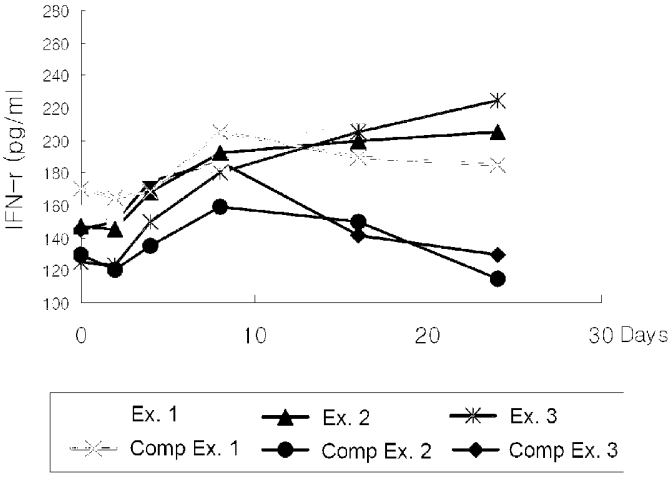
(54) Title: FORMULATION OF SEC1 MUTATED PROTEIN AND METHOD FOR FORMULATION OF THE SAME



(57) Abstract: Provided is a formulation of an SECl mutant protein, a toxin of Staphylococcus aureus, exhibiting excellent effects on prevention, symptom alleviation and treatment of mastitis via an improved immune function of lactating or non-lactating dairy cows, comprising solid mi- croparticles containing the SECl mutant protein as an active ingredient, a protein-stabilizing excipient, a carbohydrate-based auxiliary excipient and a lipophilic material, dispersed in a bio compatible oil and/or a fatty acid ester-based compound. The formulation of the present invention is capable of achieving effective in vivo delivery of a water-soluble mutant protein which is an active ingredient while maintaining activity thereof. In addition, the formulation of the present invention exhibits excellent therapeutic effects on prevention and treatment of mastitis via an enhanced immunopotentiating effects due to superior antibody-producing ability when administered to dairy cows. Further, the formulation of the present invention can be used as an injectable preparation due to excellent injectability.

WO 2006/059846 A1

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Description

FORMULATION OF SEC1 MUTATED PROTEIN AND METHOD FOR FORMULATION OF THE SAME

Technical Field

[1] The present invention relates to a formulation of a *Staphylococcal* enterotoxin C1 (SEC1) mutant protein, one of toxins of *Staphylococcus aureus*, exhibiting prevention, symptom alleviation and excellent therapeutic effects of mastitis via an improved immune function of lactating or non-lactating dairy cows and a method for formulating the same. More specifically, the present invention relates to a formulation of an SEC1 mutant protein comprising an effective amount of an SEC1 mutant protein, a protein-stabilizing excipient, a carbohydrate-based auxiliary excipient, a lipophilic material, and a biocompatible oil and/or a fatty acid ester-based compound, by which the SEC1 mutant protein can be easily administered via injection and efficacy and stability thereof are maximized, and a method for formulating the same.

Background Art

[2] *Staphylococcal* enterotoxin C1(SEC1) mutant protein, a toxin of *Staphylococcus aureus*, is a protein in which cysteine, an amino acid at a position 95 of a mutant toxin C1 of *Staphylococcus aureus*, was substituted with serine, and is known to have a probability of effective application thereof as a vaccine inducing promotion of non-specific cellular immunity as well as antibody production of specific humoral immunity (Terence N. Turner et al (1992), Infection and Immunity 62(2), pp 694-697; Carolyn J. Hovde et al (1994), Molecular Microbiology 13(5), pp 897-909; and Marcy L. Hoffann et al (1994), Infection and Immunity 62(8), pp 3396-3407). A method for preparing an SEC1 mutant protein is disclosed in Korean Patent No. 382239, Australian Patent No. 2001-11759 and the like.

[3] The SEC1 mutant protein exhibiting such prevention, symptom alleviation and therapeutic effects of mastitis, as disclosed in Korean Patent No. 382239, can be mass-produced using Escherichia coli as a host. However, similar to other protein medicines, the SEC1 mutant protein also suffers from problems associated with maintenance of protein stability such as protein denaturation upon long-term storage (more than 2 weeks) and aggregation of protein in dispersion media (for example, oil). In addition, the SEC1 mutant protein, like ordinary proteins, is also labile to heat, pH, salts and organic solvents (Weiqi Lu et al. PDA L. Pharm. Sci. Tech. 49, 13-19 (1995)).

[4] Meanwhile, Korean Patent No. 359252, assigned to the present applicant, discloses a method for preparing microparticles of SEC1 mutant protein using 3% carboxymethylcellulose and 2% lecithin via spray drying. However, such a method

suffers from several problems as follows. That is, preparation of the SEC1 mutant protein by means of spray drying exhibits a low yield of about 10 to 30% and thus is not suitable for commercialization via industrial-scale production. In addition, during a spray drying process, the SEC1 mutant protein is exposed to a high internal temperature of 50 to 70℃ which may cause denaturation of the protein. As such, there is a need for development of a formulation capable of achieving effective in vivo delivery of the SEC1 mutant protein after administration thereof while maintaining the protein at a stable state for a sufficient period of time even when exposed to an external environment, and development of a technique for large-scale production of the SEC1 mutant protein.

[5] Therefore, a great deal of research has been made toward formulation of the SEC1 mutant protein into solid microparticles utilizing biodegradable polyesters, for example polyglycolide and polylactide and polymers thereof. Such formulations exhibit protection effects of active drug against degrading enzymes in the body and sustained efficacy of the drug for a predetermined period of time, thus capable of further maximizing effects thereof. However, since biodegradable polymers are soluble only in organic solvents upon performing a formulation process, thus causing severe denaturation of proteins which in turn leads to difficulty of practical application thereof. In addition, many attempts have been made into formulations for oral administration using liposome as another type of formulation, but such formulations exhibit disadvantages such as instability of a particle structure and being non-economic as animal preparations, thus failing to achieve practical application thereof.

Disclosure of Invention

Technical Problem

[6] Therefore, the present invention has been made to solve the above problems, and other technical problems that have yet to be resolved.

[7] The present inventors have conducted a variety of extensive and intensive study and experimentation to solve problems as described above and have found that an SEC1 mutant protein formulation, comprising an effective amount of an SEC1 mutant protein as an active ingredient, and prepared by mixing the SEC1 mutant protein with a protein-stabilizing excipient containing particular ingredients, a carbohydrate-based auxiliary excipient and a lipophilic material to prepare solid microparticles and dispersing the resulting microparticles in a biocompatible oil and/or a fatty acid ester-based compound (a dispersion medium), can prevent denaturation occurring upon long-term storage of the SEC1 mutant protein in a solution state, aggregation in the dispersion media and instability of the protein due to a variety of external factors, and is capable of achieving prevention, symptom alleviation and maximized therapeutic

effects of mastitis in the body as well as commercialization thereof via industrial-scale production. The present invention has been completed based on these findings.

[8] Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to provide a formulation which is capable of easily administering a water soluble SEC1 mutant protein via injection while maintaining stability thereof and is capable of maintaining activity of the protein for a prolonged period of time in vivo when it is administered.

[9] It is another object of the present invention to provide a method for preparing such a formulation enabling commercialization thereof via effective industrial-scale production.

Technical Solution

[10] In accordance with an aspect of the present invention, the above and other objects can be accomplished by the provision of an SEC1 mutant protein formulation comprising solid microparticles containing 0.001 to 50% by weight of a *Staphylococcal* enterotoxin C1 (SEC1) mutant protein, one of toxins of *Staphylococcus aureus*, as an active ingredient, 0.1 to 90% by weight of a protein-stabilizing excipient, 0.1 to 90% by weight of a carbohydrate-based auxiliary excipient and 0.1 to 10% by weight of a lipophilic material, dispersed in a biocompatible oil and/or a fatty acid ester-based compound.

[11] The formulation in accordance with the present invention is particularly suitable for injection and exhibits long-lasting efficacy and excellent stability of the drug.

[12] The SEC1 mutant protein, as described hereinbefore, is an active ingredient exhibiting excellent effects on prevention, symptom alleviation and treatment of mastitis of dairy cows, via an improved immune function of lactating or non-lactating dairy cows, and can be prepared by various methods known in the art. The content of the active ingredient, as defined above, is in the range of 0.001 to 50% by weight, based on the weight of solid microparticles. Where the content of the active ingredient is too low, it is difficult to exert pharmacological effects thereof. In contrast, where the content of the active ingredient is too high, it may cause occurrence of aggregation and denaturation thereof in water-insoluble solvents. More preferably, the content of the active ingredient is in the range of 0.01 to 20% by weight.

[13] In order to enhance physicochemical stability of the water-soluble mutant protein and in order to prepare stabilized solid microparticles, the formulation in accordance with the present invention contains various specific ingredients.

[14] Among such ingredients, the protein-stabilizing excipient is an ingredient which enables formation of the active ingredient SEC1 mutant protein into particles while maintaining stability thereof.

[15] In order to confirm excipients which enables preparation of the SEC1 mutant protein into stabilized solid microparticles, the present inventors have selected feasible excipient candidates from a various kinds of excipients known to have protein stabilizing effects and have carried out confirmation experiments on whether these excipient candidates have effects on formation of solid microparticles and protein stabilization. Taking into consideration problems exhibited by spray drying microgranulation, a microgranulation process was carried out via lyophilization.

[16] TABLE 1 below shows whether solid microparticles are formed or not when lyophilizing a mixture of the SEC1 mutant protein and excipients, and experimental results on percentage change in protein purity when the formulation containing such solid microparticles dispersed in oil was stored under room temperature conditions (25℃, 60% RH) and under severe conditions (40℃, 75% RH) for 4 weeks, respectively. In addition, for comparison, TABLE 1 also shows the results obtained when the water-soluble SEC1 mutant protein alone was dispersed in oil.

[17] [TABLE 1]

[18]

Excipients	Formation of solid microparticles (formed: O / failed: X)	Percentage change in purity of SEC1 mutant protein (%)	
		RT conditions	Severe conditions
Triblock copolymer (PEO-PPO-PEO : Pluronic F68)	O	- 0.62	- 0.93
Sodium chloride	O	0	- 0.04
Sucrose	O	0	- 2.25
Lactose	O	0	- 0.48
Maltose	O	- 3.52*	- 6.51*
Glycine	O	- 12.4**	- 41.9**
Mannitol	O	0	- 4.77*
Polyethyleneglycol 8000	O	0	- 0.05
PVPP (Polyvinylpoly pyrrolidone)	O	- 4.02*	- 5.57*
Potassium chloride	O	- 15.6**	- 43.5**
Polymethylmethacrylate (PMMA)	O	0	- 1.14
Tetramethylglucose (TMG)	O	- 0.25	- 2.23
Silica	X	-	-
Calcium phosphate	X	-	-
Glucose	O	0	- 1.54
Polyethyleneglycol:Sodium chloride (2:8 ratio)	O	- 0.01	- 0.09
Polyethyleneglycol:Sodium chloride (5:5 ratio)	O	- 0.14	- 0.67
Polyethyleneglycol: Sodium chloride (8:2 ratio)	O	- 0.04	- 0.65
Water-soluble SEC1 mutant protein	O	- 7.21*	- 49.5**

[19] As can be seen from TABLE 1, silica and calcium phosphate, among excipients used in experiments, have failed to form solid microparticles of water-soluble SEC1 mutant protein. Among solid microparticle-forming excipients, sodium chloride and polyethyleneglycol 8000 exhibited the best stability on the SEC1 mutant protein. Meanwhile, lactose, triblock copolymer (Pluronic), polymethyl methacrylate, glucose, sugar and tetramethylglucose generally exhibit excellent stability on the SEC1 mutant protein.

[20] As can be seen from the above results, examples of the preferred protein-stabilizing excipients that can be used in the formulation of the present invention include, but are not limited to, sodium chloride, polyethyleneglycol (for example, PEG 8000), dis-accharides (for example, lactose, maltose and sucrose), glucose, tetramethylglucose, Pluronic (a triblock copolymer) and any combination thereof. Inter alia, polyethyleneglycol is more preferable and a mixture of polyethyleneglycol and sodium

chloride is particularly preferable.

[21] As previously defined, the content of protein-stabilizing excipient is in the range of 0.1 to 90% by weight, based on the weight of solid microparticles. Where the content of the excipient is too low, it is difficult to exert effects due to addition thereof. In contrast, where the content of the excipient is too high, it may cause damage to proteins during a lyophilization process and occurrence of aggregation and denaturation of particles in water-insoluble solvents after lyophilization. More preferably, the content of the excipient is in the range of 30 to 60% by weight.

[22] As another ingredient used in the formulation in accordance with the present invention, the carbohydrate-based auxiliary excipient serves to maximize immunopotency of the active ingredient SEC1 mutant protein while assisting action of the protein-stabilizing excipient. Examples of the carbohydrate-based auxiliary excipients utilizable in the present invention include, but are not limited to, sodium carboxymethylcellulose, carboxymethylcellulose, hydroxypropylcellulose, chitosan, alginate, xylose, galactose, fructose, saccharose, dextran, chondroitin sulfate and any combination thereof. Among other things, particularly preferred is carboxymethyl cellulose.

[23] The content of the carbohydrate-based auxiliary excipient, as previously defined, is in the range of 0.1 to 90% by weight, based on the weight of solid microparticles. Where the content of the auxiliary excipient is too low, it is difficult to exert effects due to addition thereof. In contrast, where the content of the auxiliary excipient is too high, this may lead to failure of formation of solid particles during a lyophilization process. More preferably, the content of the auxiliary excipient is in the range of 0.5 to 50% by weight.

[24] Meanwhile, lipophilic materials may be added to the formulation in accordance with the present invention. The lipophilic materials serve to improve dispersibility of microparticles containing the active ingredient SEC1 mutant protein, thereby improving injectability of the formulation. Examples of the lipophilic materials that can be used in the present invention include, but are not limited to, phosphatidylserine, phosphatidylethanolamine, lecithin, phosphatidylcholine-based materials (for example, stearoyl phosphatidylcholine and arachidonyl phosphatidylcholine), myristic acid, palmitic acid, stearic acid, sorbitan monooleate, polysorbate, glyceryl stearate, sorbitan palmitate, sorbitan stearate and any combination thereof. Particularly preferred are phosphatidylcholine-based materials.

[25] The content of the lipophilic material, as previously defined, is in the range of 0.1 to 10% by weight, based on the weight of solid microparticles. Where the content of the lipophilic material is too low, it is difficult to sufficiently exert addition effects thereof. In contrast, where the content of the lipophilic material is too high, this may

lead to failure of formation of solid particles after completion of lyophilization. The content of the lipophilic material is preferably in the range of 0.1 to 5% by weight.

[26] As one of media (dispersion media) capable of dispersing the solid microparticles containing the SEC1 mutant protein, protein-stabilizing excipient, carbohydrate-based auxiliary excipient and lipophilic material to make an injectable formulation, the biocompatible oils that can be used in the present invention preferably include, but are not limited to, edible oil, mineral oil, squalene, squalane, mono-, di- and triglyceride, and any combination thereof. Examples of edible oils include soybean oil, corn oil, olive oil, safflower oil, cottonseed oil, peanut oil, sesame oil and sunflower oil. Particularly preferred is soybean oil.

[27] As another material that can be used as a dispersion medium of solid microparticles, the fatty acid ester-based compound preferably include, but is not limited to, monoglyceride, diglyceride, triglyceride, isopropylpalmitate, isopropylmyristate, benzoic acid, ethyl linoleate and any combination thereof. Particularly preferred is isopropylmyristate.

[28] The biocompatible oils and fatty acid ester-based compounds may be used, alone or in combination. Combined use thereof as the dispersion medium is more preferable in terms of improved injectability and maximized dispersion effects. This fact can also be confirmed from the results of Experimental Example 3 which will be illustrated hereinafter. In particular, among biocompatible oils and fatty acid ester-based compounds, combined use of soybean oil and isopropylmyristate provides better injectability of the formulation.

[29] Upon combined use, the content of the biocompatible oil may be, for example, in the range of 1 to 99% by weight, based on the total weight of the dispersion medium. In this connection, when isopropylmyristate is used as the fatty acid ester-based compound, the content thereof is in particular preferably in the range of 20 to 40% by weight.

[30] An amount of microparticles added relative to the dispersion medium may be determined taking into consideration an optimal single-injection dose, injectability of the dispersion and the like and is preferably in the range of 1 to 99% by volume on the basis of the total volume. If necessary, it is possible to use the formulation in which the above dispersion was re-dispersed in physiological saline.

[31] Meanwhile, other ingredients known in the art may be further added to the formulation in accordance with the present invention within a range they do not damage effects of the invention and it should be construed that those ingredients are also encompassed within the scope of the present invention.

[32] In accordance with another aspect of the present invention, there is provided a method for preparing an SEC1 mutant protein formulation, comprising:

[33] (a) mixing an SEC1 mutant protein, a protein-stabilizing excipient, a carbohydrate-based auxiliary excipient and a lipophilic material;

[34] (b) lyophilizing the resulting mixture to prepare solid microparticles; and

[35] (c) dispersing the solid microparticles in a biocompatible oil and/or a fatty acid ester-based compound.

[36] In step (b), solid microparticles are fabricated to have a particle diameter of about 5 to 200 □. Where the particle diameter is too small, aggregation of microparticles occurs, thus making it difficult to achieve sufficient dispersion and leading to deterioration of sustained-release properties of the active ingredient. Conversely, where the particle diameter is too large, precipitation of microparticles occurs in the dispersion medium, thus undesirably making it difficult to maintain the dispersed state.

[37] A lyophilizing method for preparing the microparticles is well known in the art and therefore details thereof will be omitted herein.

Brief Description of the Drawings

[38] The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

[39] FIG. 1 is a graph showing results of determination on γ-IFN levels in blood collected after injection of formulations of Examples and Comparative Examples into mice, respectively, using a mouse cytokine ELISA kit;

[40] FIG. 2 is a graph showing results of determination on changes in the number of somatic cells in milk collected prior to administration, and 2, 4, 6 and 10 weeks post administration, a total of five times, following injection of a formulation of Example 1 into lactating dairy cows having more than 510^5 somatic cells/ml of milk; and

[41] FIG. 3 is a graph showing results of determination on the number of somatic cells in milk collected after injection of a formulation of Example 1 and Lavac Staph™ (*Staphylococcus Aureus* Bacterin)(Boehringer Ingelheim) into lactating dairy cows having more than 5×10^5 somatic cells/ml of milk, respectively.

Mode for the Invention

[42] Now, the present invention will be described in more detail with reference to the following examples. These examples are provided only for illustrating the present invention and should not be construed as limiting the scope and spirit of the present invention.

[43]

[44] Examples 1 through 10 and Comparative Examples 1 through 4: Preparation of SEC1 mutant protein formulations

[45] SEC1 mutant protein formulations were prepared according to the following

formula given in TABLE 2 below. For example, in Example 1, an SEC1 mutant protein, sodium chloride, carboxymethylcellulose and phosphatidylcholine were mixed together, the resulting mixture was lyophilized to prepare solid microparticles having an average particle diameter of about 50 to 80 □, and the solid microparticles were dispersed in soybean oil, thereby preparing a desired formulation.

[46] [TABLE 2]

[47]

Formulations	Ingredients	Composition (wt%(w/v))
Ex. 1	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	1.26
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Ex. 2	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Polyethyleneglycol 8000	1.26
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Ex. 3	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	1.008
	Polyethyleneglycol 8000	0.252
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Ex. 4	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	1.26
	Phosphatidylcholine	0.35
	Soybean oil:Isopropylmyristate	Balance (80:20)
Ex. 5	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	1.26
	Phosphatidylcholine	0.35
	Soybean oil:Isopropylmyristate	Balance (60:40)
Ex. 6	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	1.26
	Phosphatidylcholine	0.35
	Soybean oil: Isopropylmyristate	Balance (40:60)
Comp. Ex. 1	SEC1 mutant protein	0.14
	Sodium chloride	1.26
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Comp. Ex. 2	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Potassium chloride	1.26
	Phosphatidylcholine	0.35

[48]

	Soybean oil	Balance
Comp. Ex. 3	SEC1 mutant protein	0.14
	Soybean oil	Balance
Comp. Ex. 4	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	1.26
	Soybean oil	Balance
Ex. 7	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	0.21
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Ex. 8	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	0.42
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Ex. 9	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	3.78
	Phosphatidylcholine	0.35
	Soybean oil	Balance
Ex. 10	SEC1 mutant protein	0.14
	Carboxymethylcellulose	1.40
	Sodium chloride	7.56
	Phosphatidylcholine	0.35
	Soybean oil	Balance

[49]

[50] Experimental Example 1: Stability of formulation containing injectable SEC1 mutant protein

[51] In order to confirm whether the SEC1 mutant protein in the formulation exhibits in vivo activity upon using formulations of Examples 1 through 3 and Comparative Examples 1 through 3 as injectable formulations, experiments were carried out using mice as follows. Specifically, each formulation was added to soybean oil such that a concentration of the SEC1 mutant protein was diluted to 40 □. The diluted formulations were intraperitoneally injected into 4-week old, male Balb/c mice and blood was collected 0, 2, 4, 8, 16 and 24 days post-administration. Thereafter, γ-IFN levels in blood thus collected were determined using a mouse cytokine ELISA kit. The results thus obtained are shown in FIG. 1.

[52] As can be seen from FIG. 1, until 24 days after administration of formulations, much higher values of γ-IFN in conjunction with excellent sustainability were observed in order of Example 1, Example 3 and Example 2, as compared to the case in which 40 □ of a raw material alone was administered. Whereas, Comparative Example

1 showed a significant increase in the value of γ-IFN until 8 days of administration, but exhibited tendency of a decrease in the value of γ-IFN after 8 days. Upon comparing the results between Example 1 and Comparative Example 1, it can be seen that the carbohydrate-based auxiliary excipient, carboxymethylcellulose maximizes immunopotentiating effects of the SEC1 mutant protein in vivo. In addition, upon comparing the results between Example 1 and Comparative Examples 2 and 3, formulations of Comparative Examples 2 and 3 exhibited an increase in the value of γ-IFN up to 8 days of administration, followed by a sharp decrease, thus representing that the composition of the present invention is essential for stability of the SEC1 mutant protein.

[53]

[54] Experimental Example 2: Injectability of formulation containing injectable SEC1 mutant protein

[55] In order to quantitatively evaluate whether solid microparticles containing the SEC1 mutant protein of the present invention were homogeneously dispersed in a non-aqueous solution or a mixed solution of a non-aqueous solution and an aqueous solution, a force applied at the time of injection using a syringe (injectability) was measured for the respective formulations (dispersions). Specifically, when pushing syringes (a 18 gauge needle), each having filled with 3 ml of respective dispersions, at a constant speed of 80 mm/min, the force necessary to extrude the contents from syringe (injectability) was measured on day 0 and day 28 of storage, respectively. For comparison, soybean oil was used as a control. In addition, a formulation in which phosphatidylcholine alone was excluded from composition ingredients of Example 1 was separately prepared and used as Comparative Example 4. Injectability of these dispersions are given in TABLE 3 below.

[56] [TABLE 3]

[57]

Formulations	Injectability (kgf)	
	0 day	28 days
Ex. 1	0.3	0.3
Ex. 2	0.3	0.3
Ex. 3	0.3	0.3
Comp. Ex. 1	0.3	0.3
Comp. Ex. 2	0.3	0.7
Comp. Ex. 3	0.3	Not injectable
Comp. Ex. 4	0.3	Not injectable
Control (soybean oil)	0.3	0.3

[58] As can be seen from TABLE 3, among 28-day dispersions after storage of formulations, dispersions of Examples 1 through 3 and Comparative Example 1 exhibited excellent dispersibility and thus were easily injected similar to soybean oil as the

control. Whereas, dispersions of Comparative Examples 2 and 3 were shown to suffer from difficulty of injection or being not injectable (non-injectability). In particular, it can be seen that the dispersion containing no lipophilic material used in the present invention (Comparative Example 4) was not injectable.

[59]

[60] Experimental Example 3: Injectability of formulation containing injectable SEC1 mutant protein

[61] Experimental conditions were the same as in Experimental Example 2 and injectability of the respective dispersions was measured 0 and 24 weeks after storage thereof, respectively. For comparison, the results on injectability of the respective dispersions are shown in TABLE 4 below, using the formulation of Example 1, and formulations of Examples 4 through 6 in which soybean oil and isopropylmyristate were mixed.

[62] [TABLE 4]

[63]

	Ratio (%)		Injectability (kgf)	
	Soybean oil	Isopropylmyristate	0 day	24 week
Ex. 1	100	0	0.3	0.8
Ex. 4	80	20	0.4	0.4
Ex. 5	60	40	0.3	0.3
Ex. 6	40	60	0.3	0.6

[64] As can be seen from TABLE 4, among 24-week dispersions after storage of formulations, dispersions of Examples 4 through 6 exhibited significantly improved effects in injectability and dispersibility of Example 1. Based on the results thus obtained, it can be seen that combination of the biocompatible oil (in particular, soybean oil) with the fatty acid ester-based compound provides further improved injectability. In particular, when the fatty acid ester-based compound is isopropylmyristate, it can be said that use of isopropylmyristate in an amount of 20 to 40% by weight based on the total weight of the dispersion medium provides more preferred results.

[65]

[66] Experimental Example 4: Stability of injectable SEC1 mutant protein with respect to addition concentration of sodium chloride

[67] TABLE 5 below presents protein contents determined when formulations, prepared by lyophilizing a mixture of an SEC1 mutant protein and excipients to obtain solid microparticles and dispersing the solid microparticles in oil, were stored under room temperature conditions (25℃, 60% RH) and under severe conditions (40℃, 75% RH) for 24 weeks, respectively. In addition, for comparison of changes in protein contents between the respective formulations with respect to contents of sodium chloride, the

results for protein contents in formulations of Examples 7 through 10 in conjunction with Example 1 are set forth in TABLE 5.

[68] [TABLE 5]

[69]

	Contents of SEC1 mutant protein (%)		
	0 time	RT conditions	Severe conditions
Ex. 1	98	98	97
Ex. 7	97	94	89
Ex. 8	98	95	90
Ex. 9	98	85	74
Ex. 10	99	82	65

[70] As can be seen from TABLE 5, the formulation of Example 1 exhibited stable results without changes in protein contents for 24 weeks under room temperature conditions and under severe conditions, while the formulations of Example 7 through 10 exhibited a tendency of decreases in protein contents. These results represent that the content of sodium chloride constituting solid microparticles of SEC1 mutant protein affects stability of the protein. Therefore, it can be seen that the particularly preferred content of sodium chloride is less than 60% by weight when sodium chloride is used as the protein-stabilizing excipient. Nonetheless, the above experimental results have confirmed that formulations of the present invention including the formulations of Example 7 through 10 generally ensure excellent stability of the SEC1 mutant protein even when they are stored under severe conditions (40℃, 75% RH) for a prolonged period of time (24 weeks).

[71]

[72] Experimental Example 5: Antibody-producing ability of formulation containing injectable SEC1 mutant protein

[73] Based on the results of Experimental Example 1, in order to examine biological activity of the SEC1 mutant protein upon intraperitoneal administration of a formulation of Example 1 into subject animals, 0.3 ml (40 □) of a diluted formulation, prepared by 10-fold diluting 0.3 ml (400 □) of the formulation of Example 1 in soybean oil, and 0.03 ml (4 □) of a diluted formulation, prepared by 100-fold diluting 0.3 ml (400 □) of the formulation of Example 1 in soybean oil, were administered to mice via intraperitoneal injection at intervals of 2 weeks, thrice. 14 days after the first, second and third administration, respectively, blood was collected from mice (10 animals/ administration) followed by isolation of sera, and the titer of antibody specific for SEC1 mutant protein was analyzed using peroxidase-conjugated goat anti-mouse IgG (ICN. #55550). The results thus obtained are given in TABLE 6 below.

[74] [TABLE 6]

[75]

Protein content (μg)/mouse	Antibody titer (ED50)		
	After 1st immunization (D)	After 2nd immunization (D-14)	After 3rd immunization (D-28)
400	512	819200	3276800
40	512	409600	3276800
4	128	102400	1638400

OD(A450 to A650 nm)

[76] As can be seen from TABLE 6, the formulation of Example 1 exhibited excellent antibody-producing ability with respect to contents of the SEC1 mutant protein in mice in vivo.

[77]

[78] Experimental Example 6: Somatic cell-reducing effects of formulation containing injectable SEC1 mutant protein

[79] As a somatic experiment of subject animals in order to verify immunopotentiating effects in dairy cows, a formulation of Example 1 was administered to 295 lactating dairy cows having more than 5×10^5 somatic cells/ml of milk via intramuscular injection and milk was collected 0, 2, 4, 6 and 10 weeks after administration of the formulation, a total of five times. Changes in the number of somatic cells in the collected milk were measured. The results thus obtained are shown in FIG. 2.

[80] As can be seen from FIG. 2, the formulation of Example 1 has continuously exhibited reduction effects of somatic cells in milk, starting from 4 weeks of administration up to 10 weeks.

[81]

[82] Experimental Example 7: Comparison for somatic cell-reducing effects of formulation containing injectable SEC1 mutant protein

[83] This example is a somatic experiment of subject animals for comparison and verification of immunopotentiating effects of a formulation of Example 1 in dairy cows. For this, Lavac Staph™, a *Staphylococcus aureus* vaccine against mastitis in dairy cows (available from Boehringer Ingelheim), was used as a Comparative Example. Experiment was carried out using 295 lactating dairy cows having more than 5×10^5 somatic cells/ml of milk. The formulation of Example 1 was intramuscularly injected into 278 dairy cows and the comparative formulation was intramuscularly injected into 17 dairy cows. Thereafter, milk was collected and the number of somatic cells in the milk was measured. The results thus obtained are shown in FIG. 3.

[84] As can be seen from FIG. 3, the formulation of Example 1 has exhibited better results in a reduction rate of somatic cells in milk, as compared to Lavac Staph™ of Comparative Example.

[85]

[86] Experimental Example 8: Comparison for yields of SEC1 mutant protein microparticles between lyophilization and spray drying

[87] Microparticles of an SEC1 mutant protein were prepared by means of a lyophilization method having the most ideal drying temperature (eutectic point) conditions under which stability of the SEC1 mutant protein is maintained with formation of microparticles, and a spray drying method disclosed in Korean Patent No. 359252, respectively. Experimental conditions and the results thus obtained are given in TABLE 7.

[88] [TABLE 7]

[89]

	Lyophilization	Spray drying
Ingredients	Concentration	
SEC1 mutant protein	2 (g)/120 (ml)	2 (g)/120 (ml)
Sodium carboxymethylcellulose	20 (g)/2 (L)	20 (g)/2 (L)
Lecithin	5 (g)/500 (ml)	5 (g)/500 (ml)
Sodium chloride	22.5 (g)	-
Amounts produced (g)	49.2 (g)	2.99 (g)
Yield (%)	99.3 (%)	11.1 (%)

[90] As can be seen from TABLE 7, yield (%) of microparticles by the spray drying method was about 11%, while yield (%) of microparticles by the lyophilization method in accordance with the present invention was about 99%, thus representing a significant difference therebetween. That is, in producing the SEC1 mutant protein, it can be seen that preparation of SEC1 mutant protein microparticles via lyophilization is only suitable for mass production, thus making it possible to enter commercialization.

Industrial Applicability

[91] As apparent from the above description, a formulation containing an SEC1 mutant protein in accordance with the present invention is capable of achieving effective in vivo delivery of a water-soluble mutant protein while maintaining activity thereof by inclusion of a protein-stabilizing excipient, a carbohydrate-based auxiliary excipient, a lipophilic material and a dispersion medium. In addition, the formulation in accordance with the present invention exhibits excellent effects on prevention and treatment of mastitis of dairy cows via an enhanced immunopotentiating effects due to superior antibody-producing ability when administered to dairy cows. Further, the formulation of the present invention can also be used as an injectable preparation due to excellent injectability.

[92] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope

and spirit of the invention as disclosed in the accompanying claims.

Claims

[1] An SEC1 mutant protein formulation comprising solid microparticles containing 0.001 to 50% by weight of a *Staphylococcal* enterotoxin C1 (SEC1) mutant protein, a toxin of *Staphylococcus aureus*, as an active ingredient, 0.1 to 90% by weight of a protein-stabilizing excipient, 0.1 to 90% by weight of a carbohydrate-based auxiliary excipient and 0.1 to 10% by weight of a lipophilic material, wherein the solid microparticles are prepared via lyophilization and are dispersed in a biocompatible oil and/or a fatty acid ester-based compound.

[2] The formulation according to claim 1, wherein the protein-stabilizing excipients is selected from the group consisting of sodium chloride, polyethyleneglycol, disaccharides, glucose, tetramethylglucose, Pluronic (triblock copolymer) and any combination thereof.

[3] The formulation according to claim 1, wherein the carbohydrate-based auxiliary excipient is selected from the group consisting of sodium carboxymethylcellulose, carboxymethylcellulose, hydroxypropylcellulose, chitosan, alginate, xylose, galactose, fructose, saccharose, dextran, chondroitin sulfate and any combination thereof.

[4] The formulation according to claim 1, wherein the lipophilic material is added to improve injectability of the formulation and is selected from the group consisting of phosphatidylserine, phosphatidylethanolamine, lecithin, phosphatidylcholine-based materials, myristic acid, palmitic acid, stearic acid, sorbitan monooleate, polysorbate, glyceryl stearate, sorbitan palmitate, sorbitan stearate and any combination thereof.

[5] The formulation according to claim 1, wherein the biocompatible oil is selected from the group consisting of soybean oil, cottonseed oil, olive oil, sesame oil, corn oil, peanut oil, palm oil, mineral oil, squalene, squalane, mono-, di- and triglyceride, and any combination thereof.

[6] The formulation according to claim 1, wherein the fatty acid ester-based compound is selected from the group consisting of monoglyceride, diglyceride, triglyceride, isopropylpalmitate, isopropylmyristate, benzoic acid, ethyl linoleate and any combination thereof.

[7] The formulation according to claim 1, wherein the solid microparticles are dispersed in a mixture of the biocompatible oil and fatty acid ester-based compound.

[8] The formulation according to claim 2, wherein the protein-stabilizing excipient is sodium chloride and/or polyethyleneglycol.

[9] The formulation according to claim 3, wherein the carbohydrate-based auxiliary

excipient is carboxymethyl cellulose.

[10] The formulation according to claim 4, wherein the lipophilic material is 0.1 to 10% by weight of the phosphatidylcholine-based material.

[11] The formulation according to claim 8, wherein the content of sodium chloride and/or polyethyleneglycol as the protein-stabilizing excipient is in the range of 30 to 60% by weight, based on the weight of solid microparticles.

[12] The formulation according to claim 5, wherein the biocompatible oils is soybean oil.

[13] The formulation according to claim 7, wherein the fatty acid ester-based compound is isopropylmyristate.

[14] The formulation according to claim 1, wherein the solid microparticles have an average particle diameter of 5 to 200 □.

[15] The formulation according to claim 13, wherein the content of isopropy-lmyristate is in the range of 20 to 40% by weight, based on the total weight of the dispersion medium.

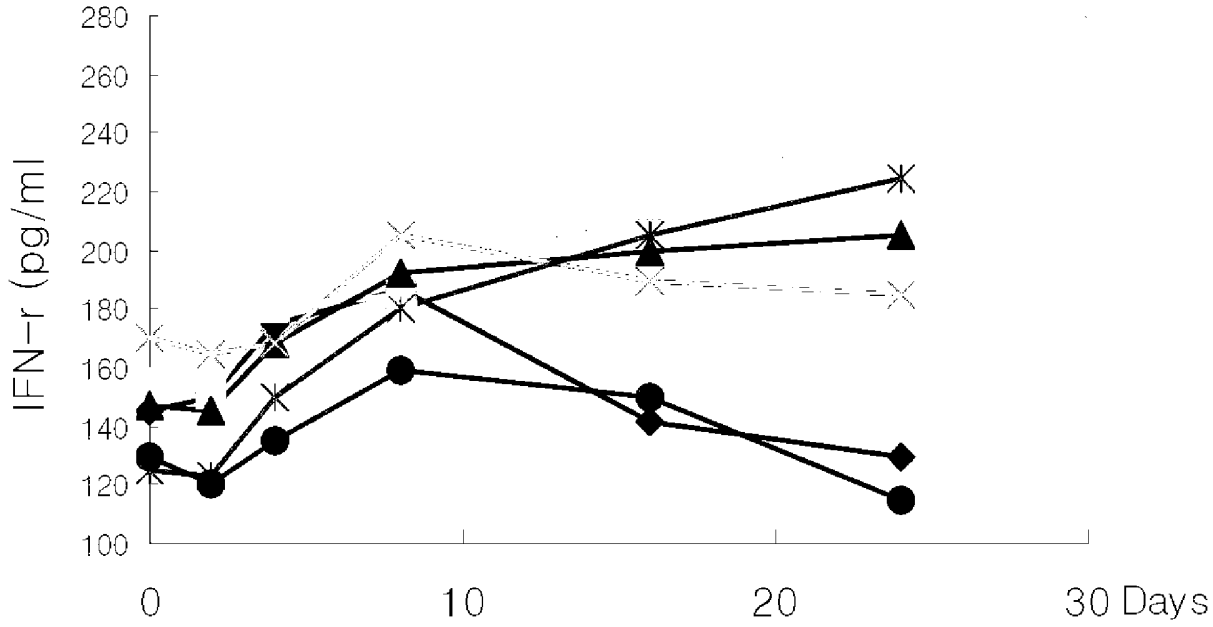
[16] A method for preparing an SEC1 mutant protein formulation of claim 1, comprising:

(a) mixing an SEC1 mutant protein, a protein-stabilizing excipient, a car-bohydrate-based auxiliary excipient and a lipophilic material;

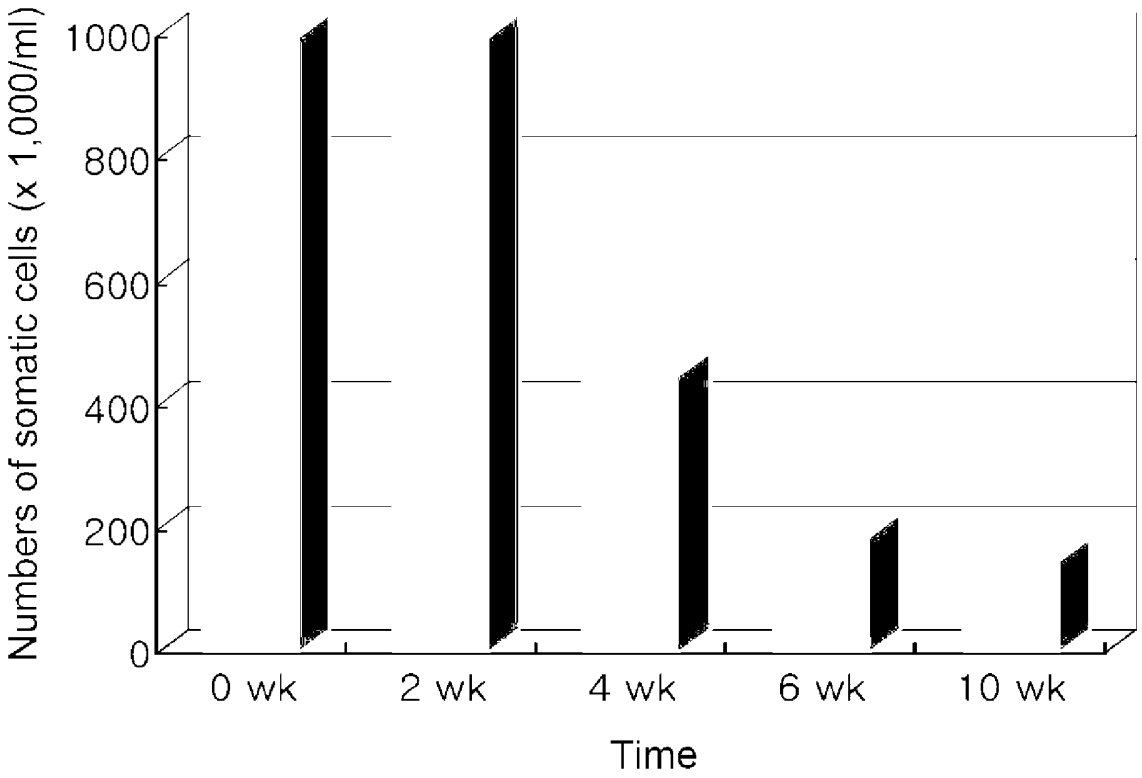
(b) lyophilizing the resulting mixture to prepare solid microparticles; and

(c) dispersing the solid microparticles in a biocompatible oil and/or a fatty acid ester-based compound.

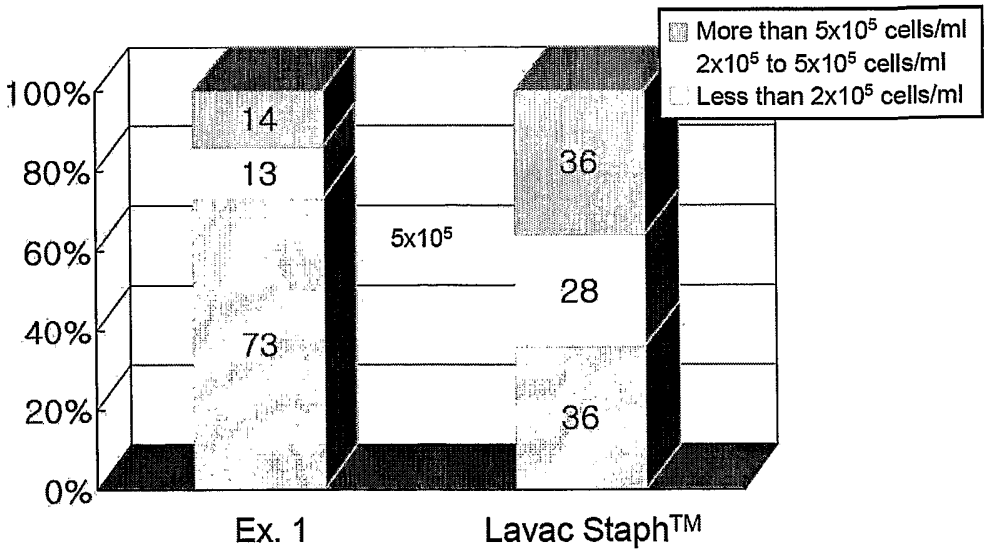
[Fig. 1]


IFN-r (pg/ml
280
260
240
220
200
180
160
140
120
100
0
10
20
30 Days
Ex. 1
Ex. 2
Ex. 3
Comp Ex. 1
Comp Ex. 2
Comp Ex. 3

[Fig. 2]


Numbers of somatic cells (x 1,000/ml)
1000
800
600
400
200
0
0 wk
2 wk
4 wk
6 wk
10 wk
Time

[Fig. 3]


More than 5x10^5 cells/ml
2x10^5 to 5x10^5 cells/ml
Less than 2x10^5 cells/ml
100%
80%
60%
40%
20%
0%
14
13
73
5x10^5
36
28
36
Ex. 1
Lavac Staph™

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/003871

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/16(2006.01)i, A61K 38/04(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC8 A61K,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
CA on CD, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/41682 A1 (LG Chemical LTD.) 20 July 2000 see the whole document	1 - 16
A	US 5,753,234 A (LG Chemical LTD.) 19 May 1998 see the whole document	1 - 16
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A	YEH MK et al. "Oral immunogenicity of the inactivated Bivrio cholerae whole-cell vaccine encapsulated in biodegradable microparticles," J. Controlled Release, 2002, Vol.82, pp237-247. see the whole document	1 - 16

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 FEBRUARY 2006 (14.02.2006)	**14 FEBRUARY 2006 (14.02.2006)**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	KIM, KYOUNG MI
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-8161

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
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