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X 72

Clarke, Modet & Co

COLOMBIA
E-MAIL clarkemodet@clarkemod
SANTAFE DE BOGOTA, A.A.

SEÑORES
DIVISION DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
E S D

COD 01

RE SOLICITUD Nº 00093684
FECHA SOLICITUD DICIEMBRE 7, 2000
PATENTE TRATAMIENTO ENZIMÁTICO
SOLICITANTE CHEMGEN CORPORATION

ASUNTO PRESENTACIÓN PRIORIDAD

DILIA RODRIGUEZ D ALEMAN, en mi condición de apoderada de la sociedad solicitante, en esta oportunidad por el presente escrito me permito presentar

- Copia certificada de la prioridad de Estados Unidos reivindicada Nº 60/169,935 de Diciembre 10 de 1999

- Traducción al Español de la mencionada prioridad

Esta prioridad está siendo presentada dentro del término de 3 meses que exige el Convenio de Paris, toda vez que la solicitud fué radicada el 7 de Diciembre de 2000

Ruego a usted continuar con el presente trámite

Señor Jefe,

DILIA RODRIGUEZ D ALEMAN
/srr
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ESTADOS UNIDOS DE AMÉRICA

A QUIEN INTERESE

DEPARTAMENTO DE COMERCIO DE ESTADOS UNIDOS

Oficina de Patentes y Marcas de Estados Unidos

12 de diciembre de 2000

EL PRESENTE TIENE COMO OBJETO CERTIFICAR QUE LOS ANEXOS AL PRESENTE INSTRUMENTO SON UNA FIEL COPIA DE LOS REGISTROS QUE REPOSAN EN LA OFICINA DE PATENTES Y MARCAS DE ESTADOS UNIDOS DE LOS DOCUMENTOS CORRESPONDIENTES A LA SOLICITUD DE PATENTE QUE APARECE A CONTINUACIÓN, Y QUE ELLOS CUMPLEN CON LOS REQUERIMIENTOS EXIGIDOS PARA QUE SE LES CONFIERA UNA FECHA DE RADICACIÓN SEGÚN EL CÓDIGO 35 USC 111.

SOLICITUD NÚMERO: *60/169,935*

FECHA DE RADICACION: *10 de diciembre de 1999*

AUTORIZADO POR EL COMISIONADO DE PATENTES Y MARCAS

(FIRMADO) N. WOODSON, FUNCIONARIO DE CERTIFICACIONES

Certificado legal No. 016754/0200

Sellos de barras: JC639 U.S. PTO 12/10/99 y JC541 U.S.PTO 60/169935

EN LA OFICINA DE PATENTES Y MARCAS DE ESTADOS UNIDOS

Solicitante: David M. Anderson

Titulo: TRATAMIENTO DE ENZIMAS PARA INFECCIONES

Solicitante No.: Sin asignar

Fecha de radicación: 10 de diciembre de 1999

Examinador: Sin asignar aún

74-79

Unidad de arte: Sin asignar aún

TRANSMISIÓN DE SOLICITUD DE PATENTE PROVISIONAL

Commisionado Asistente para Patentes

Casilla SOLICIUD DE PATENTE PROVISIONAL

Washington, D.C. 20231

Apreciado señor:

Junto con el presente se transmite para su radicación, según el C.F.R. 37, para. 1.53(c), la solicitud de patente provisional de:

David M. Anderson, de Rockville, Maryland

Lin Liu, de Gaithersburg, Maryland

Douglas W. Fodge, de Derwood, Maryland

Se anexa lo siguiente:

Especificaciones, Reivindicación(es) y Extracto (22 páginas).

La tarifa de radicación se calcula a continuación:

	Tasa	Tasas totales
Tarifa básica	US$150,00	US$150,00
Se aplican las tarifas de entidades pequeñas (sustraer el 50% de lo anterior)		US$0,00
TARIFA TOTAL DE RADICACIÓN:		US$150,00

Se adjunta un cheque por un valor de US$150,00, que cubre la tarifa de radicación.

Por medio del presente se autoriza al Comisionado Asistente para cobrar cualquier tarifa adicional que se pueda requerir en relación con la solicitud referente a C.F.R. 37 paras. 1.16-1.17, o acreditar cualquier sobrepago, en la Cuenta de Depósito No. 19-

0741. Si con el presente se incluye el pago no apropiado, por ejemplo, un cheque por la cantidad incorrecta, sin firmar, posfechado, o de otra manera inapropiado o informal, o incluso totalmente faltante, el Comisionado Asistente está autorizado para cobrar la cantidad impagada en la Cuenta de Depósito No. 19-0741.

Sírvase dirigir toda su correspondencia al apoderado o agente que se suscribe a continuación, a la dirección indicada enseguida.

Presentado respetuosamente,

(Firmado por Stephen A. Bent, Apoderado del solicitante

Registro No. 29,768)

Por: Patricia Granados, 33,683

Fecha: 11 de diciembre de 1999

FOLEY & LANDER

Washington Harbour

3000 K Street, NW, Suite 500

Washington, DC 20007-5109

Tel: (202) 672-5404, Fax: (202) 672-5399

SE REIVINDICA LO SIGUIENTE:

1. Una composición que comprende: (i) una enzima que divide un enlace que produce la liberación de una proteína de superficie celular o carbohidrato y (ii) un portador fisiológicamente aceptable de dicha enzima, donde dicha composición se encuentra en una forma adecuada para su administración oral.

2. Una composición conforme con la reivindicación 1, en la cual la mencionada enzima es seleccionada de entre un grupo conformado por esfingomielinasas y fosfolipasas.

3. Una composición conforme con la reivindicación 2, en la cual la mencionada enzima es una fosfolipasa de tipo C o de tipo D.

4. Una composición conforme con la reivindicación 3, en la cual la mencionada enzima es fosfolipasa C especifica de fosfatidilinositol.

5. Una composición conforme con la reivindicación 1, en la cual la mencionada enzima es seleccionada de entre el grupo conformado por esterasas, cerebrosideasas y carbohidrasas que dividen un enlace que produce la liberación de una proteína de superficie celular o carbohidrato.

6. Una composición conforme con cualquiera de las reivindicaciones 1 a 5, donde el mencionado portador es un comestible en el cual se ha incorporado la enzima mencionada.

7. Una composición conforme con la reivindicación 6, en la cual el mencionado comestible es un alimento animal compuesto por material de grano, una fuente de proteína, vitaminas, aminoácidos y minerales.

8. Una composición conforme con la reivindicación 7, en la cual el mencionado material de grano es maíz, sorgo, trigo, cebada o avena.

9. Una composición conforme con la reivindicación 7, en la cual la fuente de proteína es fríjol o arveja.

10. Una composición conforme con cualquiera de las reivindicaciones 1 a 5, en la cual la mencionada composición se encuentra en forma de dosificación sólida.

11. Una composición conforme con cualquiera de las reivindicaciones 1 a 5, en la cual la enzima mencionada está contenida dentro de una cápsula de gelatina.

12. Una composición conforme con cualquiera de las reivindicaciones 1 a 5, en la cual dicha composición está en la forma de jarabe o de solución líquida.

13. Un método para tratar o mejorar el riesgo de una infección de las vías digestivas, que comprende la administración oral a un sujeto que sufra de dicha infección o esté en riesgo de sufrirla, de una cantidad eficaz de enzima que divida un enlace que produzca la liberación de una proteína de superficie celular o carbohidrato.

14. Un método conforme con la reivindicación 13, en el cual la mencionada infección es causada por un patógeno protozoico, bacteriano, de levadura, fungal o viral.

15. Un método conforme con la reivindicación 14, en el cual la mencionada infección es causada por un patógeno protozoico del género *Eimeria*.

16. Un método conforme con la reivindicación 14, en el cual la mencionada infección es causada por un patógeno bacteriano del género *Closdridium*.

17. Un método conforme con la reivindicación 13, en el cual la mencionada infección es una infección parasitaria.

18. Un método conforme con la reivindicación 13, que comprende la administración oral, al mencionado sujeto, de una preparación de una enzima extracelular proveniente de la cepa *Bacillus cereus*.

19. Un método conforme con la reivindicación 18, en el cual la mencionada cepa del *Bacillus cereus* es ATCC 7004 o ATCC6464.

78 of 80
P. 390

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THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

December 12, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/169,935***
FILING DATE: ***December 10, 1999***

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WOODSON
Certifying Officer

7982

Atty. Dkt. No. 016754/0200

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: David M. Anderson

Title: ENZYME TREATMENT FOR INFECTION

Appl. No.: Unassigned

Filing Date: December 10, 1999

Examiner: Not yet assigned

Art Unit: Not yet assigned

PROVISIONAL PATENT APPLICATION TRANSMITTAL

Assistant Commissioner for Patents
Box PROVISIONAL PATENT APPLICATION
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(c) is the provisional patent application of:

David M. Anderson
Rockville, Maryland

Lin Liu
Gaithersburg, Maryland

Douglas W. Fodge
Derwood, Maryland

Enclosed are:

[X] Specification, Claim(s), and Abstract (22 pages).

[ ] Informal drawings _____sheets, Figures __-__).

[ ] Assignment of the invention to

[ ] Assignment Recordation Cover Sheet.

[ ] Small Entity statement.

The filing fee is calculated below:

	Rate		Fee Totals
Basic Fee	$150.00		$150.00
[ ] Small Entity Fees Apply (subtract ½ of above):		-	$0.00
TOTAL FILING FEE:		=	$150.00

[X] A check in the amount of $150.00 to cover the filing fee is enclosed.

[ ] The required filing fees are not enclosed but will be submitted in response to the Notice to File Missing Parts of Application.

[X] The Assistant Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Assistant Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date December 11, 1999

By [signature] 33,683
for Stephen A. Bent
Attorney for Applicant
Registration No. 29,768

FOLEY & LARDNER
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5109
Telephone: (202) 672-5404
Facsimile: (202) 672-5399

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Inventors: David M. Anderson
Lin Liu
Douglas W. Fodge

ENZYME TREATMENT FOR INFECTION

BACKGROUND OF THE INVENTION

In its 1998 *Revision* of the *World Population Estimates and Projections*, the United Nations Department of Economic and Social Affairs Population Division projected that the world population would reach 6 billion in 1999. The report also stated that it took only 12 years for the population to increase from 5 to 6 billion compared to 123 years to go from one to two billion. By the mid 21st century the projected population is between 7.3 and 10.7 billion. This remarkable population growth is attributable in part to the gains in food production, resulting from the application of technology and the development of intensive food-production practices. For future growth, more efficiency gains in food production will be needed.

One approach, which has made animal meat production more efficient, involves the widespread use of anti-microbial chemicals and antibiotics in animal feed. In large-scale farms, the spread of infection is very fast under the crowded production conditions. For this reason, widespread disease is controlled by prophylactic and therapeutic uses of these substances. For example, it is common practice to incorporate chemicals to control coccidia infections (*e.g.*, Rofenaid, Sacox, Decoquinate, Ampro, Monensin, Nicarbazin, Lasalocid, Coban, Stenorol, Bio-Cox and others) in animal feeds as well as anti-microbial antibiotics (*e.g.*, bacitracin, virginiamycin, tylosin, tetracycline, chlortetracycline, penicillin, oleandomycin, novobiocin, lincomycin, bambermycins, apramycin, erythromycin, neomycin and others). This practice is well known to promote growth and improve feed conversion.

The rise in multiple antibiotic resistances among human pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenza*, *Neisseria gonorrhoeae*, and *Mycobacterium tuberculosis*, has created fear that antibiotic resistance developed in microbes associated with farm animals could be migrating to human pathogens through transferable drug resistance factors. There is evidence that animals fed antibiotics are a source of bacteria with transferable resistance factors. Hooge, D.M., *Feedstuffs*, Vol. 71, No. 20, pp 59, 1999. Although the antibiotics used in animals and in humans are

generally different, there are similarities in mechanisms that could result in cross-resistance. In one case, fluroquinolones are approved for control of *E. coli* infections (colibacillosis) in some animals and also are used in human medicine. Hooge, *supra*.

There also is concern among meat-producing industries that yield loss and possible resurgence of animal disease could occur if there is a ban on use of antibiotics and anti-microbials in feed. In 1986, for example, Sweden banned the use of feed antibiotics and use decreased by 50%, but animal disease increased. Over the next eight years, the latter event was accompanied by an increased use of therapeutic antibiotics that resulted in an overall increase in the use of antibiotics as well as increased meat animal production costs. Smith, R., *Feedstuffs* Vol. 71, No.13, pp 1, 1999; Backstrom, L., *Feedstuffs* Vol 71, Nov. 22, pp8, 1999.

There are 30,000 human deaths per year caused by nosocomial infections with resistant pathogens, but many fewer deaths from food borne pathogens. None of the deaths from food-bone pathogens has been linked to antibiotic resistance. Smith, *supra*. It is not clear, therefore, that the use of antibiotics by the meat producing industries has contributed to the drug resistant human pathogen problem of the nosocomial infections. Nevertheless, the European Union has followed Sweden's lead and banned some performance-enhancing antibiotics (directive 70/524/EEC).

Another concern is that no new major classes of antibiotics have been developed in the last 30 years. This may mean that when significant antibiotic resistant pathogens develop, there may be no new antibiotics available to treat the infections. The difficulty of developing antibiotics and other issues seem to have caused the major pharmaceutical companies to move their R&D focus away from antibiotics development, especially for use in animals. New proposed regulations for registering a drug for animal use are so difficult that development is being stopped. Smith, *supra*.

In certain disease problems for animal health, infection is already pandemic. The problem of avian coccidiosis is a good example of a disease that is only managed, but not really under control. Virtually all flocks are infected and anti-coccidiosis chemicals are commonly rotated in the feed to control damage and limit the development of resistant strains. Coccidiosis costs poultry producers $350 million annually in losses and medication expenses for antibiotic drugs such as salinomycin. Suszkiw, J, *USDA Agricultural Research Service News*, October 28, 1997. It has been estimated that about $114 million would be spent on coccidiostats, in 1999, in the United States. Frost & Sullivan, *U.S. Pharmaceutical Products for Food Animals*, Report 5245-54, 1995.

There is a very clear need to find new and more effective methods to control infections in the digestive tract of animals that are grown using intensive farming practices. This need is based on a requirement to obtain better production efficiency in order to keep up with the rapidly expanding world population. Improved infection control guarantees better production. There is also a need for alternatives to antibiotic use in animal production to address the concern for possible antibiotic resistance development in human pathogens.

An enzyme-based treatment that can be added to food or to feed and that operates in a manner different from all antibiotics could eliminate the risk of stimulating the evolution of resistant pathogenic microorganisms, which present a problem for human health. Also, since an enzyme is a protein there is no possibility that dangerous chemical residue will be incorporated in the meat products, as happens with some antibiotics and anti-coccidiosis chemicals. American Feed Control Officials Inc., *Official Publication*, 1999, "Drugs and Feed Additives, Section 30.0 Enzymes," pp 206-217, ISBN 1-878341-10-3. Many treatments must be withdrawn for a period of time before harvest, to prevent carryover of dangerous levels of chemicals, but enzyme treatment would not have to be withdrawn. Accordingly, there is an urgent need for an enzyme-based antibiotic substitute.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of treatment that reduces the impact of microbial infections in the digestive tract infections.

It is another object of the present invention to ameliorating digestive tract infections by interfering with the binding of a pathogen to cells *in vivo*.

Yet another object of the invention is to provide a composition, suitable for ingestion, that includes an enzyme that causes release *in vivo* of pathogen surface components.

It also is an object of the invention to provide an approach for increasing weight gain and feed conversion in animals that are infected with pathogens which cause infections or necrotic enteritis.

It is a further object of the present invention to provide a dosage form, suitable for oral administration, that is effective in improving the condition of a subject infected by or at risk of infection by a microbial pathogen.

In accomplishing these and other objects, there has also been provided, in accordance with one aspect of present invention, a composition comprising (i) enzyme that cleaves a linkage that effects release of a cell-surface protein or carbohydrate and (ii) a physiologically acceptable carrier for the enzyme, the composition being in a form suitable for oral

administration. In a preferred embodiment, the enzyme included in the composition is a sphingomyelinase or a phospholipase, especially a type C or type D phospholipase. In another preferred embodiment, the enzyzme is selected from the group consisting of esterases, cerebrosideases, and carbohydrases that, as indicated above, cleave a linkage effecting release of a cell-surface protein or carbohydrate.

In accordance with another aspect of the present invention, a composition has been provided, having the aforementioned constituents (i) and (ii), where the carrier is a foodstuff. Thus, the composition can be an animal feed, additionally comprising grain material, such as corn, sorghum, wheat, barely or oats, and a source of protein, such as beans or peas, along with vitamins, amino acids, and minerals which are conventional to animal feeds.

In accordance with yet another of its aspects, the present invention provides a composition, as described above, that is a solid dosage form or, alternatively, is in the form of syrup or a liquid emulsion.

There is further provided a method of treating or ameliorating the risk of a digestive tract infection, comprising orally administering, to a subject suffering from or at risk for suffering said infection, an effective amount of enzyme that cleaves a linkage that effects release of a cell-surface protein or carbohydrate. In this regard, the infection affected may be caused by a protozoan, bacterial, yeast, fungal, or viral pathogen. In a preferred embodiment, the inventive method entails administering an extracellular enzyme preparation from a *Bacillus cereus* strain, such as the strain deposited, at the American Type Culture Collection (Manassas, Virginia), under accession number ATCC 7004 and ATCC6464, respectively.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. The detailed description and specific examples, while indicating preferred embodiments, are given for illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. Further, the examples demonstrate the principle of the invention and cannot be expected to specifically illustrate the application of this invention to all the examples of infections where it will be obviously useful to those skilled in the prior art.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

It has been discovered that enzymes of a particular class, characterized by the ability to cleave a linkage that effects release of a cell-surface protein or carbohydrate, display significant antibiotic activity, upon oral administration, which is effective, for example, in the

treatment and prevention of digestive tract infections. The enzyme class includes but is not limited to sphingomyelinases and phospholipases of type C and D, and enzymes of like cleavage specificity. Exemplary of this class, therefore, are enzymes that cleave and release glycoproteins or carbohydrates that are membrane- anchored via linkage to phosphatidylinositol. Thus, the enzyme phosphatidylinositol specific phospholipase C (E.C. 3.1.4.10), also known by the abbreviation "PI-PLC" or as 1-phosphatidylinositol phosphodiesterase, is a member of this class. Another example is glycosyl-phosphatidylinositol-specific phospholipase D, or GPI-PLD. Low, M.G. and A.R.S. Prasad, *Proc. Nat'l Acad. Sci.* 85: 980-984, 1988.

As described in greater detail below, the class of suitable enzymes also includes, for example, esterases, cerebrosidases, carbohydrases, and other phospholipases that are active to release cell-surface or pathogen-surface structures necessary to the infection process. More generally, enzymes useful in the present invention are those that can mediate release of pathogen- or host-surface components, like glycoproteins and carbohydrates, which are important to pathogen attachment and, hence, to the infection process.

Pursuant to the invention, moreover, suitable enzyme should be amenable to oral administration, in the sense that it can be included in an ingestable formulation that can deliver active enzyme, through the stomach to the intestine, in sufficient amount to disrupt an infection process. Such a formulation could incorporate enzyme that is resistant to degradation in the stomach, or could contain a high enough amount of enzyme that an effective portion of enzyme survives passage through the stomach. Alternatively, the formulation could shield or stabilize enzyme from degradation, for example, via encapsulation or the use of known stabilizers. Whether given enzyme meets these requirements can be ascertained, in routine fashion, via the approach of a feeding trail designed along the lines presented below in the examples.

The GPI-PLD and PI-PLC enzymes have been described from eukaryotic sources. For example, see Low, M.G., "Degradation of glycosyl-phosphatidylinositol anchors by specific phospholipases," Chapter 2, pp 35-63, [In] MOLECULAR AND CELL BIOLOGY OF MEMBRANE PROTEINS: GLYCOLIPID ANCHORS OF CELL-SURFACE PROTEINS, A.J. Turner (ed), Ellis Horwood, New York, 1990 (hereafter, "Low (1990)"); Low, M.G. and A.R.S. Prasad, *Proc. Nat'l Acad. Sci.* 85: 980-984, 1988; Essen, L.-O., *et al.*, *Nature* 380: 595-602, 1996; and Essen, L.-O., *et al.*, *Biochemistry* 36: 2753-2762, 1997. Also, PI-PLC has been described from prokaryotic sources, including extracellular production by bacteria. Among the known bacterial sources of PI-PLC are *Bacillus cereus* (Stein, M.W., and G.F. Logan, *J. Bacteriol.*

85:369-381, 1963; Stein, M.W. and G.F. Logan, *J. Bacteriol.* 90: 69-81, 1965; Ikezawa, H. *et al.*, *Biochimica et Biophysica Acta* 450: 154-164, 1976; Griffith, H., *et al.*, *Methods in Enzymology* 197: 493-502, 1991; Volwerk, J.J., *et al.*, *J. Cellular Biochemistry* 39: 315-325, 1989; and Kuppe, A., *et al.*, *J. Bacteriology* 171: 6077-6083, 1989), *Bacillus thuringiensis* (Ikezawa, H. and R. Taguchi, *Methods in Enzymology* 71:731-741, 1981; and Japanese Patent JP55034039), *Staphylococcus aureus* (Low, M.G. and J.B Finean, *Biochem J.* 162: 235-240, 1977), and *Clostridium novyi* (Taguchi, R. and H. Ikezawa, *Arch. Biochem. Biophys* 186:196-201, 1978).

Improved enzyme assay techniques for the PI-PLC enzyme have been devised. See Hendrickson, H.S., *et al.*, *Biochemistry* 31: 12169-12172, 1992; Hendrickson, H.S., *Analytical Biochemistry* 219: 1-8, 1994. An improved technique in this regard can be based on fluorescent substrate. Hendrickson, E.K., et al., *Bioorganic and Medicinal Chemistry Letters* 1: 619-622, 1991.

In accordance with the present invention, an extracellular enzyme preparation, obtained from *Bacillus cereus* and standardized for PI-PLC content (EXAMPLES 1-5), can be used to bring about very significant improvement in weight gain and feed conversion, for example, in chickens infected with a combination of *Eimeria acervulina* and *Clostridium perfringens*. This result is unexpected because *B. cereus* is an opportunistic pathogen that commonly causes food-borne gastroenteritis and *B. cereus* endophthalmitis.

Early studies reported that injection of extracellular *B. anthracis* or *B. cereus* enzyme into rabbits causes phosphaemia and even death (Stein, M.W., and G.F. Logan, *J. Bacteriol.* 85:369-381, 1963). It is surprising, therefore, that extracellular enzyme from a pathogen which causes gastroenteritis would have a curative effect, pursuant to the present invention, in relation to a disease caused by a microbial infection, such as the *Eimeria/Clostridium* co-infection mentioned above.

Bacillus cereus elaborates a variety of extracellular, membrane-active enzymes and cytolytic toxins, including PI-PLC and Cereolysin AB, which is composed of phospholipase C and spingomyelinase. Gilmore, M.S., *J. Bacteriol.* 171:744-753, 1989. In the aforementioned enzyme preparation, a PI-PLC [E.C. 3.1.4.10] which *B. cereus* produces is thought to be an active ingredient. Feeding test results, detailed below (EXAMPLE 6), demonstrate that the enzyme treatment was three times better than a control treatment with coccidiostat and antibiotic. Thus, the enzyme treatment of the present invention is an effective and commercially viable approach for the treatment of digestive tract infections.

Without ascribing definitively to any theory, the present inventors emphasize that PI-PLC enzyme has the ability to cleave the phosphatidylinositol glycolipid anchors of cell-surface proteins and other glycosyl phosphatidylinositols. Low, 1990; Low, M.G. and A.R. Saltiel, *Science* 239: 268-275, 1988. For example, the variant surface glycoproteins and other surface proteins and carbohydrates (Turco, S.J. and A. Descoteaux, *Annu. Rev. Microbiol.* 45: 65-94, 1992) of several protozoan parasites are anchored by glycosyl phosphatidylinositol lipids (GPI anchors), and are sensitive to PI-PLC digestion and release. Illustrative species belong to the genera *Schistosoma, Toxoplasma, Plasmodium, Trypanosoma, and Leismania* (Low (1990)), as well as *Eimeria, Babesia, Theileria, Giardia, Leptomonas* and *Entamoeba.* McConville, M.J. and M.A.J. Ferguson, *Biochemical J.* 294: 305-324, 1993.

This anchoring mechanism for cell surface components appears to be universal for eukaryotic cells ranging from single-celled protists to mammals. The presence of GPI anchors in *Giardia lamblia*, considered a very primitive eukaryote, suggests this kind of anchor evolved early in the eukaryotes. Consistent with this understanding is the discovery, in the archaebacteria, of a new phosphoglycerolipid GlcNα1-6-*myo*-inositol-*P*-dialkylglycerol (Nishihara, M., *et al., J. Biol. Chem.* 267: 12432-12435, 1992), which is the base for the more complex eukaryotic GPI anchor structures that have evolved. In protozoa, the GPI anchorage system is used more heavily than in higher eukaryotes, and there is evidence that GPI-anchored structures are important for parasite survival in insect and mammalian hosts. McConville and Ferguson, *supra.* For example, the frequent shedding of variant surface glycoproteins may be a mechanism to avoid immune system attack.

Species of the protozoan genus *Eimeria* present a widespread and costly problem for the poultry industry. *Eimeria tenella* contains phosphatidylinositol-anchored structures similar to glycoprotein/glycolipid of *Trypanosoma brucei.* These are thought to be important for membrane attachment and subsequent infection. Gurnett, A., *et al., Molecular and Biochemical Parasitology* 41: 177-186, 1990. The *Eimera* structures are cleaved by a trypanosome lipase and by *Bacillus thuringiensis* PI-PLC. *Id.* Thus, it was the present inventors' insight that in *vivo* treatment of parasites with a PI-PLC would help the host immune system and interfere with attachment and infection by pathogens entering the digestive tract.

In addition to maladies associated with protozoan parasites, there are a large number of more complex parasitic diseases that are caused by trematodes, cestodes and nematodes. The majority of parasitic infections are food-borne infections. In hogs, for example,

Trichinella spiralis (Trichinosis) and *Taenia solium* (Tape worm) are illustrative of complex parasites, with a food-borne stage, which pose a serious health risk Gamble, H.R., *Rev. Sci. Tech.* 16: 496-506, 1997. By way of another example, numerous species of parasites of animals and man, including many non-protozoan types of parasites, have been reported from most of the provinces of the Republic of China (Li, X., *Southeast Asian J. Trop. Med. Public Health* Vol 22 suppl.: 31-35, 1991) and in the Philippines (Eduardo, S.L., *Southeast Asian J. Trop. Med. Public Health* Vol. 22 suppl: 16-22, 1991). By decreasing binding and infection in the digestive tract, the enzyme treatment of the present invention could play a role, along with improved food-preparation practices and better sanitation, in bringing these sorts of parasitic diseases under control.

The prokaryotic bacteria do not contain surface glycoproteins and carbohydrates anchored by phosphatidylinositol (McConville and Ferguson, *supra*), but PI-PLC could still reduce bacterial infections by interfering with the attachment process. Pathogenic *E. coli* and a number of other well-known pathogenic *Enterobacteriaceae* expresses the bacterial adhesin FimH, a 29,000 Dalton (29 kD) mannose-binding lectin, presented at the distal tip of fimbriae. Abraham, S.N., *et al.*, *Nature* 336: 682-684, 1988. This adhesin has been shown to bind to CD48 of mast cells, a glycosylphosphatidylinositol-anchored molecule. Malaviya, R., *et al.*, *Proc. Nat'l Acad. Sci. USA* 96: 8110-8115, 1999.

A mechanism for PI-PLC to reduce the effect of bacterial infection relates to the liberation of the CD48 binding site from the host mast cells. Also, the FimH binding to mast cells triggers an inflammation response. In accordance with the present invention, therefore, reducing the binding sites also should reduce inflammation that could become excessive and damaging to intestinal health itself, as the inflammation response involves the release of tumor necrosis factor α. Malaviya, *supra*. Thus, via this mechanism of decreasing inflammation and the underlying secretion of tumor necrosis factor, a phospholipase treatment, according to the present invention, should relieve symptoms characterizing conditions such as irritable bowel syndrome, colitis, and Crohn's Disease. Van Deventer. S.J., *Ann. Rheum. Dis.* 58, Suppl. 1: I114-I120, November 1999.

Some cell surface receptors, of putative importance for initializing infections, are attached to membranes by mechanisms other than GPI anchors. These include structures such as cholesterol esters (Rostand, K.S. and J.D. Esko, *J. Biol. Chem.* 268: 24053-24059, 1993), the non-phosphorylated glycospingolipids (Karlsson, K.-A. *Ann Rev. Biochem* 58: 309-350, 1989) and other phospholipids such as phosphatdylethanolamine and

phosphatdylserine. Sylvester, F.A., *Infection and Immunity* 64:4060-4066, 1996. For the reasons stated above, therefore, targeting such structures with appropriate esterases, cerebrosidases, carbohydrases, and phospholipases, active to release these structures from cell surfaces, also should have beneficial effects, upon oral administration in accordance with the present invention, to treat digestive tract infections.

Against viral infections as well, the present invention should be effective, by its disruption of binding between viral particles and cells that the virus would infect *in vivo*. In light of the present inventors' discovery of the efficacy of oral administration, described here, it is interesting that pretreatment of influenza virus with phospholipase C, causing the release of about 50% of the virus phospholipid, resulted in a significant decrease in infectivity in chick embryos. Mizutani, H. and Mizutani, H., *Nature* 204:781-782, 1964. Conversely, pretreatment of cultured chick embryo fibroblast cells with phospholipase C, isolated from *Clostridium perfringens*, markedly inhibited subsequent infection of the cells by Semiliki Forest Virus. Friedman, R.M. and I. Pastan, *Proc. Nat'l Acad. Sci. USA* 59:1371-1378, 1968. While the art apprehended no therapeutic significance in these phenomena, in hindsight they are consistent with one mechanism thought to underlie the present invention, namely, the cleavage of a pathogen-surface ligand and/or its cognate cell-membrane receptor, disturbing interaction between them which is necessary to infection.

By virtue of the universal nature of membrane-linked surface proteins and carbohydrates in eukaryotes, the therapeutic methodology of the present invention, entailing the administration of enzyme, acutely or prophylactically, to cleave an anchoring linkage for such cell-surface proteins and/or carbohydrates, will find broad application in managing protozoan, bacterial, fungal, and viral infections of the digestive tract. To this end, a key aspect of the present invention is the demonstration that enzyme which is active in cleaving cell surface components can be administered orally and still be effective *in vivo*. Notably, while a representative suitable enzyme, PI-PLC, has been available since the 1960s, this approach has not been suggested heretofore.

Enzyme composition according to the invention preferably are formulated as dried, solid or liquid oral compositions. Such compositions generally will include stabilizers, such a buffer, a carbohydrate and/or a glycol. Dried, shelf-stable formulations of enzymes that are suitable, pursuant to the present invention, for incorporation in tablets or capsules, for example, can be prepared by freeze-drying, spray drying in fluidized bed dryer with inert or carbohydrate carrier, or by using evaporative techniques in conjunction with glass-forming stabilizers. Franks, F., *et al*, *Biopharm* 4: 38-55, 1991. Another approach involves salt

precipitation, for example, ammonium sulfate precipitate, as described in EXAMPLE 5, or solvent precipitate, as with acetone for powder formation, followed by drying and blending with a carrier.

Certain carbohydrates, particularly monosaccharides, disaccharides, and lower oligosaccharides are important glass-forming carbohydrates. Exemplary carbohydrates for use as carriers are xylose, fructose, glucose, sorbitol and maltotriose, among others, as described by Franks, *supra*. Choice of a carbohydrate carrier is based on compatibility with the enzyme, low hydroscopic tendency, and a favorable glass transition curve. The stabilizer trehalose is particularly suitable for producing ambient temperature-stable biologics. U.S. Patent 4,891,319; Roser, B.J., *Biopharm* 4 (8): 47-53, 1991; Colaco, C., *et al.*, *Bio/Technology* 10: 1007-1011, 1992; Aldridge, S.; *Genetic Engineering News*, March 15, pp10-11, 1995.

Enzymes for the present invention also can be formulated as liquids, for instance, as syrups with sorbitol or glycerol to lower water activity and to stabilize protein. Such solutions typically are sterile-filtered, prior to pharmaceutical use.

For use in feeds, a liquid enzyme preparation can be formulated with salt water (*e.g.*, NaCl, 15-18% w/w) to prevent microbial growth, and then can be applied to feeds, post-pelleting, by automated spraying technology. Fodge, D.W., *et al.*, *Feedstuffs*, Sept 29, 1997. Such liquid preparations may contain stabilizing carbohydrates such as sorbitol or glycerol, if compatible. Materials that are desired components of feed, such as other enzymes or vitamins that are heat-labile, also may be included for increased efficiency.

In instances where feed is utilized in a non-pelleted mash form (*i.e.*, not heat-treated), enzymes for the present invention can be provided as a dry concentrate, for addition at the feed mixer. Such dry enzyme concentrates are prepared by first concentrating the liquid enzyme preparation, using a 10 K or other suitable ultrafilter, to achieve a high percentage of enzyme content, and then by blending with a very dry carrier, such as corn grits, soy grits or even an inert material or insoluble salt that is approved for use in feeds. *Official Publication*, American feed Control Officials, *supra*, Part 582, "Substances generally regarded as safe in animal feeds."

There are a number of techniques available for generating enzymes stable enough to tolerate the pelleting process in some feed mills, while retaining sufficient activity, at lower temperatures, to function in the digestive tract. It is well known that modifying protein structure, primarily through changing the encoding DNA sequence or, secondarily, through chemical modification can render enzymes more stable against inactivation. One illustration

in this regard is the use of chemical cross-linking of enzyme crystals. Collina, A.M., *et al.*, *Organic Process Research and Development* 2 (6): 400-406, 1998. Another approach to increasing the stability of enzyme for the present invention entails changing amino acids by mutagenesis of the gene that codes for the enzyme of interest, or obtaining genes or parts of genes for shuffling. Crameri A., *et al.*, *Nature* 391, 288-291, 1998; Arnold, F. H. *Nature Biotechnol.* 16, 617-618, 1998b; Zhao, H., *et al.*, *Nature Biotechnol.* 16, 258-235, 1998; Zhao, H.and Arnold, F. H., *Protein Eng.* 12, 47-53, 1999. Mutation and selection for "directed evolution" of enzymes with the desired properties also is feasible. For example, see Giver, L., *et al.*, *Proc. Nat'l Acad. Sci. USA*, 95, 12809-12813,1998; Liao, H., *et al.*, *Proc. Nat'l Acad. Sci. USA* 83, 576-580, 1986; Cherry, J., *et al.*, *Nature Biotechnology*, 17, 379-384, 1999.

Certain protein modifications, including glycosylation, PEGylation and succinylation, also can enhance stability and alter pH optima, characteristics that could be optimized for enzyme to be used in the present invention. Thus, known protocols could be employed in this regard to make modified enzyme, for testing, according to the examples, to gauge suitability in the inventive treatment methodology.

An effective method for the production of PI-PLC arose from the cloning of the *B. cereus* gene into *B. megaterium*. The expression system (Rygus, T. and Hillen, *Appl. Microbiol. Bacteriol.* 35: 594-599, 1991) makes use of elements from the *Bacillus megaterium* xylose regulon (Rygus, T., *et al.*, *Arch. Microbiol.* 155: 535-542, 1991), and is available commercially from Bio101 Corp. (Vista, California). A fusion was created, between the PI-PLC gene leader coding sequence and the leader sequence of the *B. megaterium xylA* gene, in a plasmid stabilized with tetracycline resistance and regulated by a xylose responsive repressor. Strain construction with such a plasmid is illustrated below in EXAMPLE 7. Strains of this type, with amplified expression, provide a feasible means for producing commercially useful amounts of PI-PLC, to incorporate into animal feed in accordance with the present invention.

Some *Bacillus cereus* isolates produce antibiotics such as tunicamycin (Kamogashira, T., *et al.*, *Agric. Biol. Chem.* 52: 859-861, 1988). Another isolate of *Bacillus cereus* (ATCC 53522) is described, in U.S. patents No. 4,877,738 and No. 5,049,379, as a biocontrol agent to prevent damping off and root rot in plants. This effect is believed to result from the action of two antibiotics, one designated "Zwittermicin," which is a 396-Dalton, linear aminopolyol, and the other "Antibiotic B," an aminoglycoside. In the examples detailed below, the

possibility of involving such antibiotics was eliminated by excluding possible low molecular-weight compounds from the enzyme preparation.

In particular, EXAMPLE 3 describes how cell-free fermentation broth was concentrated by means of a membrane with a 10kD molecular-weight cutoff. The concentrate, containing protein larger than 10kD, was used for further processing. The small molecular-weight antibiotics, such as those described by Handelsman, *supra*, would pass through this filter. As described in EXAMPLE 4, moreover, ammonium sulfate precipitation was employed to precipitate high molecular weight proteins, leaving low molecular weight materials in solution. After the ammonium sulfate precipitate was re-dissolved, the resultant enzyme solution was dialyzed against buffer, yet another treatment that removes low molecular-weight antibiotics. Finally, as described in EXAMPLE 5, the protein was precipitated a second time, again with ammonium sulfate, to relegate any remaining, low molecular-weight compounds to the solution.

The combined use of these four treatments strongly militate against the possibility that the antibiotic effect observed is due to a low molecular-weight antibiotic, produced by ATCC 6464 or ATCC 7004. The fermentation broth also was tested, with an *E. coli* test strain, for the presence of antibiotic, as described in EXAMPLE 8, but no antibiotic activity was detected.

The present invention is further described below by reference to the following illustrative examples.

EXAMPLE 1. **Preparation of Frozen Stock cultures of *Bacillus cereus* ATCC 6464 and ATCC 7004**

Vials with lyophilized cells from the ATCC were opened and inoculated into a seed medium composed of Amberferm 4015 (Red Star) 10 g/*L*, Amberex 695 (Red Star) 5 g/*L*, and sodium chloride 5 g/*L*; pH 7.0 and grown at 30° C. The initial culture was streaked on LB Broth agar plates and a resulting single colony was inoculated back into 20 m*L* of seed medium in a 250 m*L* baffle flask (Bellco) and grown with shaking at 30° C. When the culture density reached OD 600 nm of 1.5, sterile glycerol was added to approximately 10 % v/v and vials containing 1.8 m*L* of culture were frozen at -80° C.

EXAMPLE 2. Growth of ATCC 6464 and ATCC 7004 isolates of *Bacillus cereus* for the production of phosphatidlyinositiol specific phospholipase C

Two Biostat C fermentors, 30 liters each, were batched with medium of the following composition, in tap water: Nutrient Broth No.2 (Oxoid) at 25 g/L, Tryptone (Difco) at 10 g/L, Yeast extract (Difco) at 10 g/L and Mazu DP10P Mod11 antifoam (BASF) at 0.1 mL per liter. The initial batch volume was 9.5 liters and the broth was sterilized at 121°C for 40 minutes. The initial pH was adjusted to 7.0 with ammonia gas after sterilization.

Seed cultures were prepared in 500 m*L* of the same medium in 4-liter baffle shake flasks with aspirator connection (Bellco) with attached silicone tubing with connectors for inoculation. The flasks were sterilized in an autoclave at 121° C for 50 minutes prior to inoculation with 1.8 m*L* of frozen stock culture prepared from the ATTCC shipment (EXAMPLE 1). The seed flask cultures were grown at 30° C for 5.5 hours with shaking at 200 RPM in a controlled environment incubator shaker (NBS model G-25). Prior to inoculation the ATCC 7004 culture had OD 600nm of 1.53 and pH 6.58. The ATCC 6464 culture had OD 600nm of 1.28 and pH 6.79.

The 500 m*L* seed cultures were inoculated into the 30 L fermentors and operated under the following conditions: temperature 30° C, mixing RPM 600, air flow 10 liters per minute, pressure 0.5 Bar. The initial culture OD 600nm was 0.81. At six hours the fermentation was stopped with final OD 600nm of 22.1 (ATCC 7004) and OD 24.2 (ATCC 6464). The fermentations were run without pH control and the final pH was 8.17 (ATCC 7004) and pH 8.13 (ATCC 6464). Broth was removed from the fermentors and cooled to 8° C prior to further processing.

The fermentors were run a second time using virtually an identical procedure with both ATCC *Bacillus cereus* isolates. In this case the seed cultures were used at six hours with OD 600 nm of 2.86 (ATCC 7004) and OD 1.98 (ATCC 6464), and a pH of 6.69 (ATCC 7004) and pH 6.65 (ATCC 6464). The main fermentations were run for 6.5 hours with final OD 35.9 (ATCC 7004) and OD 33.6 (ATCC 6464). Final pH was 8.38 (ATCC 7004) and 8.47 (ATCC 6464) at the time of cooling.

EXAMPLE 3. Filtration Methods for Cell Removal and PI-PLC enzyme concentration

Cells were removed and washed from each of the four fermentor batches described in EXAMPLE 2, using two A/G Technology (UFP-500-K-6A) 3 mm hollow-fiber, 500,000-molecular weight cut off (500 K) filters, attached end to end. Chilled broth was pumped

through the filters with a peristaltic pump at about 2 liters per minute with recycling back to the holding reservoir. The permeate containing the enzyme was collected in a reservoir chilled on ice.. The initial cell containing broth volume of about 9 liters was concentrated down to about 2 liters at which point diafiltation was started with 10 m*M* Tris-HCl pH 8.5. After a total volume of about 14 liters of permeate was collected, the cell washing was terminated.

The 500K permeate (about 14 liters from each fermentor) was concentrated with two A/G Technology (UFP-10-C-4XTCA) 0.5 mm hollow fiber 10,000 molecular weight cut-off filters (10 K) attached end to end. The same pumping method with recycle of concentrate was used except the permeate was discarded, and the final concentrate with a volume of about 500 m*L* from each fermentor was saved for the next processing step.

EXAMPLE 4. Further purification and concentration of PI-PLC enzyme

The 10 K ultrafilter concentrate (EXAMPLE 3) derived from each of the *Bacillus cereus* fermentations was adjusted to 80% of saturation with ammonium sulfate and mixed on ice for 60 minutes. The solution was centrifuged for 15 minutes at 6000 RPM in a Sorvall GSA rotor. The supernatant was discarded, and the precipitate was dissolved in a minimal volume of 10 m*M* Tris-HCl, 0.2 m*M* EDTA (pH 7.5) and then dialyzed in the cold against the same buffer.

The protein in each concentrate was measured using a dye-binding assay (BioRad) and the level of PI-PLC was measured (Hendrickson, E.K., J.L. Johnson, and S. Hendrickson, A fluorescent substrate for assay of phosphatidylinositol-specific phospholopase C, *Bioorganic & Medicinal Chemistry Letters*: 1, No. 11, pp 619-622, 1991) using an HPCL based detection method and the fluorescent substrate (Molecular Probes, Inc. P-3764, 1-pyrenebutyl *myo*-inositol-1-phosphate, lithium salt). Table I below summarizes the assay data and predicts the approximate purity and total enzyme on a pure basis, obtained for each of the four preparations. The enzyme preparations were stored frozen at -20° C until further processing.

95 48

TABLE I

Summary of Analysis of PI-PLC Crude Enzyme Preparations

Enzyme Prep.	ATCC Strain Number	Protein mg/L	Specific Activity U/mg[1]	Total mg protein in preparation	Estimated % Putity[2]	Total mg PI-PLC on pure basis
1	7004	1.86	1.75	325	2.92	9.49
2	6464	1.44	0.52	345	0.867	2.99
3	7004	1.35	4.42	208	7.36	15.3
4	6464	2.06	1.72	256	1.95	5.00

[1] *U* = unit (micromole/minute)

[2] If 100% pure protein has a specific activity of 60 U/mg (Hendrickson, *supra*)

EXAMPLE 5. Pooling and Concentration of Enzyme Preparations prior to application to Animal Feed

The enzyme preparations 1, 2, 3 and 4 were pooled with a total volume of 675 m*L*. Ammonium sulfate (492 grams) was added and the solution was mixed on ice for several minutes. The solution was centrifuged to collect precipitate. The pellet fraction was dissolved in a minimal amount of 20 m*M* phosphate buffer (pH 7.0).

The resulting solution was 70.9 m*L* with a density of 1.057 grams/cc. The protein concentration was measured at approximately 25.5 mg/m*L*. The PI-PLC activity was measured at approximately 1.13 U/mg, with an estimated purity of the PI-PLC at 1.88%. The solution was frozen at -20° C until it was thawed, in its entirety, and used to treat 200 pounds of chicken feed uniformly.

EXAMPLE 6. Chicken Feeding Trial with Pathogen Challenge

A feeding trial starting with one-day-old male broiler chickens was conducted at the PARC Institute Research Farm #1 in Queen Anne, Maryland. A typical uniform chicken feed diet of "starter feed," designed to meet or exceed the National Research Council's NUTRIENT REQUIREMENTS FOR POULTRY 9th ed. (1994), was prepared and fed in mash form. The chickens were divided in cages (12 inches x 24 inches floor space) in four treatment groups (see TABLE II) with each treatment group repeated four times and six birds per cage repeat. Water and feed were provided *ad libitum* throughout the 21 day test period. A randomized block design was used to allocate chicks to cages and cages to treatment groups. All cages, feeders and waterers were sanitized prior to the beginning of the test. Lighting was

continuous (24 hour per day) with incandescent lamps. Body weights were determined at day one and day 21. Feed consumption was measured at day 21.

TABLE II

Description of Treatment groups

Test Group #	Test Description	Test Material	Replications	Chicks per replication
T1	Negative Control	none	4	6
T2	Positive Control	Coccidiostat and antibiotic treatment	4	6
T3	PI-PLC Enzyme	0.34 g enzyme/ton (pure basis)	4	6
T4	PI-PLC Enzyme	0.34 g enzyme/ton (pure basis)	4	6

At day 5, all the chickens were infected with 200,000 oocysts per bird of *Eimeria acervulina* by oral gavage. On day 7, all birds were further infected with 500,000 *Clostridium perfringens* through the water supply. In the negative control (Test Group T1) there was no treatment for infection. In the positive control (Test Group T2) a coccidiostat and antibiotic were added to the feed. This was the anti-coccidiosis treatment Sacox (salinomycin at 60 g/ton) and the antibiotic BMD-50 (50 g/ton). For treatment groups T3 and T4, the PI-PLC enzyme treated feed (about 0.34 grams PI-PLC on a pure basis) was used beginning at day five at the time of the oral gavage with *Eimeria acervulina*. All the feed was prepared in one uniform batch, then divided for addition of antibiotic (Test Group T2) or enzyme (Test Groups T3 and T4). Results of bird weight analysis are presented in TABLE III and feed/gain calculations are presented in TABLE IV.

TABLE III

Average Body Weight (Grams) at 21 Days

Rep	Treatment T1	T2	T3	T4
1	323.00	341.67	377.83	366.67
2	326.67	321.33	383.83	361.17
3	299.33	337.33	378.83	361.33
4	211.67	254.00	374.33	403.40
Mean	290.17	313.58	378.71	373.14
STAT	b	b	a	a
S.D.	46.52	35.22	3.40	17.61
C.V.	16.03	11.23	0.90	4.72

TABLE IV

Average Feed Conversion (0-21 days)
Corrected for Weight of Mortality Birds

Rep	Treatment T1	T2	T3	T4
1	1.486	1.569	1.407	1.445
2	1.562	1.532	1.423	1.421
3	1.524	1.562	1.432	1.406
4	1.760	1.462	1.438	1.404
Mean	1.583	1.531	1.425	1.419
STAT	b	b	a	a
S.D.	0.11	0.04	0.01	0.02
C.V.	6.68	2.78	0.82	1.17

In both of the foregoing tables, means in a row without a common letter are significantly different ($P<0.05$), per Duncan's test for significance.

EXAMPLE 7. Cloning the *B. cereus* PI-PLC gene and expressing it in *B. megaterium*

The gene coding for phosphatidylinositol specific phospholipase C (PI-PLC) has been sequenced (Kuppe, A., *et al., J. Bacteriol.* 171:6077-6083,1989). Therefore, PCR technology was used to clone it from *Bacillus cereus* (ATCC 6464) chromosomal DNA. An expression vector, pMBGA (BIO 101, Vista, CA), for *Bacillus megaterium* was used. The PCR primers (primer-1: 5'-GACTAGTAATAAGAAGTTAATTTG and primer-2: 5'-

CGGGATCCATATTGTTGGTTATTGG) were designed with a *Spe*I site in primer-1 and a *Bam*HI site in primer-2.

The PCR-amplified PI-PLC gene was ligated into the pMEGA *Spe*I-*Bam*HI site and yielded a plasmid pCG682. PI-PLC protein was fused with the first three amino acids of *xylA* gene product through using the *Spe*I site in the expression vector. The expression of the PI-PLC gene was under the regulation of *xylA* promoter. The shake flask fermentation was used to evaluate the phosphatidylinositol specific phospholipase C production in *Bacillus megaterium*. LB broth with 10 μg/ml tetracycline (20 m*L*) was inoculated with 0.2 ml of seed culture and incubated in a 37°C shaker at 250 rpm. At about 0.5 O.D. 600nm, 5 g/*L* of D-(+) xylose was added to induce the *xylA* promoter. After three hours, supernatant was harvested by centrifugation. The phosphatidylinositol specific phospholipase C activity was measured by fluorescent substrate method (Hendrickson, H.S., *et al.*, *Biochemistry* 31: 12169-12172, 1992; Hendrickson, H.S., *Analytical Biochemistry* 219: 1-8, 1994), using 1-pyrenebutyl *myo*-inositol-1-phosphate substrate (Molecular Probes, Eugene, OR) and HPLC detection.

TABLE 5

Measurement of PI-PLC Expression		
Test Material	Xylose addition	Specific activity (unit/mg of protein)
Cell Lysate Fractions		
B. megaterium/pCG682	-	0
B. megaterium/pCG682	+	0.436
B. megaterium/pCG682	-	0
B. megaterium/pCG682	+	0.365
Fermentation Broth Fractions		
B. megaterium/pCG682	-	0
B. megaterium/pCG682	+	4.087
B. megaterium/pCG682	-	0
B. megaterium/pCG682	+	4.56

These data show that most of the PI-PLC is extracellular and that expression occurs only after D-(+)-xylose addition. This recombinant strain is estimated to have at least 15 times the productivity (mg/L/OD) of the average wild strain, as grown in EXAMPLE 2.

<u>EXAMPLE 8</u>. *Bacillus cereus* fermentation broth did not contain antibiotic activity

Fermentation with *Bacillus cereus* (ATCC7004) was conducted according to the method described in EXAMPLE 2 except that the initial volume was increased to 20 liters. A test for the presence of antibiotic was conducted with *E. coli* MG1655 using final fermentation broth or partially purified PI-PLC prepared according to the method described in EXAMPLES 3-5.

While certain representative embodiments and details have been shown for the purpose of illustrating the invention, it will be apparent to those skilled in the art that various changes and modifications may be made therein without departing from the scope of the invention.

WHAT WE CLAIM IS:

1. A composition comprising (i) enzyme that cleaves a linkage that effects release of a cell-surface protein or carbohydrate and (ii) a physiologically acceptable carrier for said enzyme, wherein said composition is in a form suitable for oral administration.

2. A composition according to claim 1, wherein said enzyme is selected from the group consisting of sphingomyelinases and phospholipases.

3. A composition according to claim 2, wherein said enzyme is a type C or type D phospholipase.

4. A composition according to claim 3, wherein said enzyme is phosphatidylinositol-specific phospholipase C.

5. A composition according to claim 1, wherein said enzyme is selected from the group consisting of esterases, cerebrosideases, and carbohydrases that cleave a linkage that effects release of a cell-surface protein or carbohydrate.

6. A composition according to any of claims 1 – 5, wherein said carrier is a foodstuff into which said enzyme is incorporated.

7. A composition according to claim 6, wherein said foodstuff is an animal feed comprised of grain material, a source of protein, vitamins, amino acids, and minerals.

8. A composition according to claim 7, wherein said grain material is corn, sorghum, wheat, barley, or oats.

9. A composition according to Claim 7, wherein the source of protein is beans or peas.

10. A composition according to any of claims 1 – 5, wherein said composition is in a solid dosage form.

11. A composition according to claim 1 - 5, wherein said enzyme is contained in a gelatin capsule shell.

12. A composition according to any of claims 1 –5, wherein said composition is in the form of syrup or a liquid emulsion.

13. A method of treating or ameliorating the risk of a digestive tract infection, comprising orally administering, to a subject suffering from or at risk for suffering said infection, an effective amount of enzyme that cleaves a linkage that effects release of a cell-surface protein or carbohydrate.

14. A method according to claim 13, wherein said infection is caused by a protozoan, bacterial, yeast, fungal, or viral pathogen.

15. A method according to claim 14, wherein said infection is caused by a protozoan pathogen of the genus *Eimeria*.

16. A method according to claim 14, wherein said infection is caused by a bacterial pathogen of the genus *Clostridium*.

17. A method according to claim 13, wherein said infection is a parasitic infection.

18. A method according to claim 13, comprising administering orally, to said subject, an extracellular enzyme preparation from a *Bacillus cereus* strain.

19. A method according to claim 18, wherein said *Bacillus cereus* strain is ATCC 7004 or ATCC6464.

ABSTRACT OF THE DISCLOSURE

Enzymes of a particular class, characterized by the ability to cleave a linkage that effects release of a cell-surface protein or carbohydrate, display significant antibiotic activity, upon oral administration, which is effective, for example, in the treatment of digestive tract infections in humans and in animals. In the latter, there are benefits of significantly improved growth rate, feed efficiency, and overall health.