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(54) **METHODS AND REAGENTS FOR
DETECTING ENDOTOXIN**

Related U.S. Application Data

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(57) **ABSTRACT**

(21) Appl. No.: **10/183,992**

A reagent containing a purified horseshoe crab Factor C,
particularly a recombinantly produced Factor C, and a
surfactant can be used in a sensitive, rapid, and reproducible
assay to detect endotoxin.

(22) Filed: **Jun. 28, 2002**

FIG. 1.

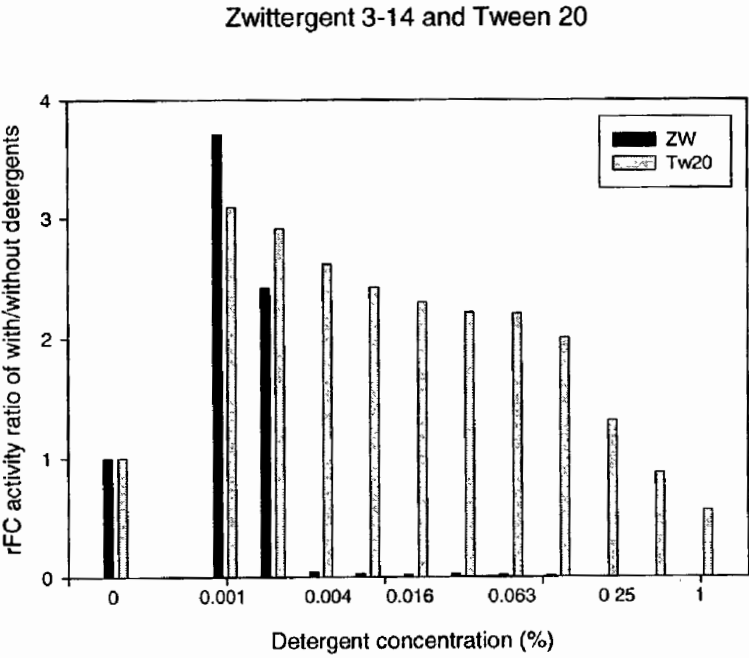


FIG. 2.

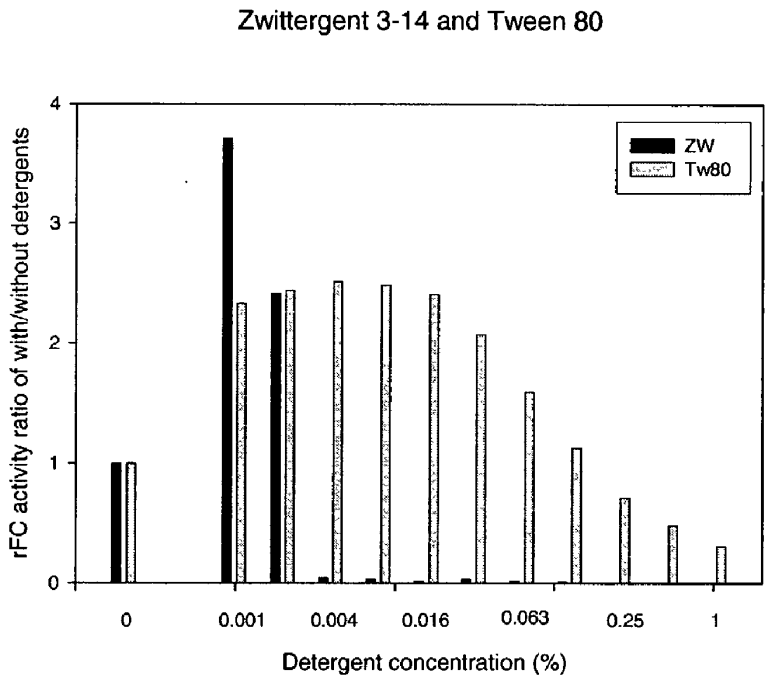


FIG. 3.

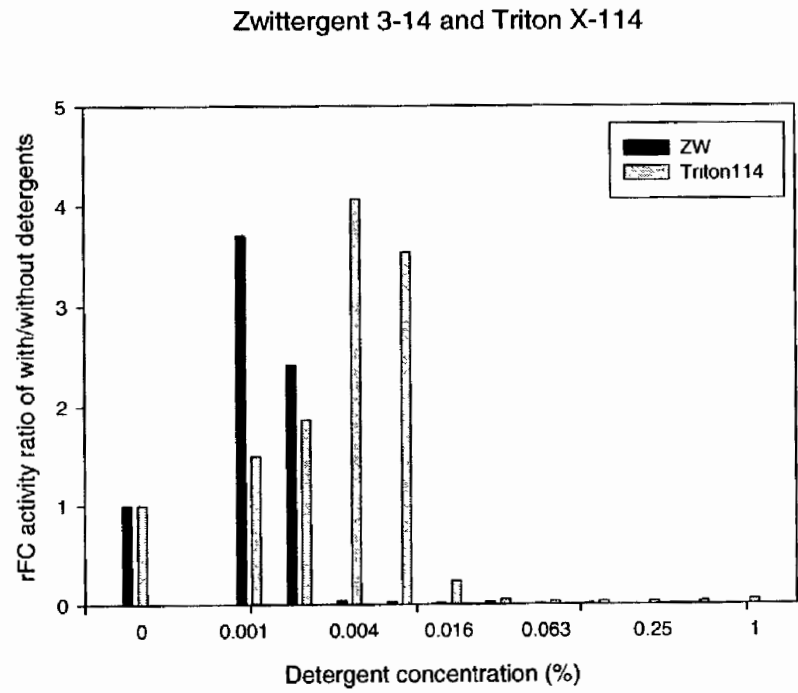


FIG. 4.

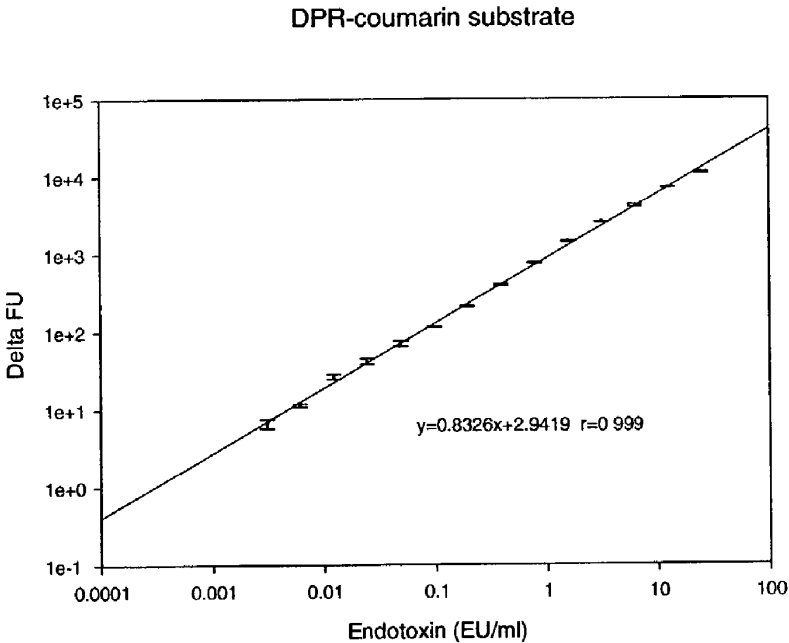


FIG. 5.

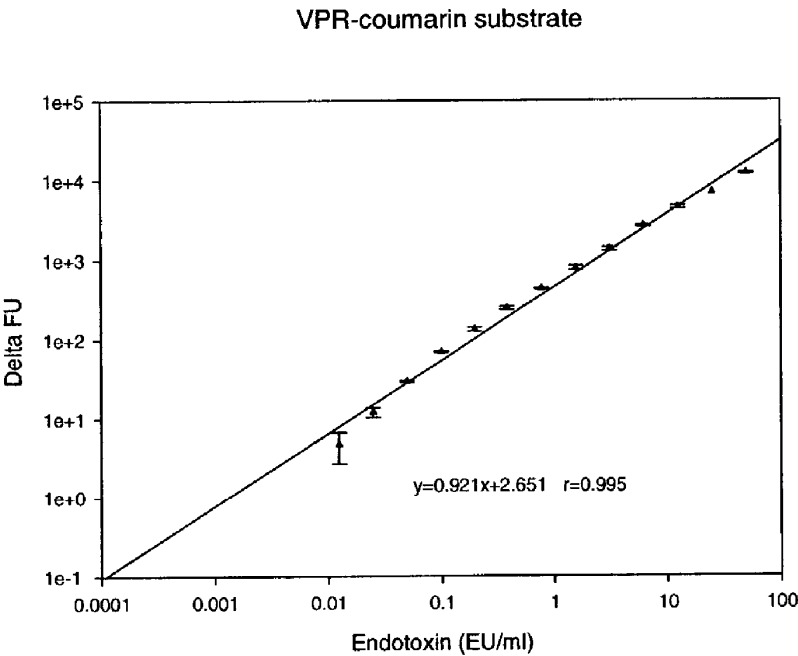


FIG. 6

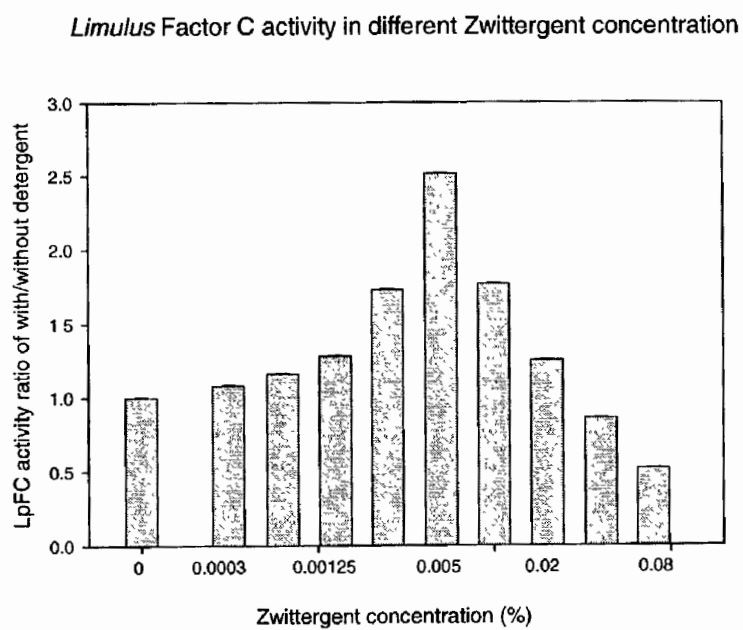


FIG. 7

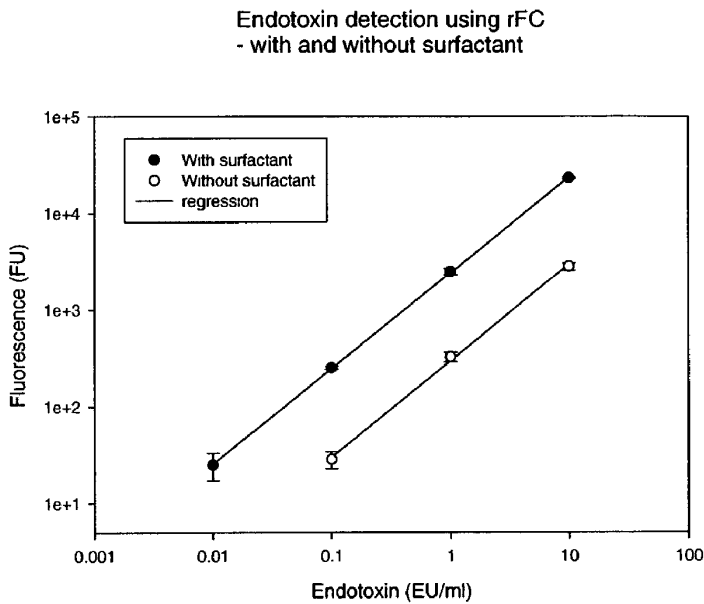


FIG. 8

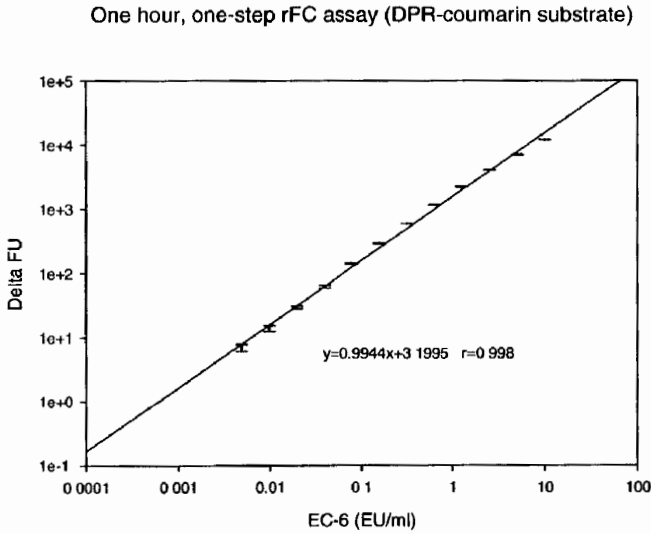


FIG. 9

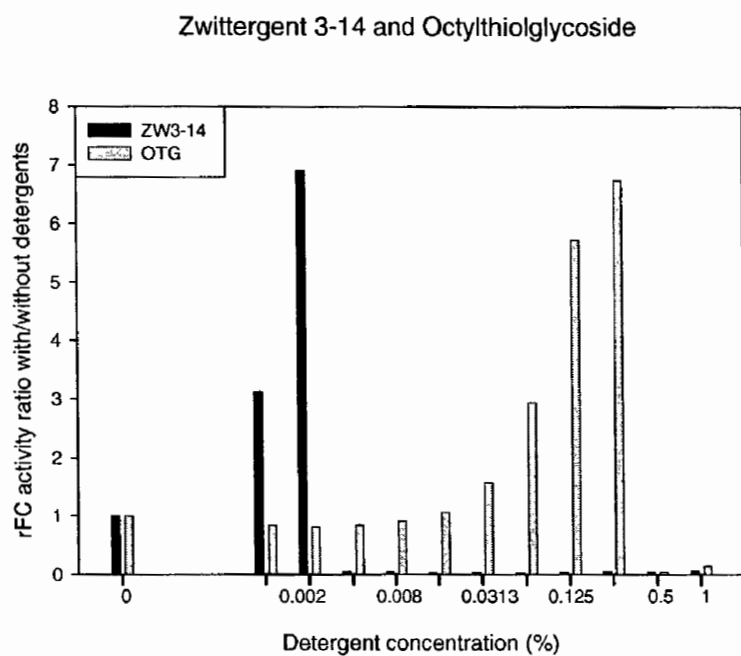


FIG. 10

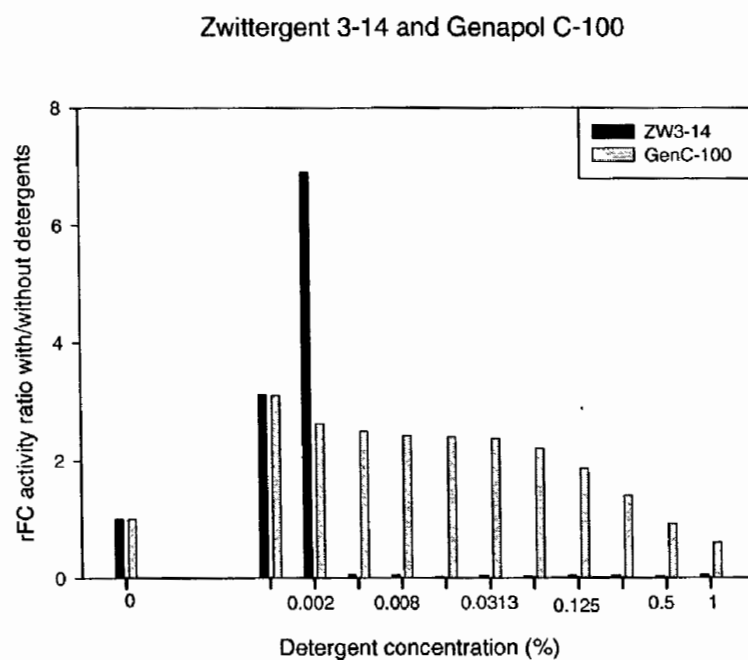
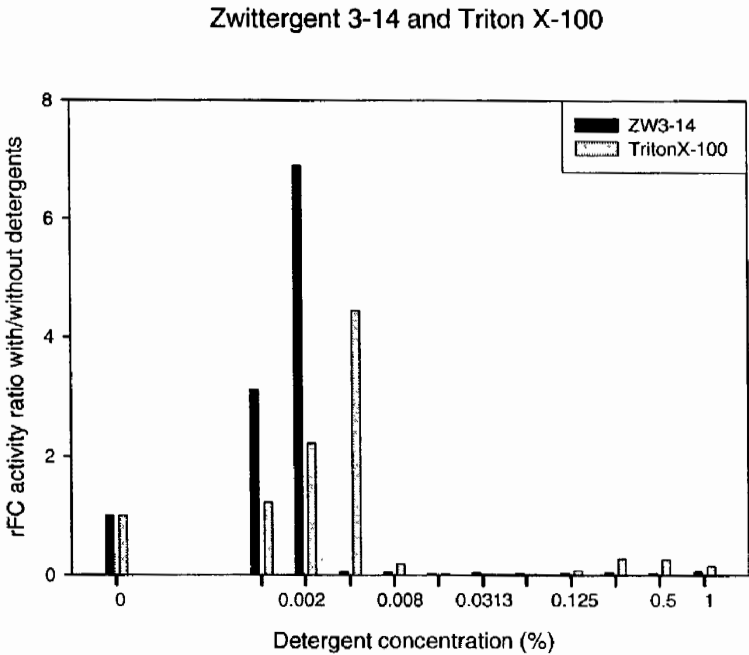


FIG. 11



METHODS AND REAGENTS FOR DETECTING ENDOTOXIN

[0001] This application claims the benefit of and incorporates by reference co-pending provisional application Serial No. 60/310,125 filed Jun. 28, 2001.

FIELD OF THE INVENTION

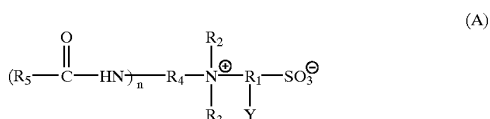
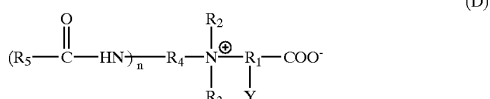
[0002] The invention relates to reagents and methods for detecting endotoxin.

BACKGROUND OF THE INVENTION

[0003] Gram negative bacterial endotoxin is a widespread contaminant of a variety of materials, such as water, food, pharmaceutical products, and parenteral preparations. The most commonly used tests for endotoxin contamination employ amebocyte lysates derived from horseshoe crab hemolymph. As populations of these animals decreases, however, it becomes increasingly important to develop rapid and reliable methods for detecting endotoxin that do not rely on the availability of horseshoe crab hemolymph.

BRIEF SUMMARY OF THE INVENTION

[0004] The invention provides reagents and methods for detecting endotoxin. One embodiment of the invention is a reagent for detecting endotoxin, comprising a purified horseshoe crab Factor C protein and a surfactant. The surfactant can be an amphoteric surfactant represented by the following formula:

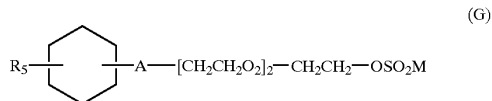


[0005] wherein R₁ is an alkylene radical having from 1 to 4 carbon atoms; Y and Y' are each (1) hydrogen, (2) lower alkyl, or (3) hydroxy lower alkyl; R₂ and R₃ are each (1) lower alkyl or (2) hydroxy lower alkyl; n is 0 or 1, when n is 0, R₄ is alkyl containing from about 8 to about 18 carbon atoms; when n is 1, R₄ is an alkylene radical having from 1 to about 6 carbon atoms; R₅ is an alkyl containing from about 8 to about 18 carbon atoms; and M is hydrogen, sodium, potassium or ammonium. The surfactant can be an anionic surfactant represented by the following formulae:



[0006] wherein R₅, Y, and M have the same meaning as set forth above; R₆ is an alkyl from 8 to 24 carbon atoms; n₁ is

an integer from 1 to 3; n_2 is 1 or 2; and Ar is phenyl or naphthyl. The surfactant can be a cationic surfactant represented by the following formula:



[0007] wherein R₅, Y, and Y' have the same meaning as set forth above. The surfactant can be a nonionic surfactant represented by the following formula:



[0008] wherein R_5 , Y, and Y' have the same meaning as set forth above and R_7 and R_8 are each methyl or ethyl. The surfactant can be those nonionic surfactants selected from the group consisting of the condensation product of about 10 to 30 moles of ethylene oxide with the monoester of a hexahydric alcohol containing 6 carbon atoms with the ester group containing 10 to 20 carbon atoms.

[0009] Even another embodiment of the invention is a method of detecting endotoxin in a test sample. A test sample is contacted with (1) a reagent comprising (a) a purified horseshoe crab Factor C protein and (b) a surfactant to form a test sample-reagent mixture and (2) a Factor C substrate to form a contacted test sample. Cleavage of the Factor C substrate generates a detectable signal. The contacted test sample is assayed for the presence or absence of the detectable signal. An amount of the detectable signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

[0010] A further embodiment of the invention is a method of detecting endotoxin in a test sample. A test sample is contacted with (1) a reagent comprising (a) a recombinant *Carcinoscorpius rotundicauda* Factor C protein and (b) a surfactant and (2) N-t-BOC-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin to form a contacted test sample. The Factor C protein is made by the method of culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant. The contacted test sample is assayed for the presence or absence of a fluorescent signal. An amount of the detectable signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

[0011] Another embodiment of the invention is a method of detecting endotoxin in a test sample. A test sample is contacted with a reagent comprising a purified horseshoe crab Factor C protein and a surfactant as described above to form a test sample-reagent mixture. The test sample-reagent mixture is contacted with a Factor C substrate, wherein cleavage of the Factor C substrate generates a detectable signal. The contacted test sample-reagent mixture is assayed for the presence or absence of the detectable signal. An amount of the detectable signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

[0012] Even another embodiment of the invention is a method of detecting endotoxin in a test sample. A test

sample is contacted with a reagent comprising a recombinant *Carcinoscorpius rotundicauda* Factor C protein and a surfactant. The Factor C protein is made by the method of culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant. The test sample-reagent mixture is contacted with N-t-BOC-Asp(OBzl)-Pro-Arg-7-Amido-4-methyl coumarin. The contacted test sample-reagent mixture is assayed for the presence or absence of a fluorescent signal. An amount of the fluorescent signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

[0013] Yet another embodiment of the invention is a kit for detecting endotoxin. The kit comprises a reagent that comprises (a) a purified horseshoe crab Factor C protein and (b) a surfactant as described above, together with instructions for a method of detecting endotoxin in a test sample. The method comprises the steps of (1) contacting a test sample with a reagent comprising (a) a purified horseshoe crab Factor C protein and (b) a surfactant as described above to form a test sample-reagent mixture; (2) contacting the test sample-reagent mixture with a Factor C substrate, wherein cleavage of the Factor C substrate generates a detectable signal; and (3) assaying the contacted test sample-reagent mixture for the presence or absence of the detectable signal, wherein an amount of the detectable signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

BRIEF DESCRIPTION OF THE FIGURES

[0014] FIG. 1. Graph showing effect of Zwittergent 3-14 and Tween 20 on recombinant Factor C activity.

[0015] FIG. 2. Graph showing effect of Zwittergent 3-14 and Tween 80 on recombinant Factor C activity.

[0016] FIG. 3. Graph showing effect of Zwittergent 3-14 and Triton X-1 14 on recombinant Factor C activity.

[0017] FIG. 4. Plot showing endotoxin detection using a DPR (N-t-BOC-Asp(OBzl)-Pro-Arg-7-Amido-4-methyl)-coumarin substrate.

[0018] FIG. 5. Plot showing endotoxin detection using a VPR (N-t-BOC-Val-Pro-Arg-7-Amido-4-methyl)-coumarin substrate.

[0019] FIG. 6. Graph showing Limulus Factor C activity at different Zwittergent concentrations.

[0020] FIG. 7. Graph showing endotoxin sensitivity in the presence and absence of surfactant.

[0021] FIG. 8. Graph showing results of a one hour, one-step endotoxin assay, using DPR-coumarin as a substrate.

[0022] FIG. 9. Graph showing effect of Zwittergent 3-14 and octylthiolglycoside on recombinant Factor C activity.

[0023] FIG. 10. Graph showing effect of Zwittergent 3-14 and Genapol C-100 on recombinant Factor C activity.

[0024] FIG. 11. Graph showing effect of Zwittergent 3-14 and TX-100 on recombinant Factor C activity.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The invention is a reagent for detecting endotoxin and a method of using the reagent. The reagent comprises a

purified horseshoe crab Factor C protein and a surfactant. This reagent can be used in conjunction with a substrate for Factor C that, upon cleavage, generates a detectable signal to detect endotoxin in a test sample. The presence of the surfactant enhances the activation of the purified Factor C by endotoxin by as much as 3-7-fold, permitting more rapid and sensitive measurement of endotoxin levels in a test sample. The reagent preferably contains a recombinant Factor C, thus eliminating the need for a continuous supply of horseshoe crab hemolymph.

[0026] Purified Factor C

[0027] One component of the reagent of this invention comprises a purified horseshoe crab Factor C protein. Purified native Factor C from any of the four known horseshoe crab species, *Limulus polyphemus*, *Carcinoscorpius rotundicauda*, *Tachypleudus tridentata*, or *Tachypleudus gigas*, can be used in the practice of the invention. The native Factor C can be purified biochemically or purified Factor C can be produced recombinantly.

[0028] "Purified Factor C" as used herein means a composition of a Factor C protein as defined hereinafter that contains less than 30% by weight of non-Factor C native amebocyte lysate components from any of the four known horseshoe crab species. The composition specifically includes a cell culture supernatant which comprises recombinant Factor C protein (see below). Methods for purifying horseshoe crab Factor C from its native source are known and are disclosed, for example, in Nakamura et al., *Eur. J. Biochem.* 154, 511-21, 1986; Navas et al., *Biochem. Intl.* 21, 805-13, 1990; Tokunaga et al., *J. Biochem.* 109, 150-157, 1991; U.S. Pat. No. 5,985,590, and U.S. Pat. No. 5,716,834. Any of these methods or their equivalents can be used to obtain a purified Factor C. See also Example 11. Purity of a Factor C preparation can be assessed by any means known in the art, such as SDS gel electrophoresis.

[0029] Preferably, the purified Factor C is recombinantly produced. Amino acid sequences for native Factor C from *Tachypleus tridentata* and *Carcinoscorpius rotundicauda* are known, as are the naturally occurring coding sequences for these proteins. SEQ ID NOS:1 and 3 provide coding sequences for the *Tachypleus tridentata* Factor C amino acid sequences shown in SEQ ID NOS:2 and 4, respectively. SEQ ID NOS:5 and 7 provide coding sequences for the *Carcinoscorpius rotundicauda* Factor C amino acid sequences shown in SEQ ID NOS:6 and 8, respectively. Because of the degeneracy of the genetic code, many other sequences can be envisioned that will encode each of these native Factor C proteins, and the invention specifically contemplates use of any of these coding sequences to produce a purified Factor C.

[0030] The invention also encompasses use of purified naturally and non-naturally occurring, i.e., recombinantly produced, Factor C variants, provided that the variants retain a Factor C enzyme activity. Factor C enzyme activity can be assessed using any assay for Factor C enzyme activity known in the art. See, e.g., Tokunaga et al., *J. Biochem.* 109,150-57 and Nakamura et al., *Eur. J. Biochem.* 176,89-94,1988. The endotoxin assays described in Examples 1 and 13, below, also can be used. Naturally occurring Factor C variants include, for example, products of Factor C mRNA splice variants or mutated Factor C genes.

[0031] Non-naturally occurring Factor C variants can be constructed using base substitutions, additions, or deletions

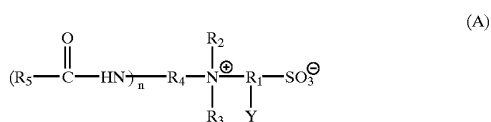
to produce proteins having Factor C activity. Non-naturally occurring Factor C variants can differ from naturally occurring Factor C by as much as 50, 75, 80, 85, 90, 95, 97, 98, or 99%, as determined using the Blast2 alignment program (Blosum62, Expect 10, standard genetic codes). Fragments of native and recombinantly produced Factor C that retain Factor C activity also can be used in the practice of the invention. Thus, "Factor C" as used herein includes naturally and non-naturally occurring proteins and protein fragments that have the properties described above.

[0032] Methods of producing proteins recombinantly are well known in the art and generally involve culturing a host cell comprising an expression vector encoding the Factor C protein in a supernatant under conditions such that the protein is expressed. The expressed protein can be recovered or, preferably, the supernatant comprising the expressed protein is used directly as the source of recombinant Factor C. Thus, "purified Factor C" specifically includes a supernatant which comprises recombinant Factor C. Host cells useful for the production of recombinant Factor C include, without limitation, yeast cells and insect cells. Recombinant production of *Carcinoscorpius rotundicauda* Factor C in *Pichia pastoris* and *Saccharomyces cerevisiae* host cells is specifically disclosed in U.S. Pat. No. 5,985,590.

[0033] A particularly preferred method of obtaining recombinant Factor C is to produce the protein in a baculovirus system, as described in Examples 2-5. Briefly, a Factor C coding sequence is cloned into pFASTBAC1. The resultant recombinant plasmid is transformed into DH10BAC competent cells that contain a bacmid with a mini-attTn7 target site and a helper plasmid. In the presence of transposition proteins provided by the helper plasmid, the mini-Tn7 transposable element on the pFASTBAC plasmid can transpose to the mini-attTn7 target site on the bacmid. Colonies containing recombinant bacmids are identified by disruption of the lacZa gene. High molecular weight DNA is prepared from selected DH10BAC clones containing the recombinant bacmid. This DNA is then used to transfect Sf9 insect cells, which will then secrete recombinant Factor C. A particularly surprising discovery of the present invention is that culture medium containing the secreted Factor C, which has been separated from the cultured cells, can be used together with a surfactant (described below) as a reagent in endotoxin assays without further purification.

[0034] Surfactant

[0035] Reagents of the invention also comprise a surfactant. Surfactants useful in the practice of the invention include those described in U.S. Pat. No. 4,322,217, although other surfactants also can be used. Useful surfactants include amphoteric surfactants that contain both an anionic and cationic group in their structure. Illustrative are the sulfobetaines represented by Formula A:



[0036] R_1 is an alkylene radical having from 1 to about 4 carbon atoms,

[0037] Y is any non-deleterious, chemically suitable substituent including (1) hydrogen, (2) substituted or unsubstituted lower alkyl, e.g., containing 1 to 4 carbon atoms such as methyl, ethyl, propyl, or hydroxy etc.;

[0038] R_2 and R_3 are each selected from substituted or unsubstituted lower alkyl containing 1 to 4 carbon atoms, e.g., such as methyl, ethyl, propyl, hydroxy ethyl, hydroxy methyl, hydroxy propyl, etc.

[0039] $n=0$ or 1,

[0040] when $n=0$, R_4 is substituted or unsubstituted alkyl, e.g., containing about 8 to about 18 carbon atoms, and

[0041] when $n=1$, R_4 is an alkylene radical having from about 1 to about 6 carbon atoms, R_5 is a substituted or unsubstituted alkyl, e.g., containing about 8 to about 18 carbon atoms.

[0042] The term "alkylene" encompasses both polymethylene radicals and other divalent saturated aliphatic radicals. Thus, there may be branching in the linkage provided by the alkylene radical. The term "lower" means a radical containing 1 to 4 carbon atoms.

[0043] Sulfobetaines that can be used in the reagent of the present invention are known in the art and have been described as zwitterionic surfactants. The preparation of such compounds is described, for example, in Fernley, *J. Am. Oil Chem. Soc.* 55, 98-103 (1978) and U.S. Pat. No. 3,280,179. In preferred sulfobetaine surfactants, R_2 and R_3 in the above structure are methyl. It is also preferred that R_1 be propylene.

[0044] One type of useful sulfobetaine surfactant has the above structure wherein n equals 0 and R_4 is an alkyl radical having from about 8 to 18 carbon atoms, preferably a straight chain alkyl radical. For these sulfobetaine surfactants, a convenient source of the R_4 component is tallow fatty alcohol, which consists of a mixture of various chain lengths, with a typical composition being approximately 66 percent C_{18} , 30 percent C_{16} , and 4 percent C_{14} and others. Another convenient source is the middle cut of distilled coconut fatty alcohol, which also consists of a mixture of various chain lengths, with a typical composition being approximately 66 percent C_{12} , 23 percent C_{14} , 9 percent C_{16} and 2 percent C_{10} .

[0045] Specific sulfobetaine surfactants of the above structure wherein n equals 0 are set forth in U.S. Pat. No. 3,539,521. A particularly preferred surfactant of this type is N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate, which is commercially available from Calbiochem-Behring Corporation under the trademark ZWITTER-GEN 3-14.

[0046] Another type of useful sulfobetaine surfactant has the above structure wherein n equals 1 and R_4 is an alkylene radical having from about 1 to about 6 carbon atoms. In these sulfobetaines wherein n equals 1, R_5 is an alkyl radical having from about 8 to about 18 carbon atoms. It is preferred that R_5 be straight chain. As previously discussed, convenient sources of alkyl radicals having from about 10 to about 18 carbon atoms are tallow fatty alcohol and coconut fatty alcohol. Specific sulfobetaine surfactants of the above structure wherein n equals 1 are set forth in U.S. Pat. No. 3,280,179.

[0047] Particularly preferred sulfobetaine surfactants are 3-(N,N-dimethyl-N-acylamidopropylammonio)-2-hydroxypropane-1-sulfonates, wherein the acyl group is derived from tallow fatty alcohol or coconut fatty alcohol, with coconut fatty alcohol preferred. It will be recognized by those skilled in the art that, in the normal preparation of these derivatives of tallow or coconut fatty alcohols, a mixture of sulfobetaines with varying carbon chain lengths for the acyl groups will result. As previously discussed, these fatty alcohols contain, for the most part, carbon chain lengths that will provide acyl groups with the desired number of carbon atoms, i.e., from about 8 to about 18 carbon atoms. Thus, these mixtures obtained from tallow or coconut fatty alcohols are useful in providing the sulfobetaine surfactant for reagents of the present invention.

[0048] A material of this type particularly preferred for use in reagents of the present invention is N-cocoamido-propyl-N,N-dimethyl-N-2-hydroxypropyl sulfobetaine. An example of this is LONZAINE CS, which is commercially available from Lonza, Inc., Fair Lawn, N.J.

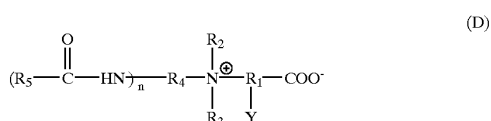
[0049] Other amphoteric surfactants include the N-long chain alkyl aminocarboxylic acids illustrated by Formula B:



[0050] N-long chain alkyl iminodicarboxylic acids illustrated by Formula C:



[0051] and N-long chain alkyl or amido betaines illustrated by Formula D:



[0052] where R_1 , R_2 , R_3 , R_4 , Y , and n have the same meaning as they have in Formula A, M is hydrogen or a salt-forming metal, and Y' has the same meaning as Y in Formula A. Y and Y' may be the same or different. Examples of specific amphoteric detergents are N-alkyl-beta-aminopropionic acid, N-alkyl-beta-iminodipropionic acid, and N-alkyl-N,N-dimethyl glycine; the alkyl group may be, for example, that derived from coco fatty alcohol, lauryl alcohol, myristyl alcohol (or a lauryl-myristyl mixture), hydrogenated tallow alcohol, cetyl, stearyl, or blends of such alcohols. The substituted aminopropionic and iminodipropionic acids are often supplied in the sodium or other salt forms, which also can be used in reagents of the invention.

[0053] Specific examples include cocobetaine sold by Witco Chemical Corporation under the name EMCOL CC 37-18, cocoamidopropyl betaine sold by Lonza Inc. under the name LONZAINE CO, and disodium N-tallow-beta-iminodipropionate sold by Henkel Corporation under the name of DERIPHAT 160.

[0054] Examples of other amphoteric detergents are the fatty imidazolines such as those made by reacting a long chain fatty acid (e.g., of 10 to 20 carbon atoms) with diethylene triamine and monohalocarboxylic acids having 2 to 6 carbon atoms, e.g. 1-coco-5-hydroxyethyl-5-carboxymethylimidazoline. Specific examples include cocoimidazoline, which is commercially available under the name AMPHOTERGE K-2 from Lonza, Inc., and capric dicarboxy imidazoline, which is commercially available under the name AMPHOTERGE KJ-2 from Lonza, Inc.

[0055] Other examples of useful surfactants include anionic synthetic surfactants, generally described as those compounds that contain hydrophilic and lipophilic groups in their molecular structure and ionize in an aqueous medium to give anions containing both the lipophilic group and hydrophilic group. The alkyl aryl sulfonates, the alkane sulfates, and sulfated oxyethylated alkyl phenols are illustrative of the anionic type of surface active compounds.

[0056] The alkyl aryl sulfonates are a class of synthetic anionic surface active agents represented by Formula E:



[0057] In Formula E, R_6 is a straight or branched chain hydrocarbon radical having from about 1 to about 24 carbon atoms, at least one R_6 having at least 8 carbon atoms; $n1$ is from 1 to 3; $n2$ is from 1 to 2; Ar is a phenyl or a naphthyl radical, and Y and M have the same meaning as in Formula B. R_6 can be, for example, methyl, ethyl, hexyl, octyl, tetraoctyl, iso-octyl, nonyl, decyl, dodecyl, octadecyl, and the like.

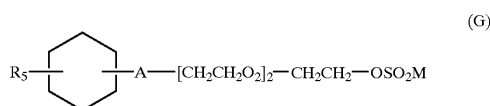
[0058] Compounds illustrative of the alkyl aryl sulfonates include sodium dodecylbenzene sulfonate, sodium decylbenzene sulfonate, ammonium methyl dodecylbenzene sulfonate, ammonium dodecylbenzene sulfonate, sodium octadecylbenzene sulfonate, sodium nonylbenzene sulfonate, sodium dodecyl-naphthalene sulfonate, sodium hexadecylbenzene sulfonate, potassium eicososyl naphthalene sulfonate, ethylamine undecylnaphthalene sulfonate and sodium docosylnaphthalene sulfonate.

[0059] The alkyl sulfates are a class of synthetic anionic surface active agents represented by Formula F:



[0060] where R_5 and M have the same meaning as in Formula B. Compounds illustrative of alkyl sulfate class of anionic surfactants include sodium octadecyl sulfate, sodium hexadecyl sulfate, sodium dodecyl sulfate, sodium nonyl sulfate, ammonium decyl sulfate, potassium tetradecyl sulfate, ammonium octyl sulfate, triethanolamine octadecyl sulfate, and ammonium nonyl sulfate.

[0061] The sulfated oxyethylated alkylphenols are a class of synthetic anionic surface active agents represented by Formula G:



[0062] where A is either oxygen, sulfur, a carbonamide group, a thiocarbonamide group, a carboxylic group, or a thiocarboxylic ester group, z is an integer from 3 to 8, and

R₅ and M have the same meaning as in Formula B. Compounds illustrative of the sulfated oxyethylated alkyl phenol class of anionic surfactants include ammonium nonylphenoxyl tetraethyleneoxy sulfate, sodium dodecylphenoxyl triethyleneoxy sulfate, ethanolamine decylphenoxyl tetraethyleneoxy sulfate, and potassium octylphenoxyl triethyleneoxy sulfate.

[0063] Other examples of useful surfactants include non-ionic surface active compounds, which can be broadly described as compounds that do not ionize but acquire hydrophilic characteristics from an oxygenated side chain, such as polyoxyethylene; the lipophilic part of the molecule may come from fatty acids, phenol, alcohols, amides, or amines. The compounds are usually made by reacting an alkylene oxide, such as ethylene oxide, butylene oxide, propylene oxide and the like, with fatty acids, straight or branched chain alcohols containing one or more hydroxyl groups, phenols, thiophenols, amides, or amines to form polyoxyalkylene glycoethers and esters, polyoxyalkylene alkylphenols, polyoxyalkylene thiophenols, polyoxyalkylene amides and the like. It is generally preferred to react from about 3 to about 30, more preferably 10 to 30, moles of alkylene oxide per mole of the fatty acids, alcohols, phenols, thiophenols, amides, or amines.

[0064] Illustrative of these nonionic surfactants are the products obtained from the reaction of alkylene oxide with an aliphatic alcohol having from 8 to 18 carbon atoms, such as octyl, nonyl, decyl, octadecyl, dodecyl, tetradecyl and the like, with monoesters of hexahydric alcohols, the ester group containing 10 to 20 carbon atoms such as sorbitan mono-laureate, sorbitan monooleate and sorbitan monopalmitate, with an alkyl phenol in which the alkyl group contains between 4 and 20 carbon atoms, such as butyl, dibutyl, amyl, octyl, dodecyl, tetradecyl, and the like, or with an alkyl amine in which the alkyl group contains between 1 to 8 carbon atoms.

[0065] Compounds illustrative of synthetic nonionic surfactants include the products obtained from condensing ethylene oxide or propylene oxide with the following: propylene glycol, ethylene diamine, diethylene glycol, dodecyl phenol, nonyl phenol, tetradecyl alcohol, N-octadecyl diethanolamide, N-dodecyl monoethanolamide, polyoxyethylene (20) sorbitan monooleate sold under the name TWEEN 80 and polyoxyethylene (20) sorbitan monolaurate sold under the name TWEEN 20.

[0066] Other nonionic surfactants include long chain tertiary amine oxides corresponding to Formula H:



[0067] wherein R₅ has the same meaning as in Formula A, and R₇ and R₈ are each methyl or ethyl radicals. The arrow in the formula is a conventional representation of a semi-polar bond. Examples of amine oxides suitable for use in this invention include dimethyldodecylamine oxide, dimethyldecylamine oxide, dimethyldodecylamine oxide, dimethyltridecylamine oxide, and dimethylhexadecylamine oxide.

[0068] Cationic surface active agents can also be used as surfactants. Such agents are those surface-active compounds which contain an organic hydrophobic group and a cationic solubilizing group. Typical cationic solubilizing groups are amine and quaternary groups. Such cationic surface-active agents are represented by Formula I:



[0069] wherein R₅, Y, and Y' have the same meaning as in Formula C.

[0070] Other examples of suitable synthetic cationic surfactants include the diamines such as those of Formula J:



[0071] wherein R is an alkyl group of about 12 to 22 carbon atoms, such as N-2-aminoethyl stearyl amine and N-2-aminoethyl myristyl amine; amide-linked amines such as those of Formula K:



[0072] such as N-2-amino ethylstearyl amide and N-amino ethyl myristyl amide; quaternary ammonium compounds wherein typically one of the groups linked to the nitrogen atom are alkyl groups which contain 1 to 3 carbon atoms, including such 1 to 3 carbon alkyl groups bearing inert substituents, such as phenyl groups and there is present an anion such as halogen, acetate, methylsulfate, etc. Typical quaternary ammonium compounds are ethyl-dimethyl-stearyl ammonium chloride, benzyl-dimethyl-stearyl ammonium chloride, benzyl-dimethyl-stearyl ammonium chloride, trimethyl stearyl ammonium chloride, trimethylcetyl ammonium bromide, dimethylethyl dilaurylammonium chloride, dimethyl-propyl-myristyl ammonium chloride, and the corresponding methosulfates and acetates.

[0073] Another suitable cationic surfactant is represented by Formula L:



[0074] wherein R₅ has the same meaning as in Formula A and each a is an integer from 1 to 15. An example is the polyethylene glycol amine of hydrogenated tallow wherein R₅ represents the tallow radical and a+a has an average value of 5.

[0075] Other useful surfactants for use in the Factor C-surfactant reagent include Triton X-100, Triton X-114, octyl-beta-D-thioglucoiside (OTG, Amresco #J575), Genapol C-100 (an alkyl polyoxyethylene C12E10; Calbiochem #345794), Tween 20, and Tween 80.

[0076] Factor C-Surfactant Reagent

[0077] To form a reagent of the invention, purified Factor C and a surfactant are combined in an aqueous solution. A suitable buffer solution for this purpose contains 30 mM Tris, pH 8.0, 30 mM NaCl, and 0.3% lactose. The concentration of purified Factor C in a reagent of the invention

preferably ranges from 0.03-3 $\mu\text{g/ml}$. The concentration of surfactant in the reagent of the invention preferably ranges from 0.001-0.003%. Optimum concentrations of purified Factor C and of the surfactant will vary, depending, for example, on the purity of the Factor C and the particular surfactant. Optimum concentrations of purified Factor C and of surfactant can be determined using routine testing. In the assay exemplified in Examples 1 and 13, the optimal concentration of the surfactant ZWITTERGENT 3-14 is 0.0025%.

[0078] Endotoxin Assay

[0079] The reagent described above can be used in an assay to detect endotoxin in a test sample. The test sample can be any sample in which it would be useful to detect endotoxin, including water, aqueous solutions such as buffers, pharmaceutical preparations (e.g., vaccines, intravenous fluids, drug preparations), biomedical imaging reagents (e.g., dyes, radioactive solutions), enzyme preparations (e.g., collagenase), tissue culture media, beverages, blood, urine, cerebrospinal fluid, lymph, serum, and solutions formed by incubating water or an aqueous solution with a solid sample, such as a foodstuff or a surgical glove.

[0080] Either a two-step or a one-step assay can be performed. To perform the two-step assay (Example 1), a test sample is incubated with a reagent containing purified Factor C and a surfactant, then a Factor C substrate that will generate a detectable signal upon cleavage is added. To perform the one-step assay (Example 13), endotoxin, the reagent containing purified Factor C and a surfactant, and a substrate are added together.

[0081] Substrates that will generate a fluorescent signal upon cleavage are particularly preferred, such as N-t-BOC-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin ("DPR," Bachem 1-1560.0050) and N-t-BOC-Val-Pro-Arg-7-Amido-4-methyl coumarin ("VPR," Bachem 1-1120.0050). Concentrations of DPR generally range from about 10-100 mM, 25-100 mM, 5-100 mM, 75-100 mM, 25-75 mM, 50-75 mM, 25-50 mM, or 50-75 mM. Concentration of VPR generally range from about 50-200 mM, 50-150 mM, 50-100 mM, 50-75 mM, 75-150 mM, or 75-100 mM.

[0082] The optimum concentration for a particular substrate varies. For example, the optimum concentration for DPR is 50 mM, whereas the optimum concentration for VPR is 100 mM. Moreover, the particular substrate can be chosen depending on the endotoxin levels expected to be present in the test sample. The VPR substrate has a better linear range at higher endotoxin concentrations, and the DPR substrate has a better linear range at lower endotoxin concentrations. Thus, DPR can be used in assays in which higher sensitivity is desired, whereas VPR is the preferred substrate when higher endotoxin levels are expected to be present and a lower sensitivity is required.

[0083] Fluorescence can be measured using any means known in the art. If a fluorimeter is used with the DPR or VPR substrates described above, the excitation is set at 360, 380, 390, or 395 nm (slit width of 5-40 nm) and the emission is measured at 440 or 460 nm (slit width of 2.5-40 nm). Measurement can be either qualitative or quantitative, by reference to a standard endotoxin concentration curve.

[0084] The assay can be carried out in a clear vessel, such as a glass or polystyrene tissue culture plate, or can be

carried out in a black vessel (e.g., a black 96-well microplate). If desired, the assay can be adapted for high-throughput screening of multiple samples using, for example, 96-well microtiter plates.

[0085] Kits

[0086] The invention also provides a kit for use in detecting endotoxin. The kit comprises a purified horseshoe crab Factor C protein and a surfactant, as described above. Instructions for using the reagent to detect endotoxin can be included. Kits also can include a Factor C substrate for use in the assay.

[0087] All patents, patent applications, and references cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

[0088] Two-Step Endotoxin Assay

[0089] Endotoxin was pre-incubated with culture medium containing recombinant *Carcinoscorpius rotundicauda* Factor C obtained from recombinant Factor C-producing Sf9 cells for 1 hour in assay buffer (30 mM Tris, pH 8.0, 30 mM NaCl, 0.3% lactose, and 0.0025% Zwittergent 3-14). The substrate N-t-Boc-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin (DPR-coumarin) was added to the mixture to a final concentration of 50 mM. The fluorescence generated from the cleavage of the substrate by activated Factor C was measured after 15-20 minutes. The results are shown in FIG. 4. Results of a similar assay carried out using the substrate N-t-Boc-Val-Pro-Arg-7-Amido-4-methyl coumarin at a final concentration of 100 mM are shown in FIG. 5.

EXAMPLE 2

[0090] Transfection of Bacmid DNA-Factor C into Sf9 Cells and Harvesting of Recombinant Viral Supernatant

[0091] Sf9 cells are seeded at a density of 5×10^6 per 35 mm well in a 6-well tissue culture plate in insect cell culture medium (ICCM) containing 50 U/ml penicillin and 50 mg/ml streptomycin. Insect-Xpress (BioWhittaker Cat. #04-10270) or Sf-900 SFM are suitable media; however, any comparable medium in which Sf9 cells grow can be used. The plates are incubated at 27° C. for 1 hour to allow the cells to settle. Meanwhile, the following are prepared (each per well): (1) 7 mg of bacmid-Factor C DNA in 100 ml ICCM (without antibiotics) and (2) 6 ml CELLFECTIN (Gibco-BRL) plus 100 ml ICCM (without antibiotics). Solutions (1) and (2) are mixed gently and incubated at room temperature for one hour. To this mixture, 0.8 ml of ICCM (without antibiotics) is added.

[0092] The adhered Sf9 cells are washed gently with 2 ml ICCM (without antibiotics). One ml of CELLFECTIN-DNA complex is added to each well and incubated at 27° C. for 5 hours. The transfection mixtures are removed completely, and 2 ml of ICCM containing antibiotics is added. After incubation at 27° C. for 96 hours, the cell culture supernatant is harvested. The supernatant is clarified by centrifugation at 5000 rpm for 10 minutes in a SIGMA 3K10 swing-out rotor,

Nr. 11133. The supernatant, containing the recombinant baculovirus, is stored at 4° C.

EXAMPLE 3

[0093] Amplification of Recombinant Viral Stock

[0094] Virus amplification can be done with Sf9 cells either in a monolayer or in suspension. For monolayer culture, the medium is decanted from a culture of 80% confluent one day-old Sf9 cells in a 75 cm² tissue culture flask. The cell monolayer is infected with 1 ml of virus (Example 2) using an MOI of 0.1-1. The virus stock is sterile-filtered using a Millipore GV millex filter (yellow; low-protein binding). Calculation of the viral inoculum is as follows:

$$\text{volume of inoculum} = \frac{\text{MOI} \times \text{total cell number}}{\text{virus titer in pfu/ml}}$$

[0095] Thus, for 1×10⁷ cells, at a virus titer of 2×10⁷ pfu/ml, the volume of the inoculum is 0.5 ml. The volume of the inoculum is adjusted to 1 ml with ICCM before it is introduced to the cells.

[0096] The flask is rocked several times to ensure that the cell monolayer is completely covered by the 1 ml viral inoculum. The flask is then incubated at 27° C. for one hour without rocking. After the incubation, the flask is placed upright, and 14 ml of fresh ICCM is added. The flask is then incubated for 3 days at 27° C.

[0097] The culture supernatant is harvested into sterile pyrogen-free tubes and centrifuged at 2000 rpm for 10 minutes at 4° C. using a Sigma 3K10 swing out rotor. For immediate use, the viral stock is stored at 4° C. For long-term storage, the viral stock is placed at -80° C.

[0098] For suspension culture, use log-phase grown Sf9 cells with viability >95% for viral amplification. Cell density and viability are determined. Sf9 cells are diluted with fresh InsectXPRESS to 1×10⁶ cells per ml. Virus to be amplified is added to the Sf9 culture, at an MOI of 0.02 (virus/cell), as follows:

Vol. of inoculum virus stock (ml) =

$$\frac{(\text{Total cells/ml}) \times (\text{Total Vol. of culture}) \times (\text{MOI})}{(\text{Virus titer in pfu/ml})}$$

[0099] Spinner flasks are incubated for three days at 27° C. +/- 2° C., with constant stirring at 90-120 rpm. The cell culture is transferred to sterile centrifuge bottles and centrifuged at 2000 rpm for 10 minutes at 4° C. The supernatant is collected into a sterile container and discard the cell pellet. The supernatant is filtered through a 0.45 μm filter into a sterile container. The supernatant is further filtered through a sterile, 0.2 μm filter into a sterile container. The finished virus stock is stored protected from light at 2-8° C. A titer determination is performed as explained in Example 4.

EXAMPLE 4

[0100] Titration of Baculovirus

[0101] Virus titer (Plaque Forming Units (PFU) per ml) can be determined with plaque assay, end-point dilution, or other viral titer kits (e.g., BacPAK Baculovirus Rapid Titer Kit by Clontech #K1599-1). Plaque assay is performed in immobilized monolayer culture. Plaque assays to be performed are determined as follows:

$$\text{Plaque assay number} = \text{Number of viral dilution} \times 4 \\ (\text{quadruplicates}) + 2 \text{ positive and } 2 \text{ negative controls.}$$

[0102] For example, 10⁵, 10⁶, 10⁷, 10⁸ for viral dilution, each dilution in quadruplicate wells, would be a 16-well plaque assay. Include two positive controls and two negative controls in the plaque assay, for a total of 20 wells.

[0103] Prepare a cell suspension of 5×10⁵ cells per ml density in 10% FBS serum media. Seed 6-well plates at 2 ml per well with above cell suspension. Cells are allowed to attach two hours before experiments. Cells are ~80% confluent.

[0104] Prepare 10-fold serial viral dilutions using 4.5 ml media and 0.5 ml of viral stock in sterile 15-ml tubes. Aspirate the medium from each well. Add viral dilution 1 ml per well and incubate at 27° C. for 2 hours.

[0105] Warm 1.3x SF-90011 (Gibco-BRL #10967-032) to 27° C. Melt 4% agarose (Gibco 18300-012) and keep in 50° C. water-bath. Add 1.3x media and 4% agarose in 3:1 ratio to make 1% agarose in culture media. Keep this agar in a 40° C. water-bath (for example, in a beaker that can be brought to a hood).

[0106] Working quickly so that the agarose mixture does not solidify, aspirate the viral solution from the cell monolayers. Add 2 ml 1% agarose to each well. Let the plates sit in the hood with the covers slightly open for 10 minutes. Invert the plates and put them in a sealed box with damp paper towels to provide humidity. Incubate at 27° C. for 5-7 days, or until plaques are well formed.

[0107] At day 7 of post-infection, add 0.5 ml 0.033% neutral red (in water), incubate 3 minutes, and discard. Alternatively, add 0.5 ml 0.1% trypan blue (in water), rock to cover, sit 3 minutes, and discard. Incubate the plates for 2 hours. With the help of a light box and a magnifier, count the plaques on each plate. Virus titer is calculated using the following formula:

$$\text{Virus titer (pfu/ml)} = \text{of plaques} \times \text{viral dilution.}$$

EXAMPLE 5

[0108] Infection of Sf9 Cells for Recombinant Factor C Production in Serum-Free Cultures

[0109] High viability Sf9 cells are used (e.g., viability of 95-100% in serum-free conditions). Culture log-phase Sf9 cells to a cell density of between 1.5×10⁶ and 2.5×10⁶ cells per ml in suspension culture. Recombinant factor C production also can be carried out by infecting Sf9 cells grown in monolayer culture. Determine cell density and viability. Dilute Sf9 cells with fresh InsectXPRESS to 1.5×10⁶ cells per ml. Add recombinant baculovirus high titer stock to the Sf9 culture, at an MOI of 1 (virus/cell), as follows:

Vol. of inoculum HTS (ml) =

$$\frac{(\text{Total cells/ml}) \times (\text{Total Vol. of culture}) \times (\text{MOI})}{(\text{Virus Titer in pfu/ml})}$$

[0110] Harvest culture supernatant seventy-two hours post-infection. Transfer the cell culture to sterile centrifuge bottles and centrifuge at 2000 rpm for 10 minutes at 4° C. Collect the supernatant into a sterile container and discard the cell pellet. Filter the supernatant through a 0.45 µm filter into a sterile container. Further filter supernatant through a sterile, 0.2 µm filter into a sterile container. This culture supernatant, which contains recombinant Factor C, can be stored at 4° C. for up to one year and can be used directly to form a reagent for endotoxin detection in accordance with the present invention.

EXAMPLE 6

[0111] Culture and Subculture of Sf9 Cells Adapted in Serum-Free Medium

[0112] Sf9 cells grow at 27° C. without CO₂. Maximal aeration is preferred. If spinner culture bottles are used, loosen the side screw-caps to increase aeration. Sf9 cells adapted to grow in serum-free medium should be subcultured at 4-5 days intervals on a routine basis.

[0113] Subculture at 5.0×10⁵ cells/ml in a 250 ml spinner or shake flask containing 100 ml of medium with loosened caps to provide maximal aeration. Stir the culture at 90-100 rpm. Cell viability should be above 90%.

[0114] Remove 4-5 day old cultures from the 27° C. incubator and swab with 70% alcohol. Take an aliquot of cell suspension to determine cell viability and total cell count using trypan blue dye. Determine the cell dilution necessary to obtain 5×10⁵ cells/ml. Swirl the stock culture flask several times and remove the appropriate volume of cell suspension based on the calculation. Inoculate a new 250 ml spinner flask containing enough pre-warmed fresh medium necessary to make up the final culture volume to 100 ml. Place the cultures back into the incubator. Set the stirring speed to 90-100 rpm.

EXAMPLE 7

[0115] Cryopreservation of Serum-Free Cultures of Sf9 Cells

[0116] Grow Sf9 cells either in a monolayer or in suspension. Harvest the cells at mid-log phase of growth (about 2 days) at a viability of >90%. Keep conditioned medium on ice and sterile-filter.

[0117] Determine the cell viability using the trypan blue dye exclusion method. Calculate the required volume of cryopreservation medium (7.5% DMSO in 50% fresh serum-free ICCM medium and 50% conditioned medium; sterile-filtered) to yield a final density of 1-2×10⁷ cells/ml. Hold the cryopreservation mixture on ice. Meanwhile, centrifuge cells from the suspension for 3 minutes at 800 rpm/500× g. Discard the sample if a lot of cells are unpeeled and in the supernatant.

[0118] Resuspend the cell pellet in the determined volume of chilled cryopreservation mix to obtain 1-2×10⁷ cells/ml. Quickly dispense 1-1.5 ml aliquots of cell suspension into cryovials. Freeze the cells at -70 to -90° C. for 4 hours on the upper gaseous phase of liquid nitrogen. Plunge the vials into liquid nitrogen.

EXAMPLE 8

[0119] Recovery of Cryopreserved Serum-Free Sf9 Cells

[0120] Thaw vial by rapid agitation in 37° C. water bath just until melted. Remove the vial from the water bath and swab with sterile 70% isopropanol. Open vial, remove contents of the vial into a sterile centrifuge tube of appropriate size; and dilute the cell suspension with 5 volumes of InsectXPRESS medium (or equivalent) that has been equilibrated to 27° C.±2° C. in an incubator. Centrifuge the diluted suspension at 125× G for 10 minutes. Discard the fluid and resuspend the cells in a volume of culture medium equal to fluid discarded. Determine cell count and viability. Adjust viable cell density to between 2×10⁵ cells per ml and 3×10⁵ cells per ml using serum free medium. Transfer flask in a 27° C.±2° C. incubator on a rotary platform shaker set for continuous shaking and 100-125 revolutions per minute. When the viable cell count of the culture doubles at least every 26 hours, the culture may be expanded as described in Example 6.

EXAMPLE 9

[0121] Adaptation of Sf9 Cells to Suspension Culture

[0122] Six to ten confluent 75 cm² monolayer T-flasks of Sf9 cells are required to initiate a 100 ml suspension culture. Dislodge cells from the bottom of the flasks by holding a flask in one hand and tapping it against the palm of the other hand. Pool the cell suspension and perform a viability count.

[0123] Dilute the cell suspension to approximately 5×10⁵ viable cells/ml at room temperature using complete serum-supplemented or serum-free growth medium contained in a 250 ml spinner flask. The top of the stirrer blade may be slightly above the cell suspension. This provides additional aeration. The side arm caps should be loosened by one-quarter turn.

[0124] Incubate the cells at 27° C. at a constant stirring rate of 100 rpm. Subculture when the viable cell count reaches 1-2×10⁶ cells/ml (3-7 days post-seeding), increase stirring speed by 5 rpm per passage until culture viability is >80%. Repeat until a stirring speed of 100 rpm is achieved for the spinner flask or 130-140 rpm is achieved for the shaker flask cultures. At this point, reduce seeding density to 3×10⁵ cells/ml during subculture.

[0125] If large clumps of cells persist (e.g., >10 cells per clump), let the spinner/shaker flask stand 2-3 minutes without stirring prior to subculturing. This allows larger clumps to settle to the bottom. Pool the upper one-third of the suspension cells for counting and seeding into new cultures. This procedure selects for a cell population that grows as single cells. It may be necessary to repeat this step 2-3 times until clumping is reduced.

EXAMPLE 10

[0126] Effect of Different Detergents and Different Concentrations of Detergents on the Recombinant Factor C Assay

[0127] Materials and Methods

[0128] The following reagents were purchased from the manufacturers indicated: Zwittergent 3-14 (Calbiochem, Cat# 693017), Tween 20 (ICN, Cat# 194841), Tween 80 (ICN, Cat# 194842), Triton X-114 (ICN, Cat# 193971), n-Octyl-beta-D-thioglycopyranoside (OTG, Amresco, Cat# J575), and Triton X-100 (ICN, Cat# 194854). The recom-

binant Factor C (rFC) assay was carried out using 20 µl of each detergent (~10× of final concentration), 150 µl of 1 EU/ml EC-6, 10 µl of rFC supernatant 0319011, 20 µl of 300 mM Tris, pH 8.0, and 20 µl of 0.55 mM DPR-coumarin substrate. The results of the assay were read at 38° C. using a Cytofluor reader set at 390/440 nm, 5 minutes per cycle, for 1 hour. The data were graphed after 30 minutes.

[0129] Results

[0130] As shown in FIGS. 1-3 and 9-11, low concentrations of Zwittergent 3-14, Tween 20, Tween 80, Triton X-114, OTG, Genapol C-100 and Triton X-100 enhanced the rFC assay 2-7 fold. The enhancing concentration range for Zwittergent 3-14, Triton X-114, OTG and Triton X-100 is narrow, while the enhancing concentration range for Tween 20, Tween 80 and Genapol C-100 is wide.

[0131] Table 1 summarizes the results obtained by testing nine detergents. These detergents can be divided into three groups: detergents that have a narrow concentration range for enhancing recombinant Factor C activity, detergents that have wide concentration range for enhancing recombinant Factor C activity, and detergents that inhibit recombinant Factor C activity.

TABLE 1

Detergents	Detergent concentration range for		CMC		
	rFC enhancing	rFC inhibition	(mM)	MW	Category
Group 1					
Zwittergent	0.001–<0.004%	>=0.004%	0.1–0.4	363.6	ZW
Triton X-100	0.001–<0.008%	>=0.008%	0.2–0.9	631	Non-ionic
Triton X-114	0.001–<0.016%	>=0.016%	0.35	537	Non-ionic
OTG	0.031–<0.5%	>=0.5%	9	308.4	Non-ionic
Group 2					
Genapol C-100	0.001–0.5%	>0.5%	/	627	Non-ionic
Tween 20	0.001–0.5%	>0.5%	0.059	1228	Non-ionic
Tween 80	0.001–0.25%	>0.25%	0.012	1310	Non-ionic
Group 3					
Deoxycholic Acid	/	>=0.0004%	1.5	414.6	Ionic
SDS	/	>=0.0004%	2.3	288.5	Ionic

[0132] The enhancing/inhibiting effect of Zwittergent 3-14 was also tested using purified Limulus factor C (FIG. 6). A similar enhancing/inhibiting effect was observed, although the relative factor C activity ratio, the enhancing and inhibiting detergent concentrations were different from those in the recombinant Factor C assay.

EXAMPLE 11

[0133] Antisera Against Limulus Factor C

[0134] We isolated the factor C from Limulus and generated antisera against the protein. The Limulus lysate was first separated with a S-100 gel filtration followed by cation exchange column. The fractions were assayed for factor C activity using the N-t-BOC-Val-Pro-Arg 7-Amido-4-methyl coumarin substrate. The Limulus factor C was purified 73 fold with 80% homogeneity as determined by enzyme assay and SDS-PAGE. The molecular weight of Limulus factor C was 117 kDa by SDS-PAGE under non-reducing conditions. Reduced SDS-PAGE showed two major bands of 79 kDa and 40 kDa, corresponding to the heavy and light chain. Tryptic peptide sequencing revealed that the Limulus factor C sequence closely matched the Asian species, with

>90% sequence identity. A specific polyclonal antibody against the Limulus factor C was generated in rabbits. The anti-factor C purified IgG inhibited the activation of factor C by LPS but had no effect on the protease activity once the factor C was activated by LPS. The factor C antisera demonstrated the importance of this protein in the initial recognition of LPS by Limulus lysate.

EXAMPLE 12

[0135] Increase in Sensitivity in Endotoxin Detection in the Presence of Surfactant

[0136] A one-step, 1-hour endpoint assay was performed. Endotoxin 055:B5, 0.01, 0.1, 1, and 10 EU/ml were used for the standard curve. Blank and endotoxin standards (100 µl each) were added to a 96-well plate. Recombinant Factor C supernatant, assay buffer (150 mM NaCl, 150 mM Tris, pH 8.0, and 1.5% lactose, with or without 0.0125% Zwittergent) and substrate solution (0.2 mM in water) were mixed at 1:4:5 ratios. This mixture was added into each blank and endotoxin standard. The fluorescence was recorded at time zero and time 1 hour. The difference in fluorescences (delta fluorescence) were normalized with the blank. Normalized

delta fluorescence was then graphed against endotoxin concentration in a log-log scale. Each data point is the result of duplicate assays.

[0137] The results are shown in FIG. 7. The endotoxin detection sensitivity increased 10-fold when surfactant was included.

EXAMPLE 13

[0138] One-Step Endotoxin Assay

[0139] A one-step endotoxin assay can be carried out in a 96-well plate, using 100 µl each of blank and endotoxin standards. One hundred microliters of a mixture of recombinant Factor C supernatant (Baculovirus-infected Sf9 cell culture medium), buffer (150 mM Tris, pH 8.0, 150 mM NaCl, 1.5% beta-lactose, and 0.0125% Zwittergent 3-14) and fluorogenic substrate (DPR-coumarin, 0.2 mM) at a ratio of 1:4:5 is added to wells of the plate. The plate is incubated at 37° C. for 1 hour. Excitation and emission are read at 390 and 440 nm, respectively, in a fluorescence microplate reader.

[0140] The results of a one-step recombinant Factor C endotoxin assay are shown in FIG. 8.

SEQUENCE LISTING

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Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
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Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Thr Cys Arg
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Asp Arg Tyr Glu Gly Ala His Cys Glu Ile Leu Lys Gly Cys Pro Leu
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Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
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Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
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Val Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Ser Phe Pro
180 185 190
Pro Lys Cys Ile Arg Glu Cys Ala Lys Val Ser Ser Pro Glu His Gly
195 200 205
Lys Val Asn Ala Pro Ser Gly Asn Met Ile Glu Gly Ala Thr Leu Arg
210 215 220
Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225 230 235 240
Cys Gln Gly Asn Gly Gln Trp Ser Gly Gln Ile Pro Gln Cys Lys Lys
245 250 255
Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Gln
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Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
275 280 285
Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asn
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Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305 310 315 320
Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
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Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
340 345 350
Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
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Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro
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Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp
385 390 395 400
Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser
405 410 415
Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly
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Arg Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Glu Glu Asn Cys Val
435 440 445
Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys
450 455 460
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<211> LENGTH: 3467
<212> TYPE: DNA
<213> ORGANISM: Tachypleudus tridentata

<400> SEQUENCE: 3

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<210> SEQ ID NO 4
<211> LENGTH: 1019
<212> TYPE: PRT
<213> ORGANISM: Tachypleudus tridentata

<400> SEQUENCE: 4

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Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
 35            40            45

Phe Asn Val Pro Met Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
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Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
 65            70            75            80

Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
 85            90            95

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
100            105            110

Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Thr Cys Arg
115            120            125

Asp Arg Tyr Glu Gly Ala His Cys Glu Ile Leu Lys Gly Cys Pro Leu
130            135            140

Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145            150            155            160

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
165            170            175

Val Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Ser Phe Pro
180            185            190

Pro Lys Cys Ile Arg Glu Cys Ala Lys Val Ser Ser Pro Glu His Gly
195            200            205

Lys Val Asn Ala Pro Ser Gly Asn Met Ile Glu Gly Ala Thr Leu Arg
210            215            220

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
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Cys Gln Gly Asn Gly Gln Trp Ser Gly Gln Ile Pro Gln Cys Lys Lys
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Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Gln
260            265            270

Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
275            280            285

Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asn
290            295            300

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305            310            315            320

Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
325            330            335

Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
340            345            350

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
355            360            365

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Phe	Leu	Gly	Ser	Asp	Leu	Asn	Gly	Ile	Lys	Ser	Glu	Glu	Leu	Lys	Ser
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Leu	Ala	Arg	Ser	Phe	Arg	Phe	Asp	Tyr	Val	Ser	Ser	Ser	Thr	Ala	Gly
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Arg	Ser	Gly	Cys	Pro	Asp	Gly	Trp	Phe	Glu	Val	Glu	Glu	Asn	Cys	Val
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Tyr	Val	Thr	Ser	Lys	Gln	Arg	Ala	Trp	Glu	Arg	Ala	Gln	Gly	Val	Cys
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Thr	Asn	Met	Ala	Ala	Arg	Leu	Ala	Val	Leu	Asp	Lys	Asp	Leu	Ile	Pro
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Ser	Ser	Leu	Thr	Glu	Thr	Leu	Arg	Gly	Lys	Gly	Leu	Thr	Thr	Thr	Trp
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Ile	Gly	Leu	His	Arg	Leu	Asp	Ala	Glu	Lys	Pro	Phe	Val	Trp	Glu	Leu
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Met	Asp	Arg	Ser	Asn	Val	Val	Leu	Asn	Asp	Asn	Leu	Thr	Phe	Trp	Ala
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Ser	Gly	Glu	Pro	Gly	Asn	Glu	Thr	Asn	Cys	Val	Tyr	Leu	Asp	Ile	Arg
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Asp	Gln	Leu	Gln	Pro	Val	Trp	Lys	Thr	Lys	Ser	Cys	Phe	Gln	Pro	Ser
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Ser	Phe	Ala	Cys	Met	Met	Asp	Leu	Ser	Asp	Arg	Asn	Lys	Ala	Lys	Cys
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Asp	Asp	Pro	Gly	Pro	Leu	Glu	Asn	Gly	His	Ala	Thr	Leu	His	Gly	Gln
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Ser	Ile	Asp	Gly	Phe	Tyr	Ala	Gly	Ser	Ser	Ile	Arg	Tyr	Ser	Cys	Glu
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Val	Leu	His	Tyr	Leu	Ser	Gly	Thr	Glu	Thr	Val	Thr	Cys	Thr	Thr	Asn
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Gln	Asn	Pro	Pro	Val	Pro	Ser	Tyr	Gly	Ser	Val	Glu	Ile	Lys	Pro	Pro
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Ser	Arg	Thr	Asn	Ser	Ile	Ser	Arg	Val	Gly	Ser	Pro	Phe	Leu	Arg	Leu
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Pro	Arg	Leu	Pro	Leu	Pro	Leu	Ala	Arg	Ala	Ala	Lys	Pro	Pro	Pro	Lys
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Pro	Arg	Ser	Ser	Gln	Pro	Ser	Thr	Val	Asp	Leu	Ala	Ser	Lys	Val	Lys
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Leu	Pro	Glu	Gly	His	Tyr	Arg	Val	Gly	Ser	Arg	Ala	Ile	Tyr	Thr	Cys
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Glu	Ser	Arg	Tyr	Tyr	Glu	Leu	Leu	Gly	Ser	Gln	Gly	Arg	Arg	Cys	Asp
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Ser	Asn	Gly	Asn	Trp	Ser	Gly	Arg	Pro	Ala	Ser	Cys	Ile	Pro	Val	Cys
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Glu	Lys	Trp	Ile	Val	Thr	Ala	Ala	His	Cys	Val	Thr	Tyr	Ser	Ala	Thr				
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Tyr	Arg	Asp	Asp	Ser	Arg	Asp	Asp	Asp	Tyr	Val	Gln	Val	Arg	Glu	Ala				
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cctaattcaa ctgaaaactc ctgttacttt gacaacacga gtccaaccaa tctgtctgcc 3420
tactgacatc acaacaagag aacacttgaa ggaggggaaca ttagcagtgg tgacaggttg 3480
gggtttgaat gaaaacaaca cctattcaga gacgattcaa caagctgtgc tacctgttgt 3540
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agttaacgtt ttctgtcat ggattaggca gttcatttga aactgatcta aatattttaa 3840
gcatggttat aaacgtcttg ttcctattat tgctttactg gtttaacca taagaaggtt 3900
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<210> SEQ ID NO 6
<211> LENGTH: 1083
<212> TYPE: PRT
<213> ORGANISM: Carcinoscorpius rotundicauda

<400> SEQUENCE: 6

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Val Phe Cys Leu Leu Cys Val Trp Arg Phe Gly Phe Cys Arg Trp Arg
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Val Phe Tyr Ser Phe Pro Phe Val Lys Ser Thr Val Val Leu Leu Gln
35 40 45
Cys Tyr His Tyr Ser Leu His Asn Thr Ser Lys Phe Tyr Ser Val Asn
50 55 60
Pro Asp Lys Pro Glu Tyr Ile Leu Ser Gly Leu Val Leu Gly Leu Leu
65 70 75 80
Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
85 90 95
Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
100 105 110
Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
115 120 125
Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys

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130	135	140
Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr		
145	150	155 160
Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys		
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Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg		
	180	185 190
Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu		
	195	200 205
Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn		
	210 215	220
Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly		
	225 230	235 240
Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro		
	245	250 255
Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly		
	260	265 270
Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg		
	275	280 285
Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr		
	290 295	300
Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn		
	305 310	315 320
Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys		
	325	330 335
Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr		
	340	345 350
Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp		
	355	360 365
Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser		
	370 375	380
Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val		
	385 390	395 400
Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala		
	405	410 415
Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His		
	420	425 430
Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His Ala Gly Lys Leu Pro		
	435	440 445
Asn Ser Gly Gly Ala Val His Val Val Asn Asn Gly Pro Tyr Ser Asp		
	450 455	460
Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser Glu Glu Leu Lys Ser		
	465 470	475 480
Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Ser Ser Ser Thr Ala Gly		
	485	490 495
Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val Asp Glu Asn Cys Val		
	500	505 510
Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg Ala Gln Gly Val Cys		
	515	520 525
Thr Asn Met Ala Ala Arg Leu Ala Val Leu Asp Lys Asp Val Ile Pro		
	530	535 540

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Asn	Ser	Leu	Thr	Glu	Thr	Leu	Arg	Gly	Lys	Gly	Leu	Thr	Thr	Thr	Trp	545	550	555	560
Ile	Gly	Leu	His	Arg	Leu	Asp	Ala	Glu	Lys	Pro	Phe	Ile	Trp	Glu	Leu	565	570	575	
Met	Asp	Arg	Ser	Asn	Val	Val	Leu	Asn	Asp	Asn	Leu	Thr	Phe	Trp	Ala	580	585	590	
Ser	Gly	Glu	Pro	Gly	Asn	Glu	Thr	Asn	Cys	Val	Tyr	Met	Asp	Ile	Gln	595	600	605	
Asp	Gln	Leu	Gln	Ser	Val	Trp	Lys	Thr	Lys	Ser	Cys	Phe	Gln	Pro	Ser	610	615	620	
Ser	Phe	Ala	Cys	Met	Met	Asp	Leu	Ser	Asp	Arg	Asn	Lys	Ala	Lys	Cys	625	630	635	640
Asp	Asp	Pro	Gly	Ser	Leu	Glu	Asn	Gly	His	Ala	Thr	Leu	His	Gly	Gln	645	650	655	
Ser	Ile	Asp	Gly	Phe	Tyr	Ala	Gly	Ser	Ser	Ile	Arg	Tyr	Ser	Cys	Glu	660	665	670	
Val	Leu	His	Tyr	Leu	Ser	Gly	Thr	Glu	Thr	Val	Thr	Cys	Thr	Thr	Asn	675	680	685	
Gly	Thr	Trp	Ser	Ala	Pro	Lys	Pro	Arg	Cys	Ile	Lys	Val	Ile	Thr	Cys	690	695	700	
Gln	Asn	Pro	Pro	Val	Pro	Ser	Tyr	Gly	Ser	Val	Glu	Ile	Lys	Pro	Pro	705	710	715	720
Ser	Arg	Thr	Asn	Ser	Ile	Ser	Arg	Val	Gly	Ser	Pro	Phe	Leu	Arg	Leu	725	730	735	
Pro	Arg	Leu	Pro	Leu	Pro	Leu	Ala	Arg	Ala	Ala	Lys	Pro	Pro	Pro	Lys	740	745	750	
Pro	Arg	Ser	Ser	Gln	Pro	Ser	Thr	Val	Asp	Leu	Ala	Ser	Lys	Val	Lys	755	760	765	
Leu	Pro	Glu	Gly	His	Tyr	Arg	Val	Gly	Ser	Arg	Ala	Ile	Tyr	Thr	Cys	770	775	780	
Glu	Ser	Arg	Tyr	Tyr	Glu	Leu	Leu	Gly	Ser	Gln	Gly	Arg	Arg	Cys	Asp	785	790	795	800
Ser	Asn	Gly	Asn	Trp	Ser	Gly	Arg	Pro	Ala	Ser	Cys	Ile	Pro	Val	Cys	805	810	815	
Gly	Arg	Ser	Asp	Ser	Pro	Arg	Ser	Pro	Phe	Ile	Trp	Asn	Gly	Asn	Ser	820	825	830	
Thr	Glu	Ile	Gly	Gln	Trp	Pro	Trp	Gln	Ala	Gly	Ile	Ser	Arg	Trp	Leu	835	840	845	
Ala	Asp	His	Asn	Met	Trp	Phe	Leu	Gln	Cys	Gly	Gly	Ser	Leu	Leu	Asn	850	855	860	
Glu	Lys	Trp	Ile	Val	Thr	Ala	Ala	His	Cys	Val	Thr	Tyr	Ser	Ala	Thr	865	870	875	880
Ala	Glu	Ile	Ile	Asp	Pro	Asn	Gln	Phe	Lys	Met	Tyr	Leu	Gly	Lys	Tyr	885	890	895	
Tyr	Arg	Asp	Asp	Ser	Arg	Asp	Asp	Tyr	Val	Gln	Val	Arg	Glu	Ala		900	905	910	
Leu	Glu	Ile	His	Val	Asn	Pro	Asn	Tyr	Asp	Pro	Gly	Asn	Leu	Asn	Phe	915	920	925	
Asp	Ile	Ala	Leu	Ile	Gln	Leu	Lys	Thr	Pro	Val	Thr	Leu	Thr	Thr	Arg	930	935	940	

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Val	Gln	Pro	Ile	Cys	Leu	Pro	Thr	Asp	Ile	Thr	Thr	Arg	Glu	His	Leu
945					950					955					960
Lys	Glu	Gly	Thr	Leu	Ala	Val	Val	Thr	Gly	Trp	Gly	Leu	Asn	Glu	Asn
			965						970					975	
Asn	Thr	Tyr	Ser	Glu	Thr	Ile	Gln	Gln	Ala	Val	Leu	Pro	Val	Val	Ala
			980				985						990		
Ala	Ser	Thr	Cys	Glu	Glu	Gly	Tyr	Lys	Glu	Ala	Asp	Leu	Pro	Leu	Thr
		995					1000					1005			
Val	Thr	Glu	Asn	Met	Phe	Cys	Ala	Gly	Tyr	Lys	Lys	Gly	Arg	Tyr	Asp
	1010					1015					1020				
Ala	Cys	Ser	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Val	Phe	Ala	Asp	Asp	Ser
1025					1030				1035						1040
Arg	Thr	Glu	Arg	Arg	Trp	Val	Leu	Glu	Gly	Ile	Val	Ser	Trp	Gly	Ser
			1045					1050						1055	
Pro	Ser	Gly	Cys	Gly	Lys	Ala	Asn	Gln	Tyr	Gly	Gly	Phe	Thr	Lys	Val
		1060					1065						1070		
Asn	Val	Phe	Leu	Ser	Trp	Ile	Arg	Gln	Phe	Ile					
	1075					1080									

<210> SEQ ID NO 7
<211> LENGTH: 3438
<212> TYPE: DNA
<213> ORGANISM: Carcinoscorpius rotundicauda

<400> SEQUENCE: 7

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aaacgagggt cgagtgttaag tgtggcgatc caggctatgt gttcaacatt ccagtgaaac	180
aatgtacata cttttatcga tggaggccgt attgtaaacc atgtgatgac ctggaggcta	240
aggatatattg tccaaagtac aaacgatgtc aagagtgtaa ggctggctct gatagttgtg	300
ttacttgtcc acctaacaaa tatggtactt ggtgtagcgg tgaatgtcag tgtaagaatg	360
gaggtatctg tgaccagagg acaggagctt gtgcatgtcg tgacagatat gaaggggtgc	420
actgtgaaat tctcaaagggt tgtcctcttc ttccatcgga ttctcagggt caggaagtca	480
gaaatccacc agataatccc caaactattg actacagctg ttcaccaggg ttcaagctta	540
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gtattcgaga atgtgccatg gtttcatctc cagaacatgg gaaagtgaat gctcttagtg	660
gtgatatgat agaagggggt actttacggt tctcatgtga tagtccctac tacttgattg	720
gtcaagaaac attaacctgt cagggtaatg gtcagtggaa tggacagata ccacaatgta	780
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cgggtaacta cttcttgatg ggttttgaca cottaaaatg taaccctgat gggctcttgt	960
caggatcaca gccatcctgt gttaaagtgg cagacagaga ggtcgactgt gacagtaaag	1020
ctgtagactt cttggatgat gttggtgaac ctgtcaggat ccactgtcct gctggctgtt	1080
ctttgacagc tgggtactgtg tggggtacag ccatatacca tgaactttcc tcagtgtgtc	1140
gtgcagccat ccatgctggc aagcttccaa actctggagg agcgggtgcat gttgtgaaca	1200
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gagcctggga aagagctcaa ggtgtgtgta ccaatatggc tgctcgtctt gctgtgctgg	1440
acaaagatgt aattccaaat tcgttgactg agactctacg agggaaaggg ttaacaacca	1500
cgtggatagg attgcacaga ctagatgctg agaagccctt tatttgggag ttaatggatc	1560
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gaactgaaac cgttaactgt acaacaaatg gcacatggag tgctcctaaa cctcgatgta	1920
tcaaagtoat cacctgccaa aacccccctg taccatcata tggttctgtg gaaatcaaac	1980
ccccaagtcg gacaaactcg ataagtcgtg ttgggtcacc tttcttgagg ttgccacggt	2040
taccctctcc attagctaga gcagccaaac ctctccaaa acctagatcc tcacaaccct	2100
ctactgtgga cttggcttct aaagttaaac tacctgaagg tcattaccgg gtagggctct	2160
gagccatcta cactgcgag tcgagatact acgaactact tggatctcaa ggcagaagat	2220
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tctacacatg aaaattctgt cattttacca taaatttggg ttctggtgtg tgctaagtcc	3360
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<210> SEQ ID NO 8
<211> LENGTH: 1019
<212> TYPE: PRT
<213> ORGANISM: Carcinoscorpius rotundicauda

<400> SEQUENCE: 8

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Ala Gln Lys Met Arg Pro Val Gln Ser Lys Gly Val Asp Leu Gly Leu
 20            25            30

Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly Asp Pro Gly Tyr Val
 35            40            45

Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe Tyr Arg Trp Arg Pro
 50            55            60

Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys Asp Ile Cys Pro Lys
 65            70            75            80

Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu Asp Ser Cys Val Thr
 85            90            95

Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser Gly Glu Cys Gln Cys
100            105           110

Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly Ala Cys Ala Cys Arg
115            120           125

Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu Lys Gly Cys Pro Leu
130            135           140

Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg Asn Pro Pro Asp Asn
145            150           155           160

Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly Phe Lys Leu Lys Gly
165            170           175

Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln Trp Ser Asn Phe Pro
180            185           190

Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser Ser Pro Glu His Gly
195            200           205

Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu Gly Ala Thr Leu Arg
210            215           220

Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly Gln Glu Thr Leu Thr
225            230           235           240

Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile Pro Gln Cys Lys Asn
245            250           255

Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn His Ala Glu His Lys
260            265           270

Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln Phe Pro Gln Gly Thr
275            280           285

Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe Leu Met Gly Phe Asp
290            295           300

Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser Gly Ser Gln Pro Ser
305            310           315           320

Cys Val Lys Val Ala Asp Arg Glu Val Asp Cys Asp Ser Lys Ala Val
325            330           335

Asp Phe Leu Asp Asp Val Gly Glu Pro Val Arg Ile His Cys Pro Ala
340            345           350

Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly Thr Ala Ile Tyr His
355            360           365

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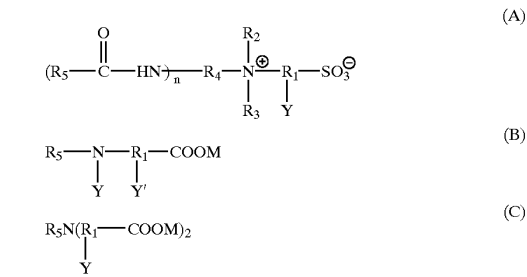
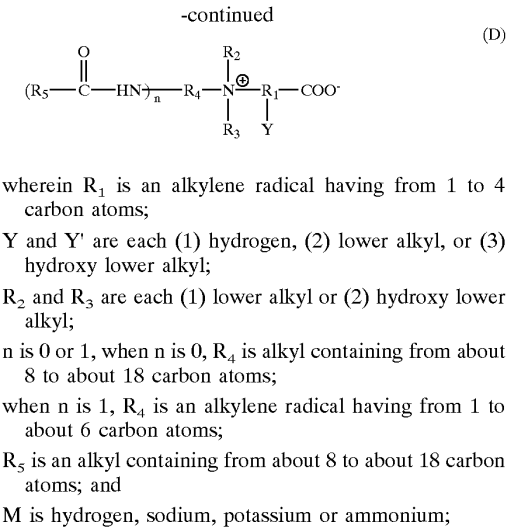
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Asn	Ser	Gly	Gly	Ala	Val	His	Val	Val	Asn	Asn	Gly	Pro	Tyr	Ser	Asp	385	390	395	400
Phe	Leu	Gly	Ser	Asp	Leu	Asn	Gly	Ile	Lys	Ser	Glu	Glu	Leu	Lys	Ser	405	410	415	
Leu	Ala	Arg	Ser	Phe	Arg	Phe	Asp	Tyr	Val	Arg	Ser	Ser	Thr	Ala	Gly	420	425	430	
Lys	Ser	Gly	Cys	Pro	Asp	Gly	Trp	Phe	Glu	Val	Asp	Glu	Asn	Cys	Val	435	440	445	
Tyr	Val	Thr	Ser	Lys	Gln	Arg	Ala	Trp	Glu	Arg	Ala	Gln	Gly	Val	Cys	450	455	460	
Thr	Asn	Met	Ala	Ala	Arg	Leu	Ala	Val	Leu	Asp	Lys	Asp	Val	Ile	Pro	465	470	475	480
Asn	Ser	Leu	Thr	Glu	Thr	Leu	Arg	Gly	Lys	Gly	Leu	Thr	Thr	Thr	Trp	485	490	495	
Ile	Gly	Leu	His	Arg	Leu	Asp	Ala	Glu	Lys	Pro	Phe	Ile	Trp	Glu	Leu	500	505	510	
Met	Asp	Arg	Ser	Asn	Val	Val	Leu	Asn	Asp	Asn	Leu	Thr	Phe	Trp	Ala	515	520	525	
Ser	Gly	Glu	Pro	Gly	Asn	Glu	Thr	Asn	Cys	Val	Tyr	Met	Asp	Ile	Gln	530	535	540	
Asp	Gln	Leu	Gln	Ser	Val	Trp	Lys	Thr	Lys	Ser	Cys	Phe	Gln	Pro	Ser	545	550	555	560
Ser	Phe	Ala	Cys	Met	Met	Asp	Leu	Ser	Asp	Arg	Asn	Lys	Ala	Lys	Cys	565	570	575	
Asp	Asp	Pro	Gly	Ser	Leu	Glu	Asn	Gly	His	Ala	Thr	Leu	His	Gly	Gln	580	585	590	
Ser	Ile	Asp	Gly	Phe	Tyr	Ala	Gly	Ser	Ser	Ile	Arg	Tyr	Ser	Cys	Glu	595	600	605	
Val	Leu	His	Tyr	Leu	Ser	Gly	Thr	Glu	Thr	Val	Thr	Cys	Thr	Thr	Asn	610	615	620	
Gly	Thr	Trp	Ser	Ala	Pro	Lys	Pro	Arg	Cys	Ile	Lys	Val	Ile	Thr	Cys	625	630	635	640
Gln	Asn	Pro	Pro	Val	Pro	Ser	Tyr	Gly	Ser	Val	Glu	Ile	Lys	Pro	Pro	645	650	655	
Ser	Arg	Thr	Asn	Ser	Ile	Ser	Arg	Val	Gly	Ser	Pro	Phe	Leu	Arg	Leu	660	665	670	
Pro	Arg	Leu	Pro	Leu	Pro	Leu	Ala	Arg	Ala	Ala	Lys	Pro	Pro	Pro	Lys	675	680	685	
Pro	Arg	Ser	Ser	Gln	Pro	Ser	Thr	Val	Asp	Leu	Ala	Ser	Lys	Val	Lys	690	695	700	
Leu	Pro	Glu	Gly	His	Tyr	Arg	Val	Gly	Ser	Arg	Ala	Ile	Tyr	Thr	Cys	705	710	715	720
Glu	Ser	Arg	Tyr	Tyr	Glu	Leu	Leu	Gly	Ser	Gln	Gly	Arg	Arg	Cys	Asp	725	730	735	
Ser	Asn	Gly	Asn	Trp	Ser	Gly	Arg	Pro	Ala	Ser	Cys	Ile	Pro	Val	Cys	740	745	750	
Gly	Arg	Ser	Asp	Ser	Pro	Arg	Ser	Pro	Phe	Ile	Trp	Asn	Gly	Asn	Ser	755	760	765	
Thr	Glu	Ile	Gly	Gln	Trp	Pro	Trp	Gln	Ala	Gly	Ile	Ser	Arg	Trp	Leu				

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770					775					780							
Ala	Asp	His	Asn	Met	Trp	Phe	Leu	Gln	Cys	Gly	Gly	Ser	Leu	Leu	Asn		
785					790					795					800		
Glu	Lys	Trp	Ile	Val	Thr	Ala	Ala	His	Cys	Val	Thr	Tyr	Ser	Ala	Thr		
			805						810					815			
Ala	Glu	Ile	Ile	Asp	Pro	Asn	Gln	Phe	Lys	Met	Tyr	Leu	Gly	Lys	Tyr		
			820					825					830				
Tyr	Arg	Asp	Asp	Ser	Arg	Asp	Asp	Asp	Tyr	Val	Gln	Val	Arg	Glu	Ala		
		835					840					845					
Leu	Glu	Ile	His	Val	Asn	Pro	Asn	Tyr	Asp	Pro	Gly	Asn	Leu	Asn	Phe		
850					855						860						
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865					870					875					880		
Val	Gln	Pro	Ile	Cys	Leu	Pro	Thr	Asp	Ile	Thr	Thr	Arg	Glu	His	Leu		
			885						890					895			
Lys	Glu	Gly	Thr	Leu	Ala	Val	Val	Thr	Gly	Trp	Gly	Leu	Asn	Glu	Asn		
			900					905					910				
Asn	Thr	Tyr	Ser	Glu	Thr	Ile	Gln	Gln	Ala	Val	Leu	Pro	Val	Val	Ala		
		915					920					925					
Ala	Ser	Thr	Cys	Glu	Glu	Gly	Tyr	Lys	Glu	Ala	Asp	Leu	Pro	Leu	Thr		
		930				935					940						
Val	Thr	Glu	Asn	Met	Phe	Cys	Ala	Gly	Tyr	Lys	Lys	Gly	Arg	Tyr	Asp		
945					950					955					960		
Ala	Cys	Ser	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Val	Phe	Ala	Asp	Asp	Ser		
			965					970						975			
Arg	Thr	Glu	Arg	Arg	Trp	Val	Leu	Glu	Gly	Ile	Val	Ser	Trp	Gly	Ser		
		980						985					990				
Pro	Ser	Gly	Cys	Gly	Lys	Ala	Asn	Gln	Tyr	Gly	Gly	Phe	Thr	Lys	Val		
		995					1000					1005					
Asn	Val	Phe	Leu	Ser	Trp	Ile	Arg	Gln	Phe	Ile							
	1010					1015											

1. A reagent for detecting endotoxin, comprising:
a purified horseshoe crab Factor C protein; and
a surfactant.
2. The reagent of claim 1 wherein the surfactant is selected from the group consisting of:

(I) amphoteric surfactants represented by the following formulae



(II) anionic surfactants represented by the following formulae:



wherein

R_5 , Y , and M have the same meaning as set forth above;

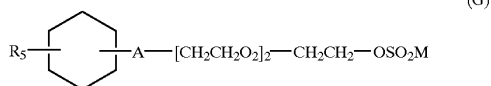
R_6 is an alkyl from 8 to 24 carbon atoms;

$n1$ is an integer from 1 to 3;

$n2$ is 1 or 2; and

Ar is phenyl or naphthyl;

(III) cationic surfactants represented by the following formula:



wherein R_5 , Y , and Y' have the same meaning as set forth above;

(IV) nonionic surfactants represented by the following formula:



wherein

R_5 has the same meaning as set forth above;

R_7 and R_8 are each methyl or ethyl; and

(V) those nonionic surfactants selected from the group consisting of the condensation product of about 10 to 30 moles of ethylene oxide with the monoester of a hexahydric alcohol containing 6 carbon atoms with the ester group containing 10 to 20 carbon atoms.

3. The reagent of claim 1 wherein the horseshoe crab is *Limulus polyphemus*.

4. The reagent of claim 1 wherein the horseshoe crab is *Carcinoscorpius rotundicauda*.

5. The reagent of claim 1 wherein the horseshoe crab is *Tachypleus tridentatus*.

6. The reagent of claim 1 wherein the horseshoe crab is *Tachypleus gigas*.

7. The reagent of claim 1 wherein the Factor C protein is made by the method of culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant.

8. The reagent of claim 7 wherein the host cell is an Sf9 cell.

9. The reagent of claim 7 wherein the horseshoe crab is *Carcinoscorpius rotundicauda*.

10. The reagent of any one of claims 1 through 9 wherein the surfactant is selected from the group consisting of ZWITTERGENT 3-14, Triton X-100, Triton X-114, octyl-beta-D-thioglucoside, Genapol C-100, Tween 20, and Tween 80.

11. A method of detecting endotoxin in a test sample, comprising the steps of:

contacting a test sample with (1) a reagent comprising (a) a purified horseshoe crab Factor C protein and (b) a surfactant and (2) a Factor C substrate, wherein cleavage of the Factor C substrate generates a detectable signal to form a contacted test sample to form a test sample-reagent-substrate mixture; and

assaying the contacted test sample for the presence or absence of the detectable signal, wherein an amount of the detectable signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

12. The method of claim 11 wherein the horseshoe crab is *Limulus polyphemus*.

13. The method of claim 11 wherein the horseshoe crab is *Carcinoscorpius rotundicauda*.

14. The method of claim 11 wherein the horseshoe crab is *Tachypleus tridentatus*.

15. The method of claim 11 wherein the horseshoe crab is *Tachypleus gigas*.

16. The method of claim 11 wherein the Factor C protein is made by the method of:

culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant

17. The method of claim 16 wherein the host cell is an Sf9 cell.

18. The method of claim 15 wherein the horseshoe crab is *Carcinoscorpius rotundicauda*.

19. The method of any one of claims 11-18 wherein the surfactant is selected from the group consisting of ZWITTERGENT 3-14, Triton X-100, Triton X-114, octyl-beta-D-thioglucoside, Genapol C-100, Tween 20, and Tween 80.

20. The method of claim 11 wherein the Factor C substrate is selected from the group consisting of N-t-BOC-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin and N-t-BOC-Val-Pro-Arg-7-Amido-4-methyl coumarin.

21. A method of detecting endotoxin in a test sample, comprising the steps of:

contacting a test sample with (1) a reagent comprising (a) a recombinant *Carcinoscorpius rotundicauda* Factor C protein, wherein the Factor C protein is made by the method of culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant and (b) a surfactant and (2) N-t-BOC-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin to form a contacted test sample; and

assaying the contacted test sample for the presence or absence of a fluorescent signal, wherein an amount of the fluorescent signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

22. The method of claim 21 wherein the host cell is an Sf9 cell.

23. A kit for detecting endotoxin, comprising:

a reagent that comprises (a) a purified horseshoe crab Factor C protein and (b) a surfactant; and

instructions for performing the method of claim 11.

24. The kit of claim 23 further comprising a Factor C substrate, wherein cleavage of the Factor C substrate generates a detectable signal.

25. The kit of claim 24 wherein the Factor C substrate is selected from the group consisting of N-t-BOC-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin and N-t-BOC-Val-Pro-Arg-7-Amido-4-methyl coumarin.

26. The kit of claim 23 wherein the purified Factor C is made by the method of culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant.

27. The kit of claim 26 wherein the host cell is an Sf9 cell.

28. A method of detecting endotoxin in a test sample, comprising the steps of:

contacting a test sample with a reagent comprising (a) a purified horseshoe crab Factor C protein and (b) a surfactant to form a test sample-reagent mixture;

contacting the test sample-reagent mixture with a Factor C substrate, wherein cleavage of the Factor C substrate generates a detectable signal; and

assaying the contacted test sample-reagent mixture for the presence or absence of the detectable signal, wherein an

amount of the detectable signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

29. A method of detecting endotoxin in a test sample, comprising the steps of:

contacting a test sample with a reagent comprising (a) a recombinant *Carcinoscorpius rotundicauda* Factor C protein, wherein the Factor C protein is made by the method of culturing a host cell comprising a vector encoding the Factor C protein in a supernatant under conditions such that the Factor C protein is expressed into the supernatant and (b) a surfactant to form a test sample-reagent mixture;

contacting the test sample-reagent mixture with N-t-BOC-Asp(Obzl)-Pro-Arg-7-Amido-4-methyl coumarin; and

assaying the contacted test sample-reagent mixture for the presence or absence of a fluorescent signal, wherein an amount of the fluorescent signal that is increased relative to a control sample that does not contain endotoxin indicates a presence of endotoxin in the test sample.

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