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(54) Title: EXPRESSION OF CHIMERIC KSAC PROTEIN AND METHOD OF PRODUCING SOLUBLE PROTEINS BY HIGH PRESSURE

(57) Abstract: The present invention encompasses vaccines or compositions comprising the chimeric KSAC protein that possesses immunogenic and protective properties, and methods of use including administering to an animal the antigenic KSAC protein thereof to protect animals. The invention also encompasses methods for making and producing the soluble, disaggregated, refolded or active proteins from inclusion bodies produced from prokaryotes or eukaryotes.

EXPRESSION OF CHIMERIC KSAC PROTEIN AND METHOD OF PRODUCING SOLUBLE PROTEINS BY HIGH PRESSURE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of US provisional application Serial No. 61/694,968 filed August 30, 2012, and US provisional application Serial No. 61/830,425 filed June 3, 2013.

FIELD OF THE INVENTION

[0002] The present invention relates to formulations for combating Leishmania infections in animals or humans. Specifically, the present invention provides pharmaceutical compositions comprising a chimeric Leishmania antigen and method of vaccination against Leishmania. The present invention also relates to methods of producing soluble or disaggregated proteins using high pressure.

BACKGROUND OF THE INVENTION

[0003] Leishmaniasis is a major and severe parasitic disease that affects humans, canines, and to a lesser degree, felines.

[0004] Leishmania and Viannia subgenera are grouped into complexes of species and subspecies based upon molecular, biochemical and immunological similarities. There are several forms of the disease named by their clinical presentation including cutaneous, mucocutaneous or visceral leishmaniasis. Each of these forms of disease is caused by different species of sand flies found in different regions of the world. Cutaneous leishmaniasis of humans is associated with members of L. aethiopica, L. major, and L. tropica complexes in the Old World and L. mexicana and L. braziliensis complexes in the New World. Visceral leishmaniasis is caused by L. donovani and L. infantum in Old World regions while L. chagasi is primarily responsible for visceral disease in the New World. Because L. infantum is the primary agent associated with canine leishmaniasis, infections in dogs often are regarded as visceral even though they tend to cause both visceral and cutaneous disease.

[0005] The agent of visceral leishmaniasis is a protozoan parasite and belongs to the *leishmania donovani* complex. This parasite is widely distributed in temperate and subtropical countries of Southern Europe, Africa, Asia, South America and Central America (Desjeux P. *et al.*, 1984, Nucl. Acids Res., 12:387-395). *Leishmania donovani infantum* (*L. infantum*) is responsible for the feline and canine disease in Southern Europe, Africa, and Asia. In South America and Central America, the agent is *Leishmania donovani chagasi* (*L. chagasi*), which is closely related to *L. infantum*. In humans, the agent is *Leishmania donovani donovani* (*L. donovani*), which is also related to *L. infantum* and *L. chagasi*.

[0006] Leishmaniasis is a slowly progressive disease that can take up to 7 years to become clinically apparent (McConkey SE *et al.*, 2002, Canine Vet J 43:607-609). Even then, signs are frequently nonspecific and a diagnosis of Leishmania is seldomly considered. Dogs are most commonly infected with L. infantum (L. donovani complex) which is responsible for viscerotropic disease in people. However, up to 90% of infected dogs present with both visceral and cutaneous lesions (Slappendel RJ *et al.*, 1998, In: Greene CE: Infectious Diseases of the Dog and Cat, pp450-458). On the other hand, many dogs appear naturally resistant to this parasite and may remain asymptomatic despite known infection (Grosjean NL *et al.*, 2002, Vet Rec 150:241-244). It is estimated that only 10% of dogs residing in endemic areas actually develop clinical disease (Lindsay DS *et al.*, 2002, Compend Cont Educ Pract Vet 24:304-312). This lower incidence of clinical disease is attributed to a genetic predisposition of certain dogs to mount a more protective cell-mediated immune response than a humoral response (Lindsay DS *et al.*, McConkey SE *et al.*, Slappendel RJ, *et al.*). Furthermore, it has been reported that up to 20% of infected dogs may mount an adequate immune response and spontaneously recover from clinical illness (McConkey SE *et al.*). In animals that mount a humoral response, IgG1 appears to correlate with clinical disease while asymptomatic dogs have higher IgG2 antibody levels (Lindsay *et al.*).

[0007] Some of the more frequently reported clinical signs of leishmaniasis include listlessness, fatigue and exercise intolerance coupled with anorexia and weight loss that eventually culminate as wasting disease (McConkey SE *et al.*). These signs may or may not be accompanied by fever, local or generalized lymphadenopathy (90%) and/or

hepatosplenomegaly (Grosjean NL *et al.*, 2003, J Am Vet Med Assoc 222:603-606; Lindsay DS *et al.*, McConkey SE *et al.*; Martínez-Subiela S *et al.*, 2002, Vet Rec 150:241-244). Articular involvement is also fairly common and may present as lameness with swollen joints or simply as a stiff gait. Less common findings include ocular lesions (<5%), chronic diarrhea (30%) and long, deformed brittle nails (20%) referred to as onychogryphosis (Lindsay DS *et al.*, Slappendel RJ *et al.*). Cutaneous lesions are present in up to 89% of infected dogs, with or without overt signs of visceral involvement. Lesions of cutaneous leishmaniasis may occur anywhere on the body but the most common sites are those which are exposed to the environment and are therefore more susceptible to bites from the sand flies. The initial papule rapidly gives rise to an ulcer. Viseral leishmaniasis is invariably fatal if not treated promptly. Viseral leishmaniasis affects the internal body organs, specifically the spleen and the liver.

[0008] Dogs are considered the major reservoir of Leishmaniasis. The disease is characterized by chronic evolution of viscero-cutaneous signs occurring in less than 50% of infected animals (Lanotte G. *et al.*, 1979, Ann. Parasitol. Hum. Comp. 54:277-95). Both asymptomatic and symptomatic dogs with detectable antibodies may be infectious (Molina R. *et al.*, 1994, Trans. R. Soc. Med. Hyg. 88:491-3; Courtenay O. *et al.*, 2002, J. Infect. Dis., 186 :1314-20). Cats may also be carriers of the protozoan parasites and are thus considered secondary potential reservoirs.

[0009] Due to a number of factors, treatment options for leishmaniasis in dogs and response to therapy are limited at best. For some undefined reason, visceral leishmaniasis is more difficult to treat in dogs than in humans. No treatment option is 100% effective in clearing parasitic infection and clinical disease often reappears with cessation of therapy (Lindsay DS *et al.*). In endemic areas, the most common treatment regimen has been a combination of allopurinol with a pentavalent antimonial such as meglumine antimonite or sodium stibogluconate (Lindsay DS *et al.*, Slappendel RJ *et al.*). However, in recent years this protocol has fallen out of favor due to increasing resistance of the parasite to the drug as well as adverse side effects associated with these compounds (Lindsay DS *et al.*). To further limit treatment options, PENTOSTAM® (sodium stibogluconate) is the only available antimonial in the United States and its distribution is regulated by the Centers for Disease Control and Prevention (CDC) in Atlanta, GA

(Lindsay DS *et al.*). Other investigations have sought to identity methods of preventing and treating leishmaniasis through, for example, administration of antigenic fusion polypeptides (see US 2009/0291099 which is hereby incorporated herein by reference in its entirety).

[0010] Different protocols have been tried but have proven no more efficacious at clearing parasitic infection or at preventing clinical relapse. In addition, each protocol is associated with potential adverse effects. Amphotericin B binds sterols and disrupts cell membrane permeability but is nephrotoxic (Lindsay DS *et al.*). When given parenterally, Paramomycin acts synergistically with antimonials causing higher levels of the antimonial for longer periods of time but is also nephrotoxic and is not currently recommended for clinical use (Lindsay DS *et al.*). Pentamidine isethionate is effective against leishmaniasis but requires at least 15 intramuscular injections and is quite painful (Lindsay DS *et al.*). Ketaconazole, miconazole, fluconazole and itraconazole are oral drugs that may be useful in containing the disease but are cost prohibitive and carry the risk of drug resistance when treating patients symptomatically. In summary, the various treatment regimens for leishmaniasis in dogs have been investigated but are not 100% efficacious; relapses are the rule rather than the exception. Ultimately, the veterinary practitioner is faced with the dilemma of treating symptomatic outbreaks of leishmaniasis in dogs at the risk of developing drug resistant strains of this parasite within the United States.

[0011] Mass detection of seropositive dogs followed by culling and/or drug treatment, or the mass application of deltamethrin-impregnated collars, was shown to have an impact in reducing human and canine Leishmaniasis prevalence in endemic areas of Southern Europe, Africa, and Asia (Maroli M. *et al.*, 2001, Med. Vet. Entomol. 15:358-63; Mazloumi Gavgani A.S. *et al.*, 2002, Lancet 360:374-9), although the efficacy of eliminating seropositive canines has been debated (Dietze R. *et al.*, 1997, Clin. Infect. Dis. 25:1240-2; Moreira Jr. E.D. *et al.*, 2004, Vet. Parasitol. 122:245-52). These control measures are either considered unacceptable, expensive or not effective (Gradoni L. *et al.*, 2005, Vaccine 23:5245-51).

[0012] Mathematical models used to compare the effectiveness of various tools for controlling Leishmaniasis suggest that a canine vaccine may be the most practical and

effective method (Dye C., 1996, Am. J. Trop. Med. Hyg. 55:125-30). Therefore, the development of vaccines able to protect canines from Leishmaniasis and/or to prevent disease progression in infected animals is highly desirable for the implementation of Leishmaniasis control programs as well for the veterinary community (Gradoni L. *et al.*).

[0013] Haynes et al. (Biotechnol. Prog., 2010, Vol. 26, No. 3, 743-749) discuss the use of high hydrostatic pressure to achieve high solubility and high refolding yields of growth hormone (GH) produced in E.coli inclusion bodies. US6,489,450, US7,064,192, US7,767,795 and US7,615,617 disclose reversing aggregation and increasing refolding of denatured proteins by application of high pressure.

[0014] There remains a need for effective and efficient methods of producing subunit (protein) vaccine for the treatment of Leishmania. The vaccine formulation and the method of producing such vaccine of the present invention fulfill this long felt need in the art.

SUMMARY OF THE INVENTION

[0015] The present invention demonstrated for the first time that a chimeric KSAC protein expressed in E. coli inclusion bodies was substantially solubilized and refolded after high pressure treatment.

[0016] The present invention showed surprising result that application of stepwise increase of pressure coupled with prolonged treatment of inclusion bodies under high pressure produced high yield of soluble, disaggregated, refolded and active proteins.

[0017] Compositions and vaccines comprising the chimeric KSAC protein are provided. Such vaccines or compositions can be used to vaccinate an animal and provide protection against Leishmaniasis. The KSAC protein may be expressed in E. coli inclusion bodies and is subsequently solubilized by high pressure treatment. The KSAC protein possesses immunogenic and protective properties.

[0018] Methods of the invention include methods for making and producing soluble, disaggregated, refolded or active proteins from inclusion bodies under high pressure for a prolonged period of time. Methods also include the methods of use including administering to an animal an effective amount of the antigenic KSAC protein thereof to elicit a protective immunogenic response.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The following detailed description, given by way of example, and which is not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying figures, incorporated herein by reference, in which:

[0020] Figure 1 is a table showing the SEQ ID NO assigned to each DNA and protein sequence.

[0021] Figures 2A-2C show the DNA and protein sequences.

[0022] Figure 3 is a graph representation of the pressure and time treatment of KSAC inclusion bodies.

[0023] Figure 4 is a schematic representation of comparison between high pressure treatment and classical chromatography refolding.

[0024] Figure 5 is the graphic representation of KSAC refolding process.

[0025] Figure 6 depicts the SDS-PAGE of KSAC refolded by exclusion chromatography.

[0026] Figure 7 depicts the HPLC plot of refolded KSAC by exclusion chromatography.

[0027] Figures 8A and 8B depict the dynamic light scattering (DLS) of refolded KSAC protein using exclusion chromatography.

[0028] Figure 9 depicts the Qdot-blot of KSAC samples treated with 3000 bar.

[0029] Figure 10 depicts the SDS-PAGE of KSAC samples treated with 3000 bar.

[0030] Figure 11 depicts the HPLC of control samples and solubilized KSAC after 3000 bar treatment.

[0031] Figure 12 depicts the superposed HPLC chromatogram of the supernatant of the 3000 bar pressure treated KSAC samples and the KSAC protein obtained with the classical refolding process.

[0032] Figure 13 depicts the superposition of the DLS data obtained with the 3000 bar pressurized protein in the buffer without urea and the protein obtained with the classical refolding process.

[0033] Figure 14 shows the effect of pressure and buffer on the protein sizes.

[0034] Figure 15 depicts the HPLC chromatogram of 4000 bar treated samples.

[0035] Figure 16 shows the DLS size distribution by number of the 5000 bar treated samples without urea.

[0036] Figure 17 shows the comparison of KSAC soluble protein content determined by HPLC and Qdot-blot.

[0037] Figures 18A and 18B depict the SDS-PAGE analysis of KSAC samples after high pressure treatments.

[0038] Figures 19A-19D depict the Q-Dot Blott analysis of KSAC samples after high pressure treatments.

[0039] Figure 20 depicts the HPLC analysis of KSAC samples after process A treatment.

[0040] Figure 21 depicts the HPLC analysis of KSAC samples after process B treatment.

[0041] Figure 22 depicts the HPLC analysis of KSAC samples after process A, process B and classical process treatments.

DETAILED DESCRIPTION OF THE INVENTION

[0042] It is noted that in this disclosure and particularly in the claims, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[0043] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, Genes V. published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8). The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicate otherwise. The word "or" means any one member of a particular list and also includes any combination of members of that list.

[0044] The term "animal" is used herein to include all mammals, birds and fish. The animal as used herein may be selected from the group consisting of equine (e.g., horse),

canine (e.g., dogs, wolves, foxes, coyotes, jackals), feline (e.g., lions, tigers, domestic cats, wild cats, other big cats, and other felines including cheetahs and lynx), bovine (e.g., cattle), swine (e.g., pig), ovine (e.g., sheep, goats, lamas, bisons), avian (e.g., chicken, duck, goose, turkey, quail, pheasant, parrot, finches, hawk, crow, ostrich, emu and cassowary), primate (e.g., prosimian, tarsier, monkey, gibbon, ape), humans, and fish. The term "animal" also includes an individual animal in all stages of development, including embryonic and fetal stages.

[0045] The terms "polypeptide" and "protein" are used interchangeably herein to refer to a polymer of consecutive amino acid residues.

[0046] The terms "nucleic acid", "nucleotide", and "polynucleotide" are used interchangeably and refer to RNA, DNA, cDNA, or cRNA and derivatives thereof, such as those containing modified backbones. It should be appreciated that the invention provides polynucleotides comprising sequences complementary to those described herein. The "polynucleotide" contemplated in the present invention includes both the forward strand (5' to 3') and reverse complementary strand (3' to 5'). Polynucleotides according to the invention can be prepared in different ways (e.g. by chemical synthesis, by gene cloning etc.) and can take various forms (e.g. linear or branched, single or double stranded, or a hybrid thereof, primers, probes etc.).

[0047] The term "genomic DNA" or "genome" is used interchangeably and refers to the heritable genetic information of a host organism. The genomic DNA comprises the DNA of the nucleus (also referred to as chromosomal DNA) but also the DNA of the plastids (e.g., chloroplasts) and other cellular organelles (e.g., mitochondria). The genomic DNA or genome contemplated in the present invention also refers to the RNA of a virus. The RNA may be a positive strand or a negative strand RNA. The term "genomic DNA" contemplated in the present invention includes the genomic DNA containing sequences complementary to those described herein. The term "genomic DNA" also refers to messenger RNA (mRNA), complementary DNA (cDNA), and complementary RNA (cRNA).

[0048] The term "gene" is used broadly to refer to any segment of polynucleotide associated with a biological function. Thus, genes or polynucleotides include introns and exons as in genomic sequence, or just the coding sequences as in cDNAs , such as an

open reading frame (ORF), starting from the start codon (methionine codon) and ending with a termination signal (stop codon). Genes and polynucleotides can also include regions that regulate their expression, such as transcription initiation, translation and transcription termination. Thus, also included are promoters and ribosome binding regions (in general these regulatory elements lie approximately between 60 and 250 nucleotides upstream of the start codon of the coding sequence or gene; Doree S M *et al.*; Pandher K *et al.*; Chung J Y *et al.*), transcription terminators (in general the terminator is located within approximately 50 nucleotides downstream of the stop codon of the coding sequence or gene; Ward C K *et al.*). Gene or polynucleotide also refers to a nucleic acid fragment that expresses mRNA or functional RNA, or encodes a specific protein, and which includes regulatory sequences.

[0049] The term "heterologous DNA" as used herein refers to the DNA derived from a different organism, such as a different cell type or a different species from the recipient. The term also refers to a DNA or fragment thereof on the same genome of the host DNA wherein the heterologous DNA is inserted into a region of the genome which is different from its original location.

[0050] As used herein, the term "antigen" or "immunogen" means a substance that induces a specific immune response in a host animal. The antigen may comprise a whole organism, killed, attenuated or live; a subunit or portion of an organism; a recombinant vector containing an insert with immunogenic properties; a piece or fragment of DNA capable of inducing an immune response upon presentation to a host animal; a polypeptide, an epitope, a hapten, or any combination thereof. Alternately, the immunogen or antigen may comprise a toxin or antitoxin.

[0051] The term "immunogenic protein or peptide" as used herein includes polypeptides that are immunologically active in the sense that once administered to the host, it is able to evoke an immune response of the humoral and/or cellular type directed against the protein. Preferably the protein fragment is such that it has substantially the same immunological activity as the total protein. Thus, a protein fragment according to the invention comprises or consists essentially of or consists of at least one epitope or antigenic determinant. An "immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of the protein, analogs thereof, or immunogenic

fragments thereof. By "immunogenic fragment" is meant a fragment of a protein which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number of epitope mapping techniques, well known in the art.

[0052] The term "immunogenic protein or peptide" further contemplates deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein. The term "conservative variation" denotes the replacement of an amino acid residue by another biologically similar residue, or the replacement of a nucleotide in a nucleic acid sequence such that the encoded amino acid residue does not change or is another biologically similar residue. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic--aspartate and glutamate; (2) basic--lysine, arginine, histidine; (3) non-polar--alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar--glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another hydrophobic residue, or the substitution of one polar residue for another polar residue, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like; or a similar conservative replacement of an amino acid with a structurally related amino acid that will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein are, therefore, within the definition of the reference polypeptide. All of the polypeptides produced by these modifications are included herein. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

[0053] The term "epitope" refers to the site on an antigen or hapten to which specific B cells and/or T cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site". Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

[0054] An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to a composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, and/or cytotoxic T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host, a quicker recovery time and/or a lowered viral titer in the infected host.

[0055] The terms “recombinant” and “genetically modified” are used interchangeably and refer to any modification, alteration or engineering of a polynucleotide or protein in its native form or structure, or any modification, alteration or engineering of a polynucleotide or protein in its native environment or surrounding. The modification, alteration or engineering of a polynucleotide or protein may include, but is not limited to, deletion of one ore more nucleotides or amino acids, deletion of an entire gene, codon-optimization of a gene, conservative substitution of amino acids, insertion of one or more heterologous polynucleotides.

[0056] The term “inclusion bodies” as used herein refers to inactive aggregates of heterologous proteins expressed in prokaryotes or eukaryotes. The terms “substantially soluble”, “substantially solubilized”, “substantially disaggregated” or “substantially refolded” are used interchangeably herein to refer to aggregated proteins in inclusion bodies that are at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% soluble in aqueous solution, or are disaggregated, or are refolded to active form after treatments. Refolding means that a fully or partially denatured protein adopts secondary, tertiary and quaternary structure like that of the native molecule.

[0057] One embodiment of the invention provides a composition or vaccine comprising a protein produced from E. coli. The protein may be a fusion protein comprising two or more immunogenic portions of Leishmania protein selected from Kinetoplastid Membrane Protein 11 (KMP11), Sterol MethylTransferase (SMT), A2 and Cysteine Proteinase (CP). The protein may be a fusion protein comprising Leishmania KMP11, SMT, A2 and CP designated as chimeric KSAC protein. In one aspect of the embodiment, the KSAC protein is solubilized from the *E.coli* inclusion bodies by a high pressure. In another aspect, the KSAC protein is substantially soluble in an aqueous solution or substantially refolded.

[0058] Moreover, homologs of aforementioned proteins or polynucleotides are intended to be within the scope of the present invention. As used herein, the term "homologs" includes orthologs, analogs and paralogs. The term "analogs" refers to two polynucleotides or polypeptides that have the same or similar function, but that have evolved separately in unrelated organisms. The term "orthologs" refers to two polynucleotides or polypeptides from different species, but that have evolved from a common ancestral gene by speciation. Normally, orthologs encode polypeptides having the same or similar functions. The term "paralogs" refers to two polynucleotides or polypeptides that are related by duplication within a genome. Paralogs usually have different functions, but these functions may be related. Analogs, orthologs, and paralogs of a wild-type polypeptide can differ from the wild-type polypeptide by post-translational modifications, by amino acid sequence differences, or by both. In particular, homologs of the invention will generally exhibit at least 80-85%, 85-90%, 90-95%, or 95%, 96%, 97%, 98%, 99% sequence identity, with all or part of the polynucleotide or polypeptide sequences described above, and will exhibit a similar function.

[0059] In one embodiment, the chimeric KSAC protein has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a polypeptide having the sequence as set forth in SEQ ID NO:2. In another embodiment, the polynucleotide encoding the chimeric KSAC protein has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a polypeptide having the sequence as set forth in SEQ ID NO:2. In yet another embodiment, the KSAC encoding polynucleotide has at

least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a polynucleotide having the sequence as set forth in SEQ ID NO:1.

[0060] The term "identity" with respect to sequences can refer to, for example, the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman). The sequence identity or sequence similarity of two amino acid sequences, or the sequence identity between two nucleotide sequences can be determined using Vector NTI software package (Invitrogen, 1600 Faraday Ave., Carlsbad, CA). When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus, RNA sequences are within the scope of the invention and can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

[0061] The polynucleotides of the disclosure include sequences that are degenerate as a result of the genetic code, e.g., optimized codon usage for a specific host. As used herein, "optimized" refers to a polynucleotide that is genetically engineered to increase its expression in a given species. To provide optimized polynucleotides coding for KSAC polypeptides, the DNA sequence of the KSAC protein gene can be modified to 1) comprise codons preferred by highly expressed genes in a particular species; 2) comprise an A+T or G+C content in nucleotide base composition to that substantially found in said species; 3) form an initiation sequence of said species; or 4) eliminate sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA, or that form secondary structure hairpins or RNA splice sites. Increased expression of KSAC protein in said species can be achieved by utilizing the distribution frequency of codon usage in eukaryotes and prokaryotes, or in a particular species. The term "frequency of preferred codon usage" refers to the preference exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the disclosure as long as the amino acid

sequence of the KSAC polypeptide encoded by the nucleotide sequence is functionally unchanged.

[0062] In another embodiment, the present invention provides a method for producing a soluble, disaggregated, refolded or active protein expressed in prokaryotes or eukaryotes comprising the steps of (i) preparing the inclusion bodies in a buffer containing no or low concentration of urea to form inclusion body suspension; and (ii) subjecting the inclusion body suspension to a high pressure for a period of time.

[0063] In yet another embodiment, the present invention provides a method of producing a soluble, disaggregated, refolded or active protein expressed in prokaryotes or eukaryotes comprising the steps of (i) preparing the inclusion bodies in a buffer containing no or low concentration of urea to form inclusion body suspension; (ii) subjecting the inclusion body suspension to a gradual increase of pressure over a period of time; and (iii) maintaining the high pressure applied to the inclusion bodies for a period of time.

[0064] In one aspect of the embodiment, the buffer may contain DiThio Threitol (DTT). In another aspect, the DTT concentration may range from about 1mM to about 100mM, about 1mM to about 90mM, about 1mM to about 70mM, about 1mM to about 60mM, about 1mM to about 50mM, or about 1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM, 55mM, 60mM, 65mM, 70mM, 75mM, 80mM, 85mM, 90mM, 95mM, 100mM.

[0065] In one aspect, urea may not be present in the buffer. In another aspect, urea may be present in the buffer at the concentration of about 1M, about 2M, about 3M, about 4M, about 5M, about 6M, about 7M, about 8M, about 9 M, and about 10M.

[0066] In another aspect of the embodiment, the high pressure may be in the range from about 1000 bar to about 5000 bar, from about 2000 bar to about 4000 bar. The high pressure may be any pressure in the range from about 2000 bar to about 4000 bar, for example, but not limiting to, 2000 bar, 2100 bar, 2200 bar, 2300 bar, 2400 bar, 2500 bar, 2600 bar, 2700 bar, 2800 bar, 2900 bar, 3000 bar, 3100 bar, 3200 bar, 3300 bar, 3400 bar, 3500 bar, 3600 bar, 3700 bar, 3800 bar, 3900 bar, and 4000 bar.

[0067] In another aspect of the embodiment, the gradual increase of the pressure may be done continuously or stepwise. In one aspect, the gradual increase of the pressure is

applied to the inclusion body suspension by continuously increasing the pressure at a constant rate over a period of time to reach the desired final high pressure. For example, the pressure is increased at the rate of about 200 bar/min – about 1000 bar/min continuously over about 2 min – about 10 min to reach 2000 bar, at the rate of about 200 bar/min – about 1000 bar/min continuously over about 3 min – about 15 min to reach 3000 bar, at the rate of about 200 bar/min – about 1000 bar/min continuously over about 4 min – about 20 min to reach 4000 bar, at the rate of about 200 bar/min – about 1000 bar/min continuously over about 5 min – about 25 min to reach 5000 bar. In another aspect, the gradual increase of the pressure is applied stepwise. For example, the pressure is increased at about 1000 bar/min for about one minute to reach 1000 bar, then the 1000 bar pressure is maintained for about one hour to relax the protein, after the relaxation period, the pressure is increased again at about 1000 bar/min for about one minute to reach the final desired high pressure of 2000 bar. The pressure may also be increased at about 1000 bar/min for about thirty seconds to reach 500 bar, the 500 bar pressure is maintained for about one hour to relax the protein, then the pressure is increased again at about 1000 bar/min for about thirty seconds to reach 1000 bar, the 1000 bar pressure is maintained for about one hour to relax the protein, the pressure is increased again at about 1000 bar/min for about thirty seconds to reach 1500 bar, the 1500 bar pressure is maintained for about one hour to relax the protein, then the pressure is increased again at about 1000 bar/min to reach the final desired pressure of 2000 bar. To reach the final desired high pressure of 3000 bar, 4000 bar, and 5000 bar, the same stepwise increase of the pressure at about 1000 bar/min for about one minute or about 30 seconds with intermediate relaxation of protein for about one hour may be employed. For example, to reach the target pressure of 3000 bar, the pressure is increased at about 1000 bar/min for about one minute to reach 1000 bar, then the 1000 bar pressure is maintained for about one hour to relax the protein, the pressure is increased again at about 1000 bar/min for about one minute to reach the pressure of 2000 bar, then the 2000 bar pressure is maintained for about one hour to relax the protein for the second time, the pressure is increased again at about 1000 bar/min for about one minute to reach the final desired pressure of 3000 bar. To reach the target pressure of 3000 bar, the pressure can also be increased at about 1000 bar/min for about thirty seconds, with a plateau of 1 hour

duration at each 500 bar, and the target pressure of 3000 bar may be reached after 5 hr. To reach the final desired pressure of 4000 bar, the pressure is increased at about 1000 bar/min for about one minute to reach 1000 bar, then the 1000 bar pressure is maintained for about one hour to relax the protein, the pressure is increased again at about 1000 bar/min for about one minute to reach the pressure of 2000 bar, then the 2000 bar pressure is maintained for about one hour to relax the protein for the second time, the pressure is increased again at about 1000 bar/min for about one minute to reach the final desired pressure of 3000 bar, then the 3000 bar pressure is maintained for about one hour to relax the protein for the third time, the pressure is increased again at 1000 bar/min for about one minute to reach the final desired pressure of 4000 bar. To reach the target pressure of 4000 bar, the pressure can also be increased at about 1000 bar/min for about thirty seconds, with a plateau of 1 hour duration at each 500 bar, and the target pressure of 4000 bar may be reached after 7 hr.

[0068] The inclusion body suspension may be treated under the high pressure for about 10 hours to about 100 hours, about 20 hours to about 100 hours. The high pressure treatment is preferably for more than 24 hours, for example, for about 25 hours to about 100 hours, about 25 hours to about 80 hours, about 25 hours to about 60 hours, about 25 hours to about 50 hours, about 25 hours, about 26 hours, about 27 hours, about 28 hours, about 29 hours, about 30 hours, about 31 hours, about 32 hours, about 33 hours, about 34 hours, about 35 hours, about 36 hours, about 37 hours, about 38 hours, about 39 hours, about 40 hours, about 41 hours, about 42 hours, about 43 hours, about 44 hours, about 45 hours, about 46 hours, about 47 hours, about 48 hours, about 49 hours, about 50 hours.

[0069] In another embodiment, the present invention provides a method for producing a soluble, disaggregated, refolded or active protein expressed in prokaryotes or eukaryotes comprising the steps of (i) preparing the inclusion bodies in a buffer containing no or low concentration of urea to form inclusion body suspension; (ii) subjecting the inclusion body suspension to a gradual increase of pressure over a period of time; (iii) maintaining the high pressure applied to the inclusion bodies for a period of time; and (iv) recovering the protein by depressurization.

[0070] Depressurization may be performed at the rate of about 83 bar/hr – 200 bar/hr.

[0071] The prokaryotes contemplated in the present invention may include *Avibacterium, Brucella, Escherichia coli, Haemophilus* (e.g., Haemophilus suis*), Salmonella* (e.g., Salmonella enteridis, Salmonella typhimurium, Salmonella infantis*), Shigella, Pasteurella*, and R*imeirella*.

[0072] In prokaryotic systems, a number of expression vectors may be selected. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as PBLUESCRIPT (Stratagene); piN vectors (Van Heeke & Schuster, *J. Biol. Chern.* 264:5503-5509 (1989)); and the like; PGEX Vectors (Promega, Madison, Wis.); In eukaryotic systems, the cell lines may be yeast (such as *Saccharomyces cerevisiae, Pichia pastoris)*, baculovirus cells, mammalian cells, plant cells. The expression vectors of eukaryotic systems include, but are not limited to, pVR1020 or pVT1012 vectors (Vical Inc., San Diego, CA), PichiaPink Vector (Invitrogen, CA, USA), pFasBac TOPO vector (Invitrogen).

[0073] The method for producing a soluble, disaggregated, refolded or active protein expressed in prokaryotes or eukaryotes provided in the present invention may be used to solubilize any proteins. The proteins may include antibodies and insulin.

[0074] In another embodiment, the present invention provides a composition or vaccine comprising the chimeric KSAC protein aforementioned and a pharmaceutically or veterinarily acceptable carrier, excipient, vehicle or adjuvant.

[0075] The pharmaceutically or veterinarily acceptable carriers or adjuvant or vehicles or excipients are well known to the one skilled in the art. The pharmaceutically or veterinarily acceptable carrier or adjuvant or vehicle or excipients that can be used for methods of this invention include, but are not limited to, 0.9% NaCl (e.g., saline) solution or a phosphate buffer, poly-(L-glutamate) or polyvinylpyrrolidone. The pharmaceutically or veterinarily acceptable carrier or vehicle or excipients may be any compound or combination of compounds facilitating the administration of the vector (or protein expressed from an inventive vector *in vitro*), or facilitating transfection or infection and/or improve preservation of the vector (or protein). Doses and dose volumes are herein discussed in the general description and can also be determined by the skilled artisan from this disclosure read in conjunction with the knowledge in the art, without any undue experimentation.

[0076] Optionally other compounds may be added as pharmaceutically or veterinarily acceptable carriers or adjuvant or vehicles or excipients, including, but not limited to, alum; CpG oligonucleotides (ODN), in particular ODN 2006, 2007, 2059, or 2135 (Pontarollo R.A. *et al., Vet. Immunol. Immunopath*, 2002, 84: 43-59; Wernette C.M. *et al., Vet. Immunol. Immunopath,* 2002, 84: 223-236; Mutwiri G. *et al., Vet. Immunol. Immunopath,* 2003, 91: 89-103); polyA-polyU, dimethyldioctadecylammonium bromide (DDA) ("Vaccine Design The Subunit and Adjuvant Approach", edited by Michael F. Powell and Mark J. Newman, *Pharmaceutical Biotechnology*, 6: p.03, p.157); N,N-dioctadecyl-N',N'-bis(2-hydroxyethyl) propanediamine (such as AVRIDINE®) (*Ibid*, p. 148); carbomer, chitosan (*see* US Patent No. 5,980,912 for example).

[0077] The pharmaceutical compositions and vaccines according to the invention may comprise or consist essentially of one or more adjuvants. Suitable adjuvants for use in the practice of the present invention are (1) polymers of acrylic or methacrylic acid, maleic anhydride and alkenyl derivative polymers, (2) immunostimulating sequences (ISS), such as oligodeoxyribonucleotide sequences having one or more non-methylated CpG units (Klinman et al., 1996; WO98/16247), (3) an oil in water emulsion, such as the SPT emulsion described on p 147 of "Vaccine Design, The Subunit and Adjuvant Approach" published by M. Powell, M. Newman, Plenum Press 1995, and the emulsion MF59 described on p 183 of the same work, (4) cation lipids containing a quaternary ammonium salt, e.g., DDA (5) cytokines, (6) aluminum hydroxide or aluminum phosphate, (7) saponin or (8) other adjuvants discussed in any document cited and incorporated by reference into the instant application, or (9) any combinations or mixtures thereof.

[0078] In one embodiment, a solution of adjuvant, especially of carbomer (*Pharmeuropa*, vol. 8, No.2, June 1996), is prepared in distilled water, advantageously in the presence of sodium chloride, the solution obtained being at an acidic pH. This stock solution is diluted by adding it to the desired quantity (for obtaining the desired final concentration), or a substantial part thereof, of water charged with NaCl, advantageously physiological saline (NaCl 9 g/l) all at once in several portions with concomitant or subsequent neutralization (pH 7.3 to 7.4), advantageously with NaOH. This solution at physiological pH is used for mixing with the vaccine, which may be especially stored in freeze-dried,

liquid or frozen form. The polymer concentration in the final vaccine composition can be from 0.01% to 2% w/v, from 0.06 to 1% w/v, or from 0.1 to 0.6% w/v.

[0079] The subunit (protein) vaccine may be combined with adjuvants, like oil-in-water, water-in-oil-in-water emulsions based on mineral oil and/or vegetable oil and non ionic surfactants such as block copolymers, TWEEN®, SPAN®. Such emulsions are notably those described in page 147 of "Vaccine Design – The Subunit and Adjuvant Approach", Pharmaceutical Biotechnology, 1995, or TS emulsions, notably the TS6 emulsion, and LF emulsions, notably LF2 emulsion (for both TS and LF emulsions, see WO 04/024027). Other suitable adjuvants are for example vitamin E, saponins, and CARBOPOL® (Noveon; see WO 99/51269; WO 99/44633), aluminium hydroxide or aluminium phosphate ("Vaccine Design, The subunit and adjuvant approach", Pharmaceutical Biotechnology, vol. 6, 1995), biological adjuvants (i.e. C4b, notably murine C4b (Ogata R T *et al.*) or equine C4b, GM-CSF, notably equine GM-CSF (US 6,645,740)), toxins (i.e. cholera toxins CTA or CTB, *Escherichia coli* heat-labile toxins LTA or LTB (Olsen C W *et al.*; Fingerut E *et al.*; Zurbriggen R *et al.* Peppoloni S *et al.*), and CpG (i.e. CpG #2395 (see Jurk M *et al.*), CpG #2142 (see SEQ. ID. NO: 890 in EP 1,221,955)).

[0080] Another aspect of the invention relates to a method for inducing an immunological response in an animal against one or more antigens or a protective response in an animal against one or more pathogens, which method comprises inoculating the animal at least once with the vaccine or pharmaceutical composition of the present invention. Yet another aspect of the invention relates to a method for inducing an immunological response in an animal to one or more antigens or a protective response in an animal against one or more Leishmania pathogens in a prime-boost administration regime, which is comprised of at least one primary administration and at least one booster administration using at least one common polypeptide, antigen, epitope or immunogen. The immunological composition or vaccine used in primary administration may be same, may be different in nature from those used as a booster. The prime-administration may comprise one or more administrations. Similarly, the boost administration may comprise one or more administrations. The prime-boost administrations may be carried out 2 to 6 weeks apart, for example, about 3 weeks apart. According to one embodiment, a semi-

annual booster or an annual booster, advantageously using the subunit (protein) vaccine, is also envisioned.

[0081] The prime-boost administration can include the subunit vaccine or composition comprising the chimeric KSAC protein aforementioned. The prime-boost administration can also include recombinant viral vectors and plasmid vectors expressing Leishmania antigens (see, for example, US2009/0324649 which is hereby incorporated herein by reference in its entirety). In one aspect of the prime-boost regime, the composition or vaccine comprising the KSAC protein is administered followed by the administration of vaccine or composition comprising a recombinant viral vector that contains and expresses any Leishmania antigens, or a DNA plasmid vaccine or composition that contains and expresses any Leishmania antigens, or an inactivated viral vaccine or composition comprising the Leishmania antigens.

[0082] Usually, one administration of the vaccine is performed at 10 to 15-week old by the subcutaneous or intramuscular route. A second or third administration can be done within the 2-6 weeks of the first administration. The animals are preferably at least 4-week old at the time of the first administration.

[0083] A variety of administration routes may be used in addition to subcutaneously or intramuscularly, such as intradermally or transdermally.

[0084] The composition or vaccine according to the invention comprise or consist essentially of or consist of an effective quantity to elicit a therapeutic response of one or more polypeptides as discussed herein; and, an effective quantity can be determined from this disclosure, including the documents incorporated herein, and the knowledge in the art, without undue experimentation.

[0085] For the composition or vaccine comprising the expressed KSAC protein of the present invention, a dose may include, from about 1μg to about 2000μg, about 5μg to about 1000μg, about 10μg to about 100μg, about 20μg to about 1000μg, about 30 μg to about 500 μg, or about 50μg to about 500μg. The dose volumes can be between about 0.1ml to about 10ml, or between about 0.2ml to about 5ml.

[0086] The invention will now be further described by way of the following non-limiting examples.

EXAMPLES

[0087] Construction of DNA inserts, plasmids and recombinant viral or plant vectors was carried out using the standard molecular biology techniques described by J. Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

Example 1 Solubilization of KSAC protein expressed in E. coli inclusion bodies

[0088] The KSAC inclusion bodies produced from E. coli were prepared in the following three buffers: 1) Tris 20mM, 50mM DiThio Threitol (DTT), pH8; 2) Tris 20mM, 50mM DiThio Threitol (DTT), pH8, urea 1M; 3) Tris 20mM, 50mM DiThio Threitol (DTT), pH8, urea 2M. The KSAC inclusion bodies prepared in the same buffers at room temperature without pressure during the entire treatment duration were used as controls.

[0089] Figure 3 shows the steps of pressure levels for protein relaxation during the pressure increase (stepwise increase of pressure). Pressurization at target pressure was applied for 48 hours, then the samples were depressurized for 24 hours.

[0090] Figure 4 shows the schematic comparison between the high pressure solubilisation and folding of recombinant proteins from inclusion bodies and the classical solubilisation and folding of recombinant proteins from inclusion bodies.

[0091] Figure 5 shows the schematic graph of KSAC refolding. Figure 6 shows the typical SDS-PAGE pattern of the KSAC protein after the KSAC protein was solubilized in 7M urea, 20mM DTT and refolded by exclusion chromatography. Figure 7 shows the typical HPLC plot of the KSAC protein. The HPLC chromatogram identifies the trimer of KSAC protein by its mass. The protein concentration and the relative purity towards total protein content were estimated. Figure 8 shows the dynamic light scattering (DLS) of refolded KSAC protein using exclusion chromatography. Fig.8A shows the distribution by number which indicates that the majority of the population has a size of 12 to 18nm. Fig.8B shows the exhaustive range of size detected that is not linked to relative population. Objects between 10 and 800nm were detected.

[0092] The Qdot-blot (Fig. 9) and SDS-PAGE (Fig. 10) analysis of the inclusion bodies pressurized at 3000 bars and the control samples showed that all proteins are in the

supernatant phase of the treated sample in all three buffers which indicates that the proteins are solubilized.

[0093] Superposition of the HPLC chromatograms of the supernatant of the controls (Fig.11 upper panel) and all the 3000 bar treated samples (Fig.11 lower panel) showed that no protein is detected in the controls while the KSAC protein and all the other proteins present in the inclusion bodies are detected after pressure treatment which means that all the proteins are solubilized after pressure treatment.

[0094] Figure 12 is the superposed HPLC chromatogram of the supernatant of the 3000 bar pressure treated KSAC samples and the KSAC protein obtained with the classical refolding process. The results show that the peaks are similar that the soluble protein obtained by high pressure treatment is organized in trimer.

[0095] Table 1 below shows quantification of KSAC protein after 3000 bar treatment by qDot-blot and HPLC.

Table 1

	2M urea		
	control	Assay pellet	Assay supernatant
Dot-blot µg/ml	347	10	797
HPLC µg/ml	-	-	723
	1M urea		
	control	Assay pellet	Assay supernatant
Dot-blot µg/ml	328	10	750
HPLC µg/ml	-	-	746
	Without urea		
	control	Assay pellet	Assay supernatant
Dot-blot µg/ml	123	15	649
HPLC µg/ml	-	-	825

[0096] The quantity of solubilized protein was about 800 µg/ml. This was very close to the estimated quantity of initial KSAC protein as inclusion bodies (1000 µg/ml). The solubilisation yield is very high (75-100%).

[0097] Figure 13 shows the superposition of the DLS data obtained with the 3000 bar pressurized protein (lighter line) in the buffer without urea and the protein obtained with the classical refolding process (darker line). The exhaustive range of size (upper panel) shows that less objects of higher size are detected in pressurized samples. The

distribution by number (lower panel) shows that the majority of the pressure-refolded population has a similar size with the population refolded by the classical process and the folding seems very similar.

[0098] Figure 14 shows that the protein sizes obtained at 3000 bar are identical to protein sizes obtained from classical chromatography refolding for all three buffers used (circled). When treated at 2000 bar, the protein sizes are identical to the protein sizes from classical chromatography refolding when urea is used in the buffer. At pressure higher than 3000 bar, high-pressure aggregates appear when no urea is used. With urea present in the buffer, the protein seems to collapse (10nm in size) which indicates that denaturation has occurred.

[0099] Figure 15 shows HPLC chromatogram of 4000 bar treated samples. The results show that proteins with sizes larger than KSAC protein appeared when no urea was present in the buffer, or with only 1M urea was added in the buffer. Protein size was as expected when 2M was added in the buffer.

[0100] Figure 16 shows the DLS size distribution by number of the 5000 bar treated samples without urea. The results indicate that three populations of proteins were detected with large sizes up to 90nm. The population whose size is similar to the chromatography refolded KSAC is a minority one.

[0101] Figure 17 shows the comparison of KSAC soluble protein content determined by HPLC and Qdot-blot. The results indicate that the maximum concentrations of solubilized proteins are obtained with 3000 bar treated samples with good consistency between HPLC and Qdot-blot technologies. For the 2000 bar treated samples, the presence of urea helps to increase the solubilization yield. For the 4000 bar treated samples, HPLC gives higher yield than Qdot-blot, indicating a loss of recognition of the antigens.

Example 2 Vaccination of dogs using KSAC vaccine

[0102] In this study, forty-nine 15-week old beagle dogs were vaccinated according to the following protocol.

Table 2

Group	Vaccination	Post vaccinal survey***	Blood sampling for

	1ml (10µg) SC at D0, D21, D42 and M12 (booster)		immunological survey
Vaccinated (n=24)	KSAC*/GLA-SE**	- D0 + 4/6h, D1, D2 - D21 + 4/6h, D22 and D23 - D42 + 4/6h, D43 and D44 - M12 + 1 day and + 3 days	D0, D28, D35, D50, D56
Controls (n=25)	Placebo		

*KSAC: purified KSAC protein in Tris buffer (20mM, pH=8), 10µg/ml
**GLA-SE: oil-in-water emulsion (4% oil)
***post vaccinal survey was performed as long as any sign was detected

[0103] After vaccination (on D77), the dogs were transferred to South Italy, in a highly endemic area for canine Leishmaniasis to testing up to 15 months (M15). Post vaccinal survey included the evaluation of rectal temperature, general condition (see Table 3), pain on palpation (presence/absence), cutaneous heat (presence/absence), itching (presence/absence) and swelling. Swellings were scored as follow; 0 = no swelling, 1 = minor swelling (only detectable), 2 = swelling but < 2cm, 3 = swelling > 2cm.

[0104] The post vaccinal survey showed that no major sign (persisting pain, important itching) was detected during the observation period.

Table 3 Clinical signs evaluated during canine Leishmaniasis survey and calculation of the Overall Clinical Score (OCS)

Clinical signs	**Measurement**	**Scoring**
Body condition (See Annex 3)	Moderate, Stout or Obese	0
	Thin	1
	Emaciated	3
General condition	Good= animal in good health which plays and is attentive	0
	Apathy= animal with lack of energy (when compared to its normal behaviour) or animal which stays lying but responds to stimuli	1
	Depression= animal which stays lying without responding to stimuli	3
Cutaneous signs	Absence	0
	Bilateral symetric alopecia	1
	Ulcers/Nodules	2
	Exfoliative dermatitis	3
Ocular signs	Absence	0

	Blepharitis and/or conjunctivitis and/or keratitis	1
	Uveitis	2
Epistaxis	Absence	0
	Presence	3
Arthritis	Absence	0
	Presence	1
Lymph node and/or spleen enlargement	Absence	0
	Bilateral enlargment of lymph nodes prescapular or retromandibular lymph nodes and of popliteal Lymph nodes– 3 And/or Spleen enlargment– 3	3

[0105] For each category, only the highest score was considered in the calculation of the Overall Clinical Score (OCS). The scores of the seven categories are summed to determine the OCS.

[0106] The OCS data indicated that no major abnormality of the OCS was detected before M15.

[0107] Infection patterns were categorized into 4 categories (Oliva *et al.* 2006, J of Clin Microbiol 44:1318-22) and regrouped in non-established (alias non active) and active infections depending on whether culture-based analysis was positive or negative.

Table 4 Terminology used for the classification of the dogs according to their parasitological, serological and clinical signs

	Healthy	PCR negative Cult negative Sero negative
Non established infection	Subpatent	PCR positive Cult negative Sero negative or positive
Active infection	Asymptomatic	PCR positive Cult positive Sero positive Healthy
	Symptomatic	PCR positive Cult positive Sero positive

		Clinical and/or biological sign(s)

PCR = qPCR on spleen aspirate
Cult = Culture on Lymph node aspirate or LDA on spleen aspirate
Sero = serology by IFAT
Clinical and/or biological sign(s) = non null OCS

[0108] The parasitological and serological assays were performed to evaluate the status of the dogs as defined by Oliva et al (2006). Nested PCR on bone marrow, culture on lymph nodes aspirates were performed according to Gradoni et al (2005, Vaccine 23:5245-5251). IFAT (ImmunoFluorescent Antibody Test) serum titers test was performed according to a technique derived from OIE (2004, Manual of diagnostic tests and vaccines for terrestrial animals, fifth Edition OIE, Paris). Quantitative PCR and LDA (Limiting Dilution Assay) on spleen aspirate were performed using methods derived from Bretagne et al (2001, Clin Diagn Lab Immunol 8:828-831) and Hill *et al.* (1983, Infect Immun 39:1087-1094), respectively.

Table 5 Results of culture on Lymph nodes aspirates and LDA performed on specimens collected at M8, M12 and M15 indicating active infections

	M8		M12		M15	
Vaccinated group (24 dogs)	Culture LN	LDA spleen	Culture LN	LDA spleen	Culture LN	LDA spleen
Neg	23	21 (2 NT*)	23	22	21	22
Pos	1	1	1	2	3	2
Control group (25 dogs)						
Neg	24	24	23	19	16	15
Pos	1	1	3	6	9	10

*NT: not tested

Table 6 Results of serological (IFAT) and parasitological assays (nPCR on Bone Marrow aspirates and quantitative PCR on spleens aspirates) performed on specimens collected at M8, M12 and M15

	M8			M12			M15		
Vaccinated	IFAT	nPCR	Q-	IFAT	nPCR	Q-	IFAT	nPCR	Q-

group (24 dogs)		BM	PCR spleen		BM	PCR spleen		BM	PCR spleen
Neg	23	23	33 (1 NT)	21	21	21	20	16	21
Pos	1	1	1	3	3	3	4	8	3
Control group (25 dogs)									
Neg	24	24	23	21	15	17	28	10	15
Pos	1	1	2	4	10	8	7	15	10

[0109] In addition to the tests done in Table 6, IFAT titers were also measured on samples collected at M0 (time corresponding to the peak of vaccine induced antibody response) on both vaccinated and control dogs, and all samples were found negative.

Table 7 Repartition of the dogs according to classes defined in Table 4

		M8		M12		M15	
		Con*	Vac**	Con*	Vac**	Con*	Vac**
	Healthy	23	23	16	21	15	21
Non established infection	Subpatent	1	0	3	1	0	0
Active infection	Asymptomatic	1	1	6	2	8	1
	Symptomatic	0	0	0	0	2	2

Con*: Controls
Vac**: Vaccinated

[0110] The results indicated a high proportion of dogs with active infections among control dogs (at M15, 40%, 10/25) and a lower proportion of dogs with active infections among vaccinated dogs (at M15, 12.5%, 3/24).

[0111] The study shows that after 15 months in the endemic area, a reduction of the proportion of dogs with active Leishmania infected was observed in vaccinated versus control dogs (i.e. vaccine efficacy). The vaccine efficacy was evaluated at 69%. These results are consistent with a significant control of parasite infection in vaccinated dogs.

[0112] The result also demonstrated that vaccination with the subunit KSAC vaccine does not interfere with the IFAT serology test. This supports the possibility to vaccinate

in endemic areas without interfering with on-going epidemiology studies and clinical assessment of the dogs.

Example 3 Comparison of different processes of solubilizing KSAC protein

[0113] The objective of the study is to compare the efficiency of solubilizing protein from inclusions bodies by different processes.

[0114] The KSAC inclusion bodies produced from E. coli were prepared in the following buffers to form inclusion bodies suspension: a) 20mM Tris buffer, 50mM DiThioThreitol (DTT), pH=8.0; b) 20mM Tris buffer, pH=8.0.

[0115] The inclusion bodies suspensions were stored in Quick Seal tubes for high pressure treatments as described below.

[0116] In process A, stepwise pressurization was applied to the inclusion bodies suspensions increasing the pressure from 0 bar to 3000 bar at 1000 bar/min, with a plateau of 1 hour duration at each 500 bar (target pressure of 3000 bar reached after 5 hr). The 3000 bar pressure was maintained for 48 hours. The samples were then depressurized from 3000 bar to 0 bar at constant rate of 125 bar/hr for 24 hrs.

[0117] In process B, the inclusions bodies suspensions were treated according to the method described in US 6,489,450. The samples were subject to pressurization at constant rate up to 2500 bar in 1 hr. The 2500 bar pressure was maintained for 6 hrs. Depressurization was performed at constant rate for 1 hr reducing the pressure from 2500 bar to 0 bar.

[0118] Samples were prepared as shown in Table 8 below.

Table 8 inclusion bodies suspensions treatment

Sample (1mg/mL KSAC inclusion bodies	buffer	High pressure treatment process
1	Tris 20mM	Process A
2	Tris 20mM + DTT 50mM	Process A
3	Tris 20mM	Control*
4	Tris 20mM + DTT 50mM	Control

5	Tris 20mM	Process B
6	Tris 20mM + DTT 50mM	Process B
7	Tris 20mM	Control
8	Tris 20mM + DTT 50mM	Control

Control *: no high pressure treatment, stored at room temperature.

SDS-PAGE Analysis

[0119] After the high pressure treatments, the samples were centrifuged to separate the supernatant and pellets, and processed for protein analysis on SDS-PAGE. The SDS-PAGE analysis is shown in Figures 18A and 18B. Each well was loaded with either 5 µl of sample (crude), 5 µl of supernatant, 5 µl of pellet resuspended in Tris buffer.

[0120] The KSAC protein amounts calculated from the band intensity on the SDS-PAGE were presented in Table 9 below.

Table 9 Comparative integration of the intensities of the bands measured on SDS gels

		Process A				Process B			
sample		I.I. KSAC band	Total protein I.I.	%KSAC /total	%KSAC-S /%KSAC-P*	I.I. KSAC band	Total protein I.I.	%KSAC /total	%KSAC-S /%KSAC-P
KSAC reference		36	49	73%		29	47	62%	
Control – no DTT	S[1]	0	0	0%	-	0	0	0%	-
	P[2]	13	26	50%		4	5	80%	
	C[3]	3	21	14%		6	16	38%	
Process – no DTT	S	0	9	0%	0%	0	2	0%	0%
	P	43	76	57%		28	39	72%	
	C	27	86	31%		14	41	34%	
Control – with DTT	S	1	8	13%	-	0	0	0%	-
	P	20	40	50%		24	36	67%	
	C	16	31	52%		22	37	59%	
Process – with DTT	S	55	127	43%	75%	18	29	62%	69%
	P	8	14	57%		8	9	89%	
	C	45	106	42%		28	47	60%	
KSAC reference		38	52	73%		29	45	64%	

S[1]: supernatant
P[2]: pellet
C[3]: crude, before centrifugation
%KSAC-S/%KSAC-P*: [%KSAC/total in supernatant] / [%KSAC/total in pellet]

[0121] The results show that there is no significant amount of KSAC detected in the supernatant of the controls or the samples treated with processes A and B when buffer containing no DTT was used. Soluble KSAC protein was found in the supernatant of the samples treated with high pressure (both processes A and B) when buffer containing DTT was used. Surprisingly, the results of protein quantification from SAS-PAGE also indicate that process A provided better solubilization when compared to process B. This surprising result was further confirmed by the more accurate calculation of the solubilization yield for each high pressure process using Q-Dot Blott and HPLC.

Q-Dot Blot Analysis

[0122] The supernatants of the samples were analyzed by Q-Dot Blott to estimate the amount of KSAC protein solubilized by the treatments. The results are shown in Figures 19A-19D and Table 10.

Table 10 Concentrations of solubilized KSAC found in the supernatants for controls and high pressure processed samples

Identification	Process A	Process B
Treatment without DTT	26.0 g/ml	12.7 g/ml
Control without DTT	0	9.9 g/ml
Treatment with DTT	632.1 g/ml	368.9 g/ml
Control with DTT	61.7 g/ml	63.9 g/ml

[0123] No significant difference was observed between the control samples (no high pressure treatment) with and without DTT. There was no soluble KSAC found in the supernatant. The Q-Dot Blott result confirmed the SDS-PAGE result.

[0124] The treatment performed using process A with DTT allowed solubilizing and refolding of the KSAC protein (detected by Q-Dot Blott). The concentration of soluble KSAC protein was found to be 632 μg/mL using process A while concentration of KSAC protein obtained using the process B was only about 369 g/mL. The solubilization yields obtained are 63% for process A and 37% for process B. The Q-Dot Blott results further demonstrate that process A is more efficient in producing soluble and refolded proteins.

HPLC Analysis

[0125] Figure 20 shows the superposition of the HPLC chromatograms of the supernatant of the control and process A treated sample. The retention time, retention volume and estimated purity obtained for the process A treated sample are show in Table 11 below.

Table 11 Retention time, retention volume and estimated purity obtained for process A treated sample

Detection	Information	Control - no processing	After Process A
UV	RT (Retention time – min)	12.7	12.4
	VR (Retention volume – mL)	6.64	6.46
	Estimated Purity (%)	17.3	85.5

[0126] Figure 21 shows the superposition of the HPLC chromatograms of the supernatant of process A treated sample and process B treated sample. The retention time, retention volume and estimated purity obtained for the process A treated sample are show in Table 12 below.

Table 12 Retention time, retention volume and estimated purity obtained for process A and B treated samples

Detection	Information	Process A	Process B
UV	RT (Retention time – min)	12.4	12.4
	VR (Retention volume – mL)	6.46	6.47
	Estimated Purity (%)	85.5	74.2

[0127] Figure 22 shows the superposition of the HPLC chromatograms of the supernatant of process A treated sample, process B treated sample and classical process treated sample (denaturation and refolding obtained by urea and DTT treatment). The retention time, retention volume and estimated purity obtained for the process A treated sample are show in Table 13 below.

Table 13 Retention time, retention volume and estimated purity obtained for process A, process B and classical process treated samples

Detection	Information	Classical process	Process A	Process B
UV	RT (Retention time – min)	12.5	12.4	12.4
	VR (Retention volume – mL)	6.50	6.46	6.47
	Peak area (mAU)	8628	25251	10506
	Estimated purity (%)	94.7	85.5	74.2

[0128] The HPLC results further confirmed that process A provided better solubilization of KSAC protein than process B judging from the peak areas (25251mAU for process A

vs 10506mAU for process B). Both process A and B allow obtaining a refolding of the KSAC protein very close to the one obtained using the classical process (solubilization using urea + DTT treatment and refolding by SEC chromatography).

[0129] The trials performed with both processes A and B did not yield significant soluble KSAC protein in the absence of DTT. The results confirmed that a reducing agent is needed during the high pressure treatment to break disulfide bonds. However, the unexpected surprising discovery is that there is no need for the removal of DTT in order to obtain a correct refolding of the protein. Contrary to the general knowledge that DTT has to be removed from the buffer in order for proteins to be refolded properly, it is surprisingly discovered by applicants that presence of DTT does not interfere with the refolding process in the high pressure treatment of present invention. The KSAC soluble proteins obtained from high pressure process of present invention were refolded correctly to form trimers in the presence of DTT.

***

[0130] Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the above examples is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

[0131] All documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

[0129] Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

[0130] Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

CLAIMS

What we claim is:

1. A composition comprising a fusion protein consisting of leishmania antigens Kinetoplastid Membrane Protein 11 (KMP11), Sterol MethylTransferase (SMT), A2 and Cysteine Proteinase (CP) wherein the fusion protein has been substantially solubilized or substantially refolded by a high pressure.
2. The composition of claim 1, wherein the fusion protein has at least 90% sequence identity to SEQ ID NO:2.
3. The composition of claim 1, wherein the fusion protein is encoded by a polynucleotide having at least 90% sequence identity to SEQ ID NO:1.
4. The composition of any one of claims1-3, wherein the high pressure is from about 1000 bar to about 5000 bar.
5. The composition of claim 4, wherein the high pressure is applied for at least 20 hours.
6. The composition of any one of claims 1-5, wherein the fusion protein is solubilized or refolded from *E. coli* inclusion bodies.
7. The composition of claim 6, wherein the *E. coli* inclusion bodies are prepared in a buffer comprising 0M to about 7M urea.
8. The composition of claim 6, wherein the *E. coli* inclusion bodies are prepared in a buffer comprising DiThio Threitol (DTT).
9. The composition of claim 7 or 8, wherein the *E. coli* inclusion bodies are further subject to a high pressure ranging from about 1000 bar to about 5000 bar.
10. The composition of claim 9, wherein the *E. coli* inclusion bodies are treated under the high pressure for about 20 hours to about 100 hours.
11. The composition of claim 10, wherein the *E. coli* inclusion bodies are depressurized at the rate of about 83bar/hr – 125bar/hr.
12. The composition of any one of claims 1-11, wherein the high pressure is increased in a stepwise process.
13. A method of treating an animal susceptible to leishmania infection comprising administering to the animal a composition comprising a fusion protein consisting of

leishmania antigens Kinetoplastid Membrane Protein 11 (KMP11), Sterol MethylTransferase (SMT), A2 and Cysteine Proteinase (CP), wherein the fusion protein has been substantially solubilized or substantially refolded by a high pressure.

14. A method of preventing leishmaniasis by inducing in an animal an immune response against Leishmaniasis, comprising the step of administering to the animal a composition comprising a fusion protein consisting of leishmania antigens Kinetoplastid Membrane Protein 11 (KMP11), Sterol MethylTransferase (SMT), A2 and Cysteine Proteinase (CP), wherein the fusion protein has been substantially solubilized or substantially refolded by a high pressure.
15. The method of claim 13 or 14, wherein the fusion protein has at least 90% sequence identity to SEQ ID NO:2.
16. The method of claim 13 or 14, wherein the fusion protein is encoded by a polynucleotide having at least 90% sequence identity to SEQ ID NO:1.
17. The method of claim 15 or 16, wherein the animal is a canine.
18. A method of producing a soluble protein expressed in prokaryotes or eukaryotes comprising the steps of (i) preparing the inclusion bodies in a buffer comprising 0M to about 7M of urea to form inclusion body suspension; (ii) subjecting the inclusion body suspension to a stepwise increase of pressure over a period of time; and (iii) maintaining the high pressure applied to the inclusion bodies for a period of time.
19. The method of claim 18, wherein the buffer further comprises DTT.
20. The method of claim 18 or 19, wherein the high pressure is in the range from about 2000 bar to about 5000 bar.
21. The method of claim 18, 19 or 20, wherein the inclusion bodies are subject to the high pressure for about 20 hours to about 100 hours.
22. The method of claim 19, 20 or 21 wherein the DTT concentration is from about 1mM to about 100mM.
23. The method of any one of claims 18 to 22, wherein the method further comprises the step of depressurization.
24. The method of claim 23, wherein the depressurization is at the rate of about 83bar/hr – 125bar/hr.

Figure 1

SEQ ID NO:	Type	Gene Description
1	DNA	KSAC DNA
2	protein	Chimeric KSAC protein
3	protein	Leishmania KMP11 protein
4	protein	Leishmania SMT protein
5	protein	Leishmania A2 protein
6	protein	Leishmania CBP protein

Figure 2A

KSAC DNA sequence (SEQ ID NO:1)

ATGGCCACCACGTACGAGGAGTTTTCGGCGAAGCTGGACCGCCTGGATGAGGAGTTCAAC
AGGAAGATGCAGGAGCAGAACGCCAAGTTCTTTGCGGACAAGCCGGATGAGTCGACGCTG
TCGCCCGAGATGAAGGAGCACTACGAGAAGTTCGAGCGCATGATCAAGGAACACACAGAG
AAGTTCAACAAGAAGATGCACGAGCACTCGGAGCACTTCAAGCAGAAGTTCGCCGAGCTG
CTCGAGCAGCAGAAGGCTGCGCAGTACCCGTCCAAGACTAGTTCCGCCGGTGGCCGTGAG
ACCGCGCCGACGAACCTGATTCGTCGCCGCAACAAGGACGAGACAAACGGGGATGTCAGC
GCCGCCGCCGACCGCTTCCGCGACCGCTTCGAGAAGGCAACCCTCGAGGAGCGCAAGGCC
GCCACCACGACGATGGTCAACGAGTACTACGACCTGGTGACGGACTTCTACGAGTACGGC
TGGGGCCAGAACTTCCATTTCGCGCCGCGCTACGCCGGCGAGACCTTCTTCGAGTCCCTC
GCGCGCCACGAGTACTTCCTGGCCGCTCGCGGCGGCTTCATGGAGGGCGACCACATCGTC
GACGTGGGCTGCGGCGTCGGCGGTCCGGCGCGCAACATGGTTCGCCTCACGCGCTGCAAC
GTCATCGGCGTCAACAACAACGATTACCAGATCAGCCGCGCTCGCCGTCATGACGCGCTC
GCCGGTATGAGCTCCAAGATCGACTACGTCAAGACCGACTTCTGCAACATGAGCTTAGCC
GACAACACCTTCGACGGCGCCTACGCCATCGAGGCCACCTGCCACGCAAAGGACAAGGTC
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TGGTGCATGACCGACAAGTACAACCCCAATGACGAGTACCACCGCACAATCAAGCACCGC
ATCGAGCTGGGCGACGGCCTGCCGGAGATGGAGACGTGCAAACAGGTGATCGAGTACATG
AAGCAGGCCGGCTTCGTGGTGGAGGAGGCCATAGACGTCATCAGTCAGTTCGAGTCCAGC
CCCATCAAGAGTATCCCGTGGTACCAGCCGCTGGTCGGCGACTATTCGTCCCTGCAGGGC
CTGCGCTCTACCCCGATTGGCCGCATCCTCACGAACGTCATGTGTCGCGTGCTGGAGTTC
GTGCGCCTAGCTCCGAAGGGCACGTACAAGGCGACGGAGATTTTGGAGGAGGCTGCGGAA
AGCCTGGTGGTGGGCGGTCAGCTCGGCATCTTCACGCCGTCCTTCTACATCCGCGCTCGC
AAGCCGTCCAAGCAGGCTGGATCCAAGATCCGCAGCGTGCGTCCGCTTGTGGTGTTGCTG
GTGTGCGTCGCGGCGGTGCTCGCACTCAGCGCCTCCGCTGAGCCGCACAAGGCGGCCGTT
GACGTCGGCCCGCTGAGCGTTGGCCCGCAGAGCGTCGGCCCGCTGAGCGTTGGCCCGCAG
GCGGTTGGCCCGCTGAGCGTTGGCCCGCAGAGCGTCGGCCCGCTGAGCGTTGGCCCGCAG
GCGGTTGGCCCGCTGAGCGTTGGCCCGCAGAGCGTTGGCCCGCTGAGCGTTGGCCCGCTG
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AGCGTTGGCCCGCAGAGCGTCGGCCCGCTGAGCGTTGGCCCGCAGAGCGTCGGCCCGCTG
AGCGTTGGTCCGCAGAGCGTTGGCCCGCTGAGCGTTGGCCCGCAGAGCGTTGACGTTAGC
CCGGTGAGCGGATCCGAATTCGATGCGGTGGACTGGCGCGAGAAGGGCGCCGTGACGCCG
GTGAAGAATCAAGGCGCGTGCGGGTCGTGCTGGGCGTTCTCGGCGGTCGGCAACATCGAG
TCGCAGTGGGCCCGTGCCGGCCACGGCTTGGTGAGCCTGTCGGAGCAGCAGCTGGTGAGC
TGCGATGACAAAGACAATGGCTGCAACGGCGGGCTGATGCTGCAGGCGTTCGAGTGGCTG
CTGCGACACATGTACGGGATCGTGTTCACGGAGAAGAGCTACCCCTACACGTCCGGCAAC
GGTGATGTGGCCGAGTGCTTGAACAGCAGTAAACTCGTTCCCGGCGCGCAAATCGACGGC
TACGTGATGATCCCGAGCAACGAAACGGTTATGGCTGCGTGGCTTGCGGAGAATGGCCCC
ATCGCGATTGCGGTCGAC

Figure 2B

GCCAGCTCCTTCATGTCTTACCAGAGCGGCGTGCTGACCAGCTGCGCTGGCGATGCACTG
AACCACGGCGTGCTGCTCGTCGGGTACAACAAGACCGGTGGGGTTCCGTACTGGGTGATC
AAGAACTCGTGGGGTGAGGACTGGGGCGAGAAGGGCTACGTGCGCGTGGTCATGGGGCTG
AACGCGTGCCTGCTCAGTGAATACCCCGTGTCCGCGCATGTGCCGCGGAGTCTCACCCCT
GGCCCGGGCACGGAGAGCGAGGAGCGCGCCCCTAAACGGGTGACGGTGGAGCAGATGATG
TGCACCGATATGTACTGCAGGGAGGGGTGCAAGAAGAGTCTTCTCACCGCGAACGTGTGC
TACAAGAACGGGGGAGGCGGCTCCTCTATGACGAAGTGCGGTCCGCAGAAGGTGCTGATG
TGCTCGTACTCGAACCCTCATTGCTTTGGTCCTGGGCTGTGCCTCGAGACTCCTGATGGC
AAGTGCGCGCCGTACTTCTTGGGCTCGATCATGAACACCTGCCAGTACACGTAA

KSAC protein sequence (SEQ ID NO:2)

MATTYEEFSAKLDRLDEEFNRKMQEQNAKFFADKPDESTLSPEMKEHYEKF
ERMIKEHTEKFNKKMHEHSEHFKQKFAELLEQQKAAQYPSK***TS***SAGGRET
APTNLIRRRNKDETNGDVSAAADRFRDRFEKATLEERKAATTTMVNEYYDL
VTDFYEYGWGQNFHFAPRYAGETFFESLARHEYFLAARGGFMEGDHIVDV
GCGVGGPARNMVRLTRCNVIGVNNNDYQISRARRHDALAGMSSKIDYVKT
DFCNMSLADNTFDGAYAIEATCHAKDKVKCYSEVFRVIKPGTCFVLYEWCM
TDKYNPNDEYHRTIKHRIELGDGLPEMETCKQVIEYMKQAGFVVEEAIDVIS
QFESSPIKSIPWYQPLVGDYSSLQGLRSTPIGRILTNVMCRVLEFVRLAPKGT
YKATEILEEAAESLVVGGQLGIFTPSFYIRARKPSKQA***GS***KIRSVRPLVVLLV
CVAAVLALSASAEPHKAAVDVGPLSVGPQSVGPLSVGPQAVGPLSVGPQSVG
PLSVGPQAVGPLSVGPQSVGPLSVGPLSVGPQSVGPLSVGSQSVGPLSVGPQS
VGPLSVGPQAVGPLSVGPQSVGPLSVGPQAVGPLSVGPQSVGPLSVGPQSVG
PLSVGSQSVGPLSVGPQSVGPLSVGPQSVGPLSVGPQSVGPLSVGPQSVGPLS
VGPQSVDVSPVS***GSEF***DAVDWREKGAVTPVKNQGACGSCWAFSAVGNIESQ
WARAGHGLVSLSEQQLVSCDDKDNGCNGGLMLQAFEWLLRHMYGIVFTE
KSYPYTSGNGDVAECLNSSKLVPGAQIDGYVMIPSNETVMAAWLAENGPIAI
AVDASSFMSYQSGVLTSCAGDALNHGVLLVGYNKTGGVPYWVIKNSWGED
WGEKGYVRVVMGLNACLLSEYPVSAHVPRSLTPGPGTESEERAPKRVTVEQ
MMCTDMYCREGCKKSLLTANVCYKNGGGGSSMTKCGPQKVLMCSYSNPH
CFGPGLCLETPDGKCAPYFLGSIMNTCQYT

- KPM11 (in red)
- Sterol Methyltransferase (in green)
- **A2 (in blue).**
- Cysteine proteinase (in brown)
- **Linkers (in italic and black)**

Figure 2C

KMP11 protein (SEQ ID NO:3)
MATTYEEFSAKLDRLDEEFNRKMQEQNAKFFADKPDESTLSPEMKEHYEKF
ERMIKEHTEKFNKKMHEHSEHFKQKFAELLEQQKAAQYPSK

SMT protein (SEQ ID NO:4)
SAGGRETAPTNLIRRRNKDETNGDVSAAADRFRDRFEKATLEERKAATTTM
VNEYVDLVTDFYEYGWGQNFHFAPRYAGETFFESLARHEYTLAARGGFME
GDHIVDVGCGVGGPARNMVRLTRCNVIGVNNNDYQISRARRHDALAGMSS
KIDYVKTDFCNMSLADNTFDGAYAIEATCHAKDKVKCYSEVFRVIKPGTCF
VLYEWCMTDKYNPNDEYHRTIKHRIELGDGLPEMETCKQVIEYMKQAGFV
VEEAIDVISQFESSPIKSIPWYQPLVGDYSSLQGLRSTPIGRILTNVMCRVLEF
VRLAPKGTYKATEILEEAAESLVVGGQLGIFTPSFYIRARKPSKQA

A2 protein (SEQ ID NO:5)
KIRSVRPLVVLLVCVAAVLALSASAEPHKAAVDVGPLSVGPQSVGPLSVGPQ
AVGPLSVGPQSVGPLSVGPQAVGPLSVGPQSVGPLSVGPLSVGPQSVGPLSV
GSQSVGPLSVGPQSVGPLSVGPQAVGPLSVGPQSVGPLSVGPQAVGPLSVGP
QSVGPLSVGPQSVGPLSVGSQSVGPLSVGPQSVGPLSVGPQSVGPLSVGPQSV
GPLSVGPQSVGPLSVGPQSVDVSPVS

CBP protein (SEQ ID NO:6)
DAVDWREKGAVTPVKNQGACGSCWAFSAVGNIESQWARAGHGLVSLSEQQ
LVSCDDKDNGCNGGLMLQAFEWLLRHMYGIVFTEKSYPYTSGNGDVAECL
NSSKLVPGAQIDGYVMIPSNETVMAAWLAENGPIAIAVDASSFMSYQSGVLT
SCAGDALNHGVLLVGYNKTGGVPYWVIKNSWGEDWGEKGYVRVVMGLN
ACLLSEYPVSAHVPRSLTPGPGTESEERAPKRVTVEQMMCTDMYCREGCK
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CAPYFLGSIMNTCQYT

Figure 3

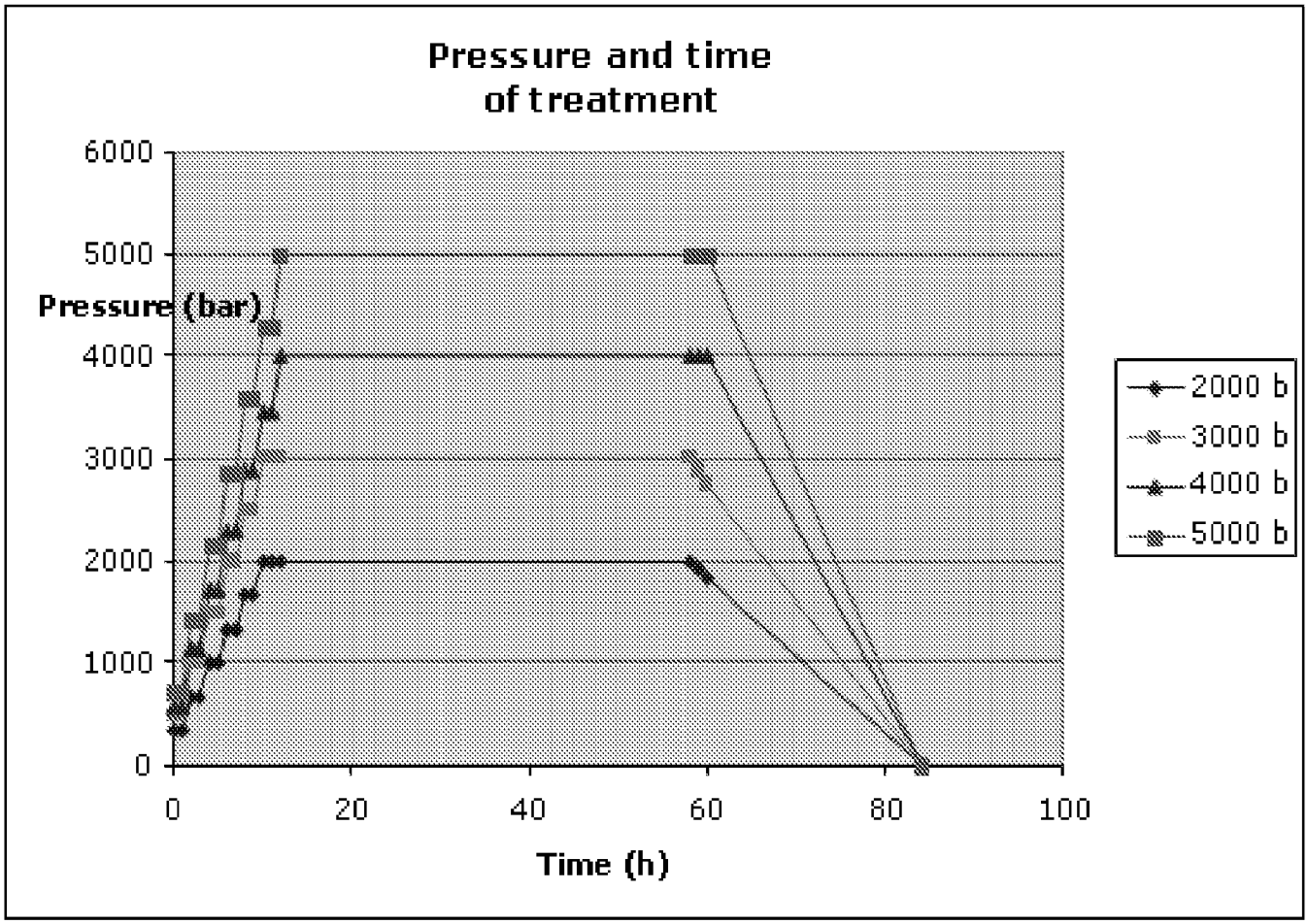

Pressure and time of treatment
Pressure (bar)
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Time (h)
2000 b
3000 b
4000 b
5000 b

Figure 4

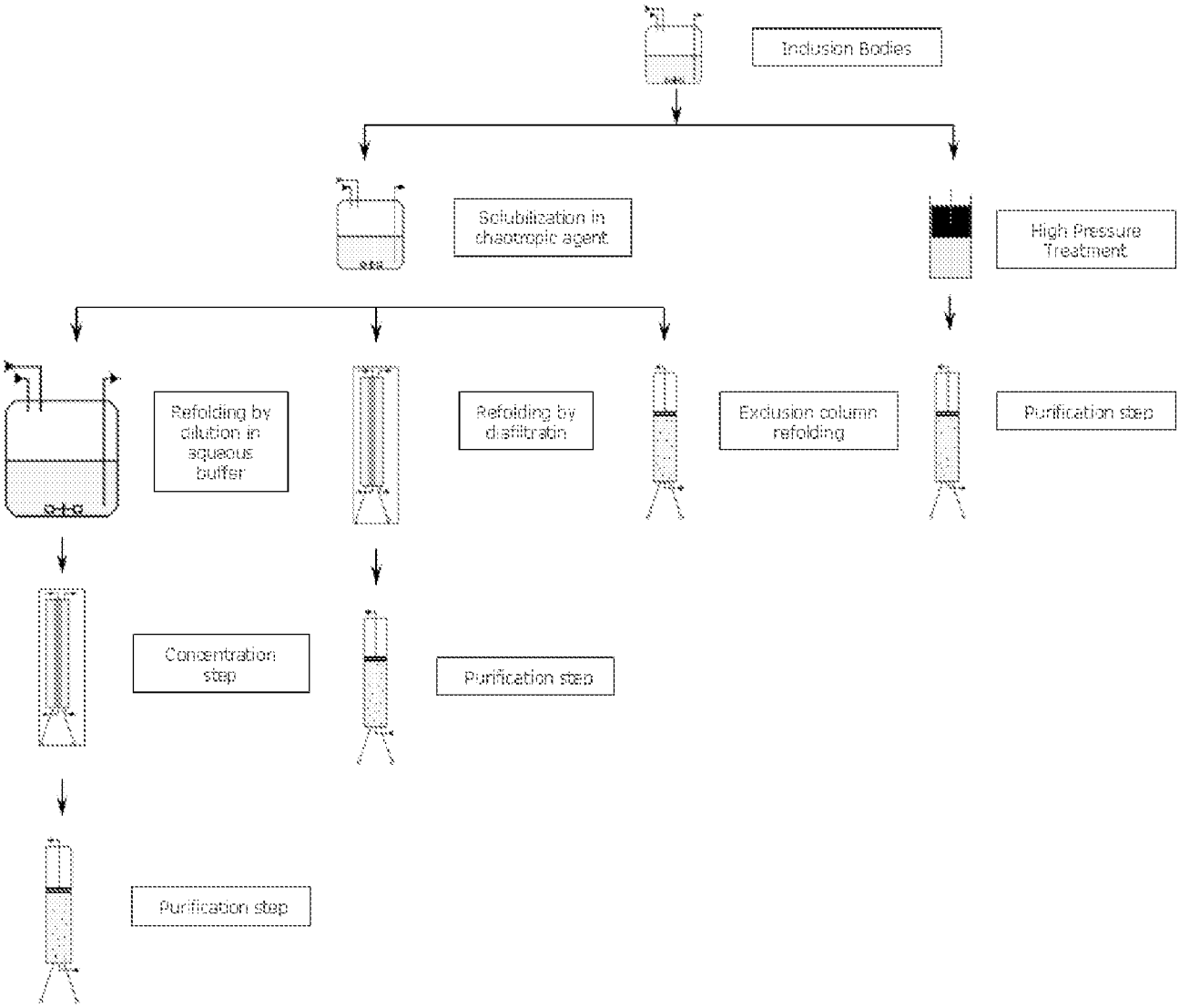

Inclusion Bodies
Solubilization in chaotropic agent
High Pressure Treatment
Refolding by dilution in aqueous buffer
Refolding by diafiltratin
Exclusion column refolding
Purification step
Concentration step
Purification step
Purification step

Figure 5

Refolding of KSAC

Chimeric Protein
4 proteins :
- KPM11
- Stérol methyl transferase
- A2
- Cystéine proteinase

PM=107KDa
Trimeric

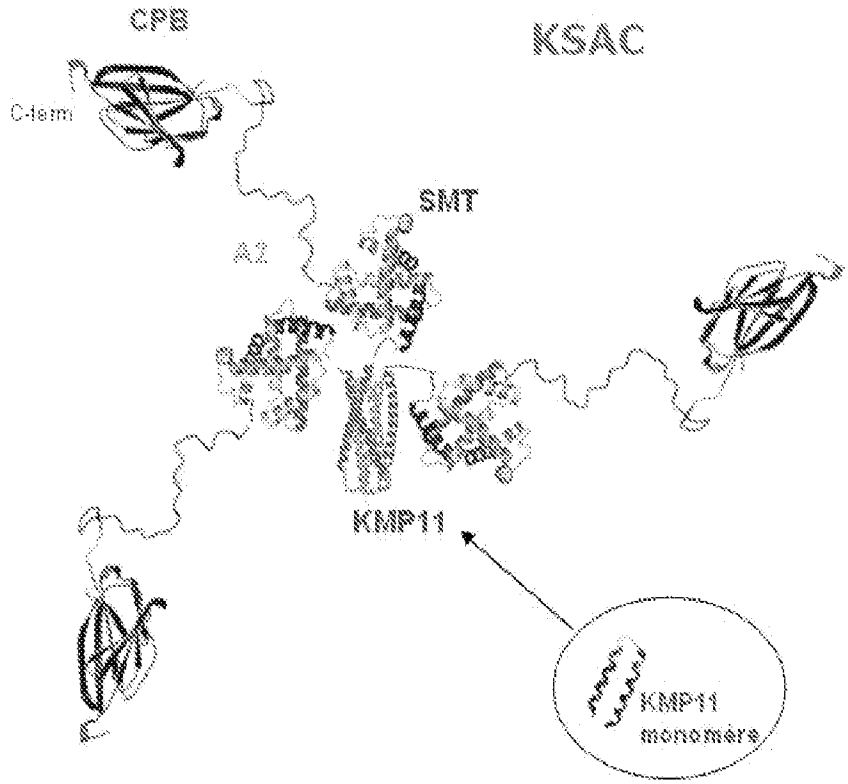


Figure 6

SDS-PAGE pattern of KSAC protein
refolded by exclusion chromatography

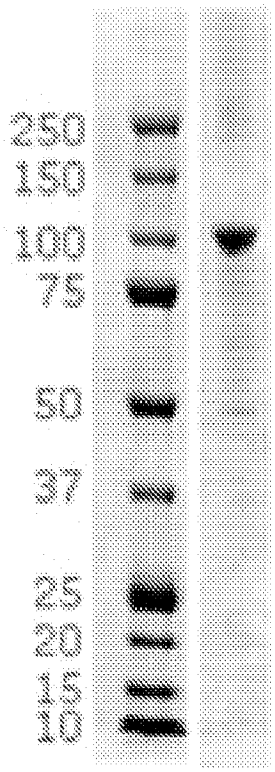


Figure 7

HPLC plot of refolded KSAC by exclusion chromatography

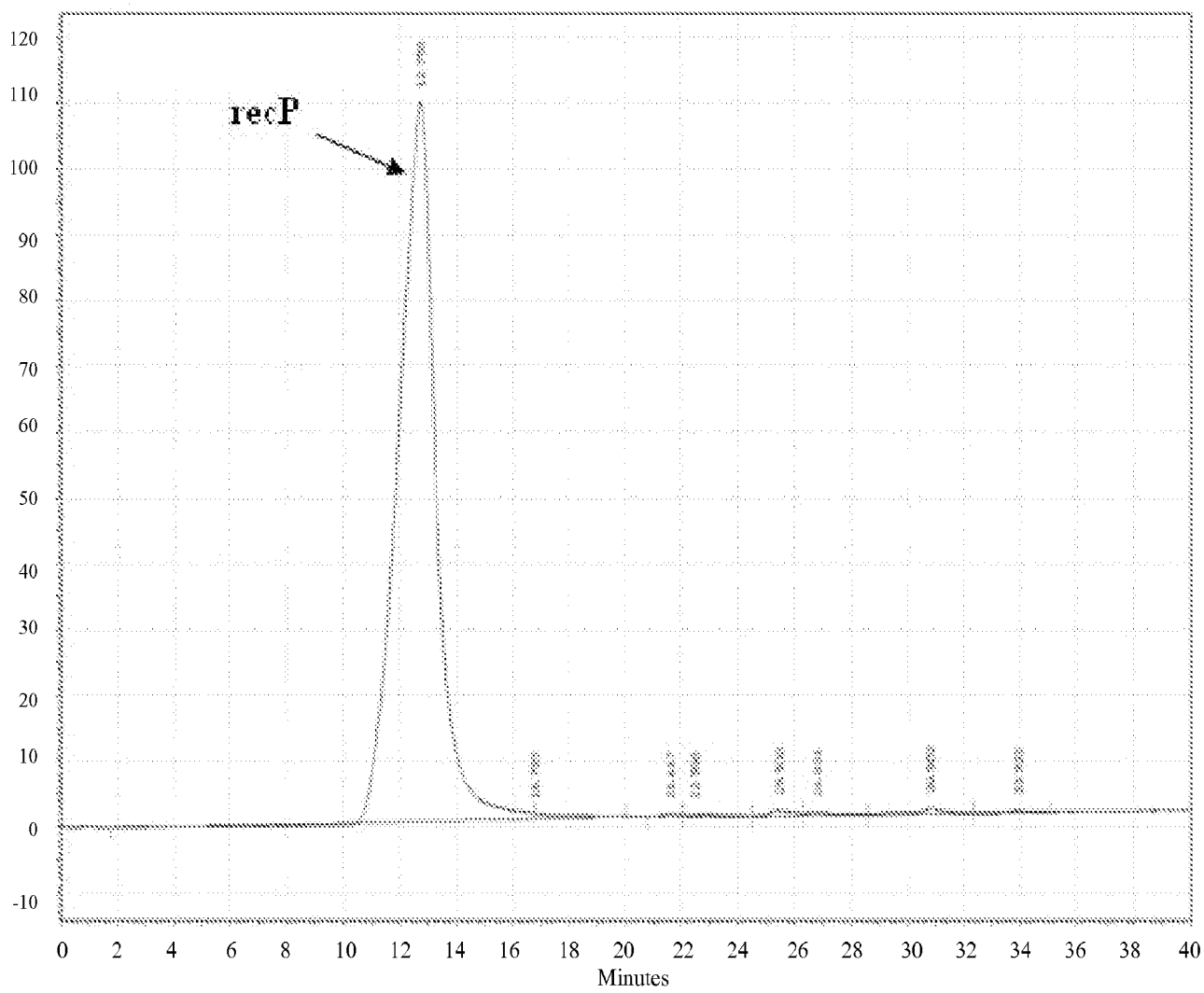


Figure 8A

Dynamic Light Scattering (DLS) of refolded KSAC protein
using exclusion chromatography

Fig. 8A

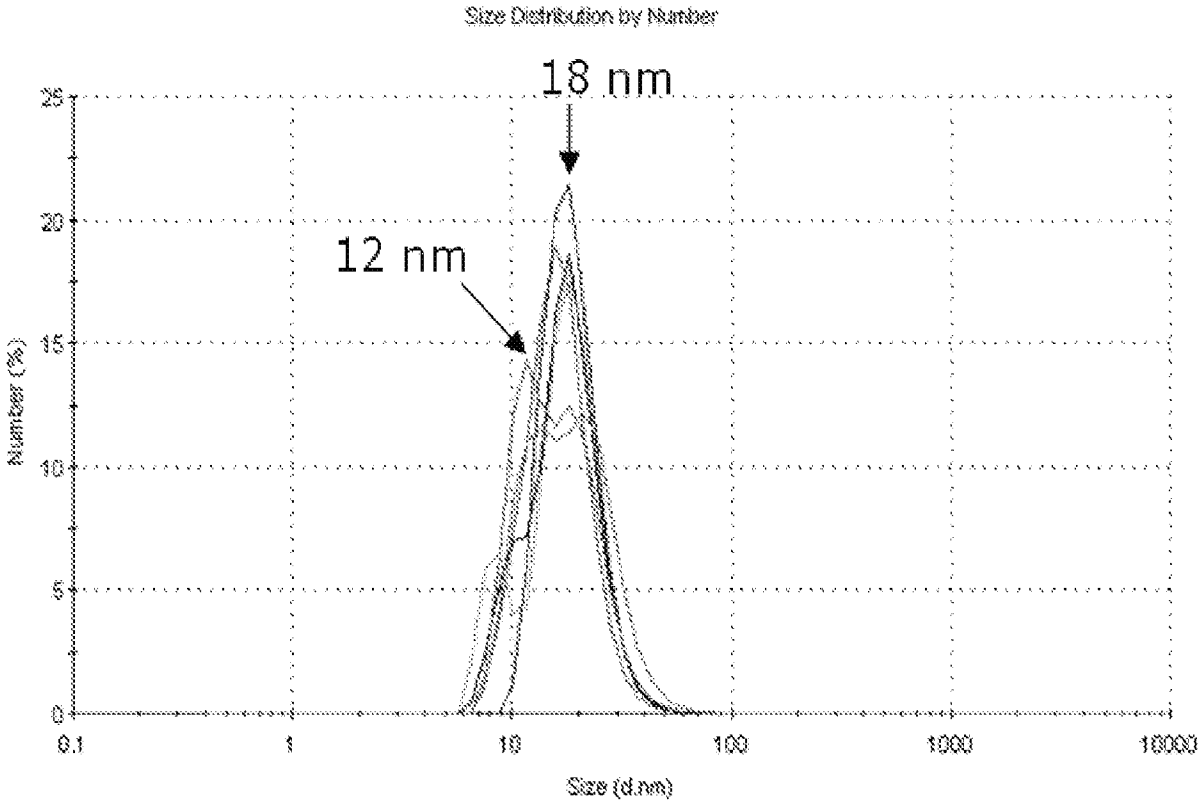


Figure 8B

Fig. 8B

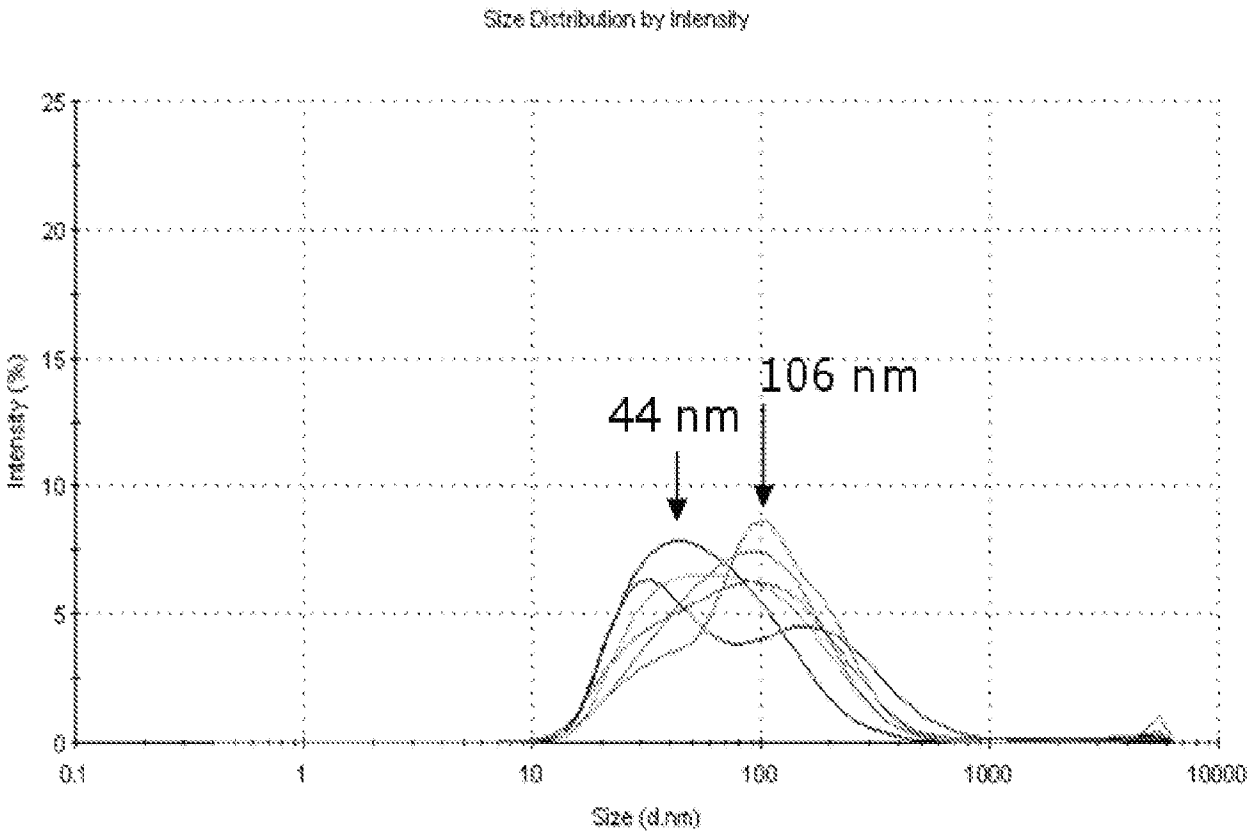


Figure 9

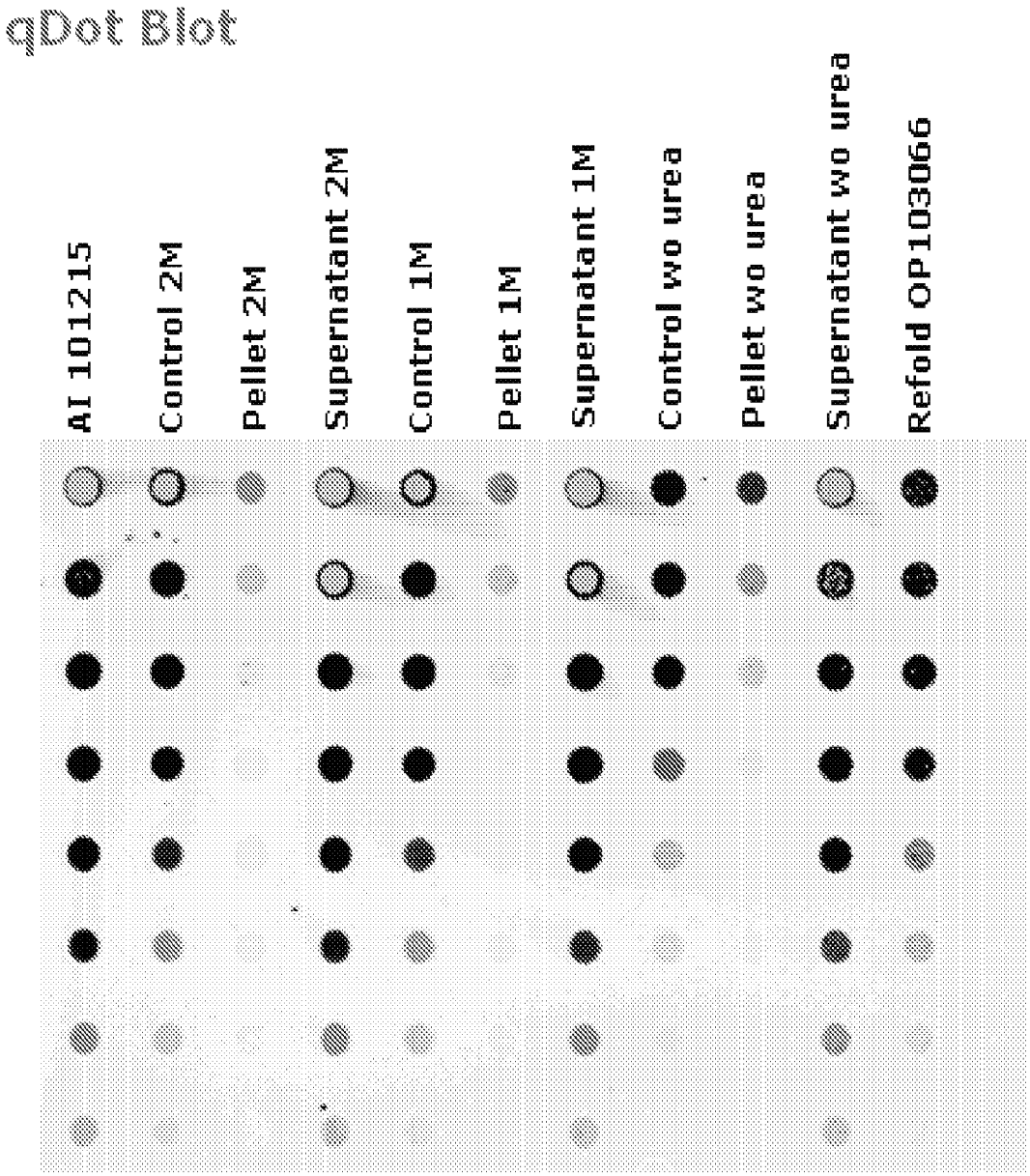

qDot Blot
AI 101215
Control 2M
Pellet 2M
Supernatant 2M
Control 1M
Pellet 1M
Supernatant 1M
Control wo urea
Pellet wo urea
Supernatant wo urea
Refold OP103066

Figure 10

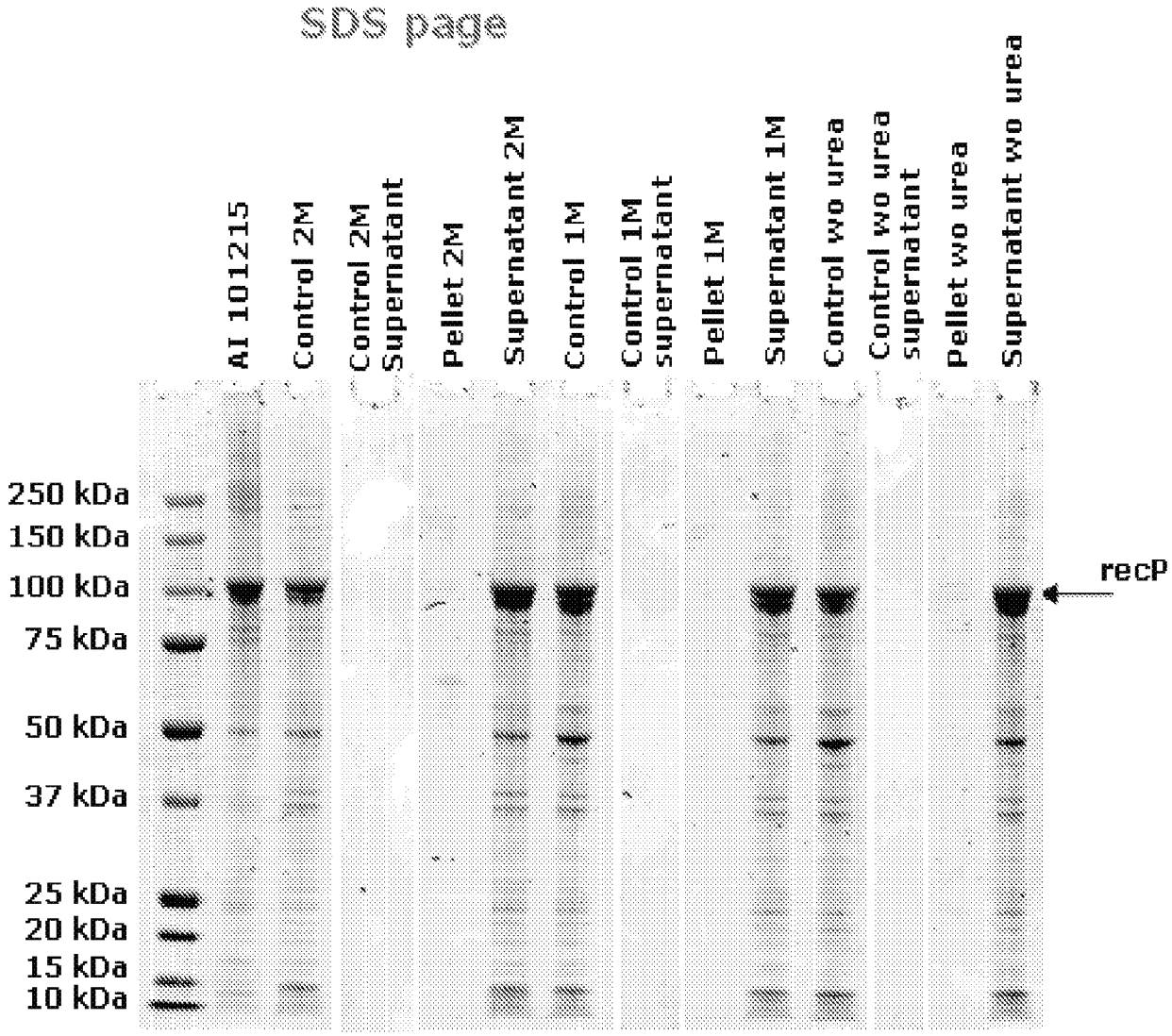

SDS page
AI 101215
Control 2M
Control 2M Supernatant
Pellet 2M
Supernatant 2M
Control 1M
Control 1M supernatant
Pellet 1M
Supernatant 1M
Control wo urea
Control wo urea supernatant
Pellet wo urea
Supernatant wo urea
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
recP

Figure 11

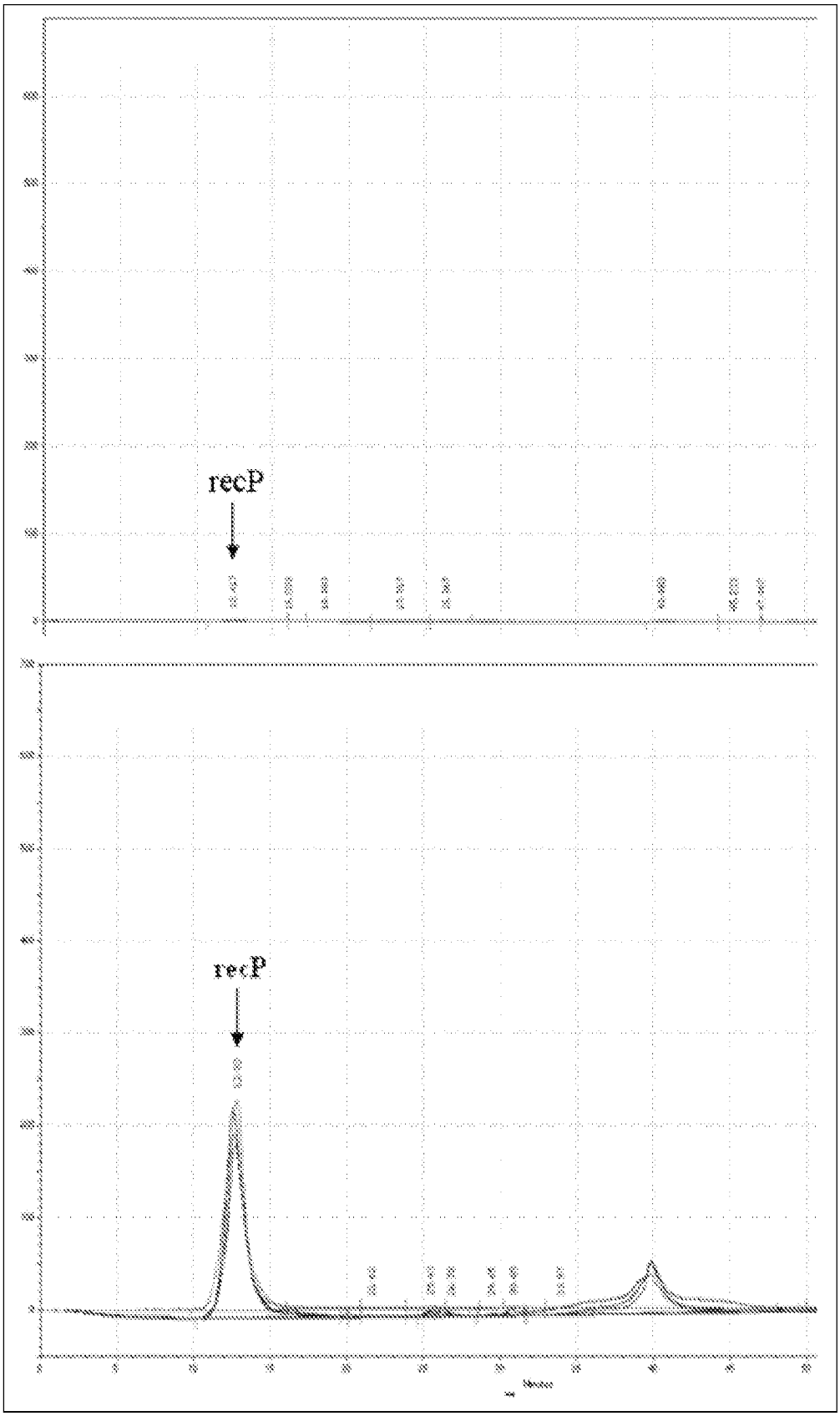


Figure 12

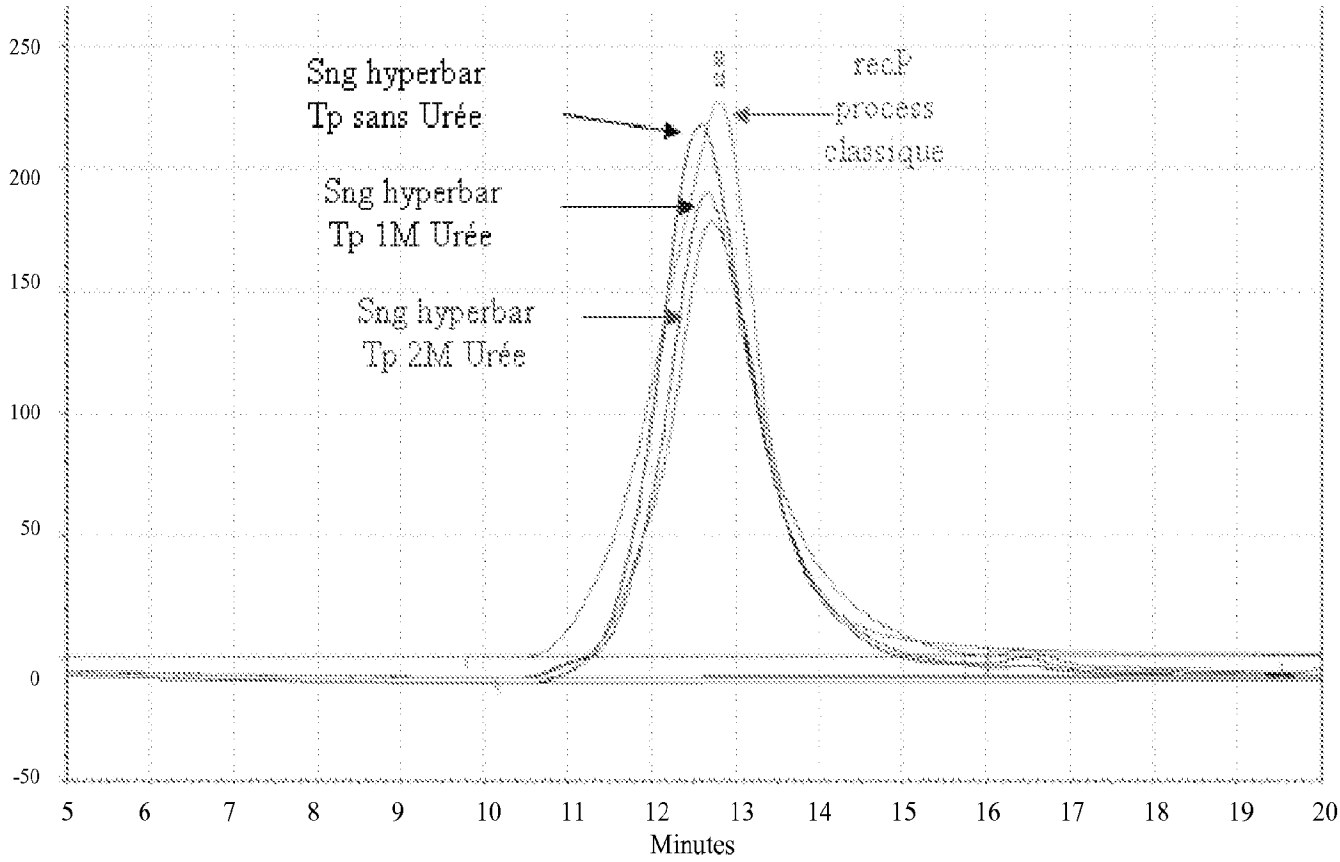
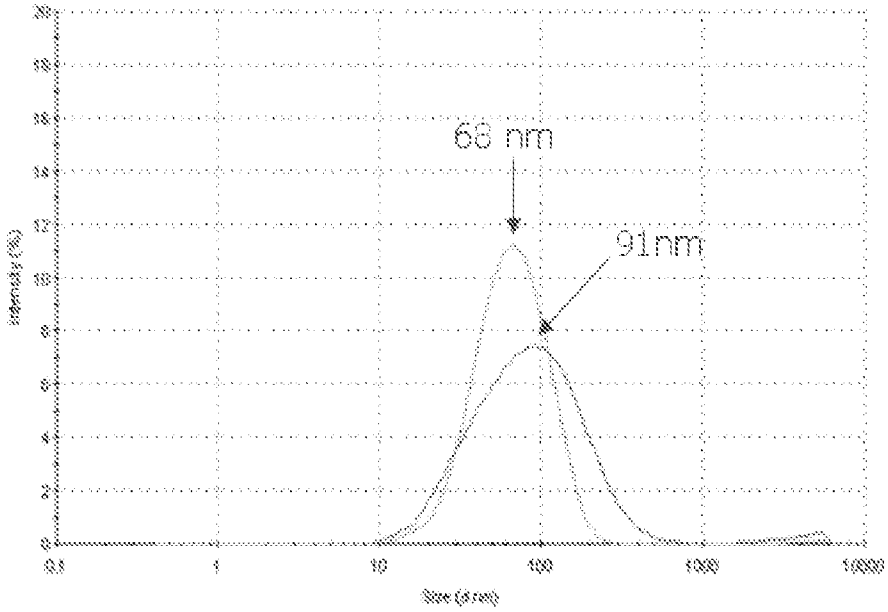

Sng hyperbar
Tp sans Urée
Sng hyperbar
Tp 1M Urée
Sng hyperbar
Tp 2M Urée
rec.
process
classique
250
200
150
100
50
0
-50
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Minutes

Figure 13

Size Distribution by Intensity



Size Distribution by Number

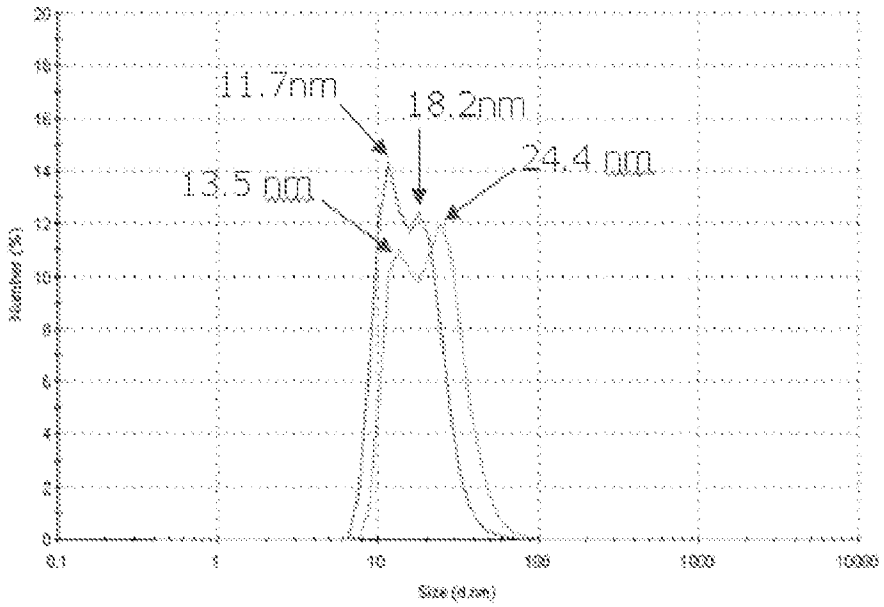


Figure 14

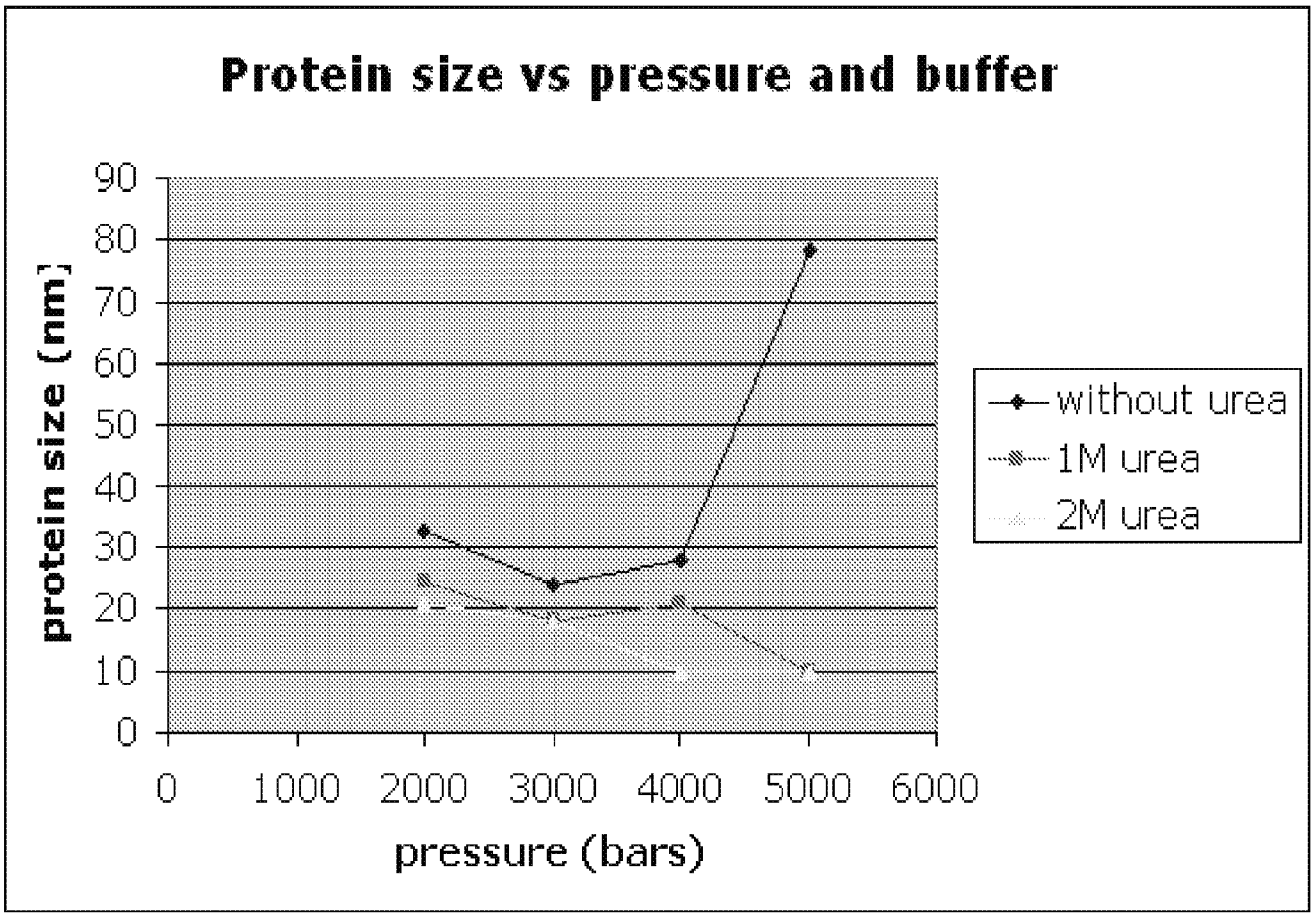

Protein size vs pressure and buffer
protein size (nm)
90
80
70
60
50
40
30
20
10
0
0
1000
2000
3000
4000
5000
6000
pressure (bars)
without urea
1M urea
2M urea

Figure 15
HPLC chromatogram of 4000 bar treated samples

Fig. 15A

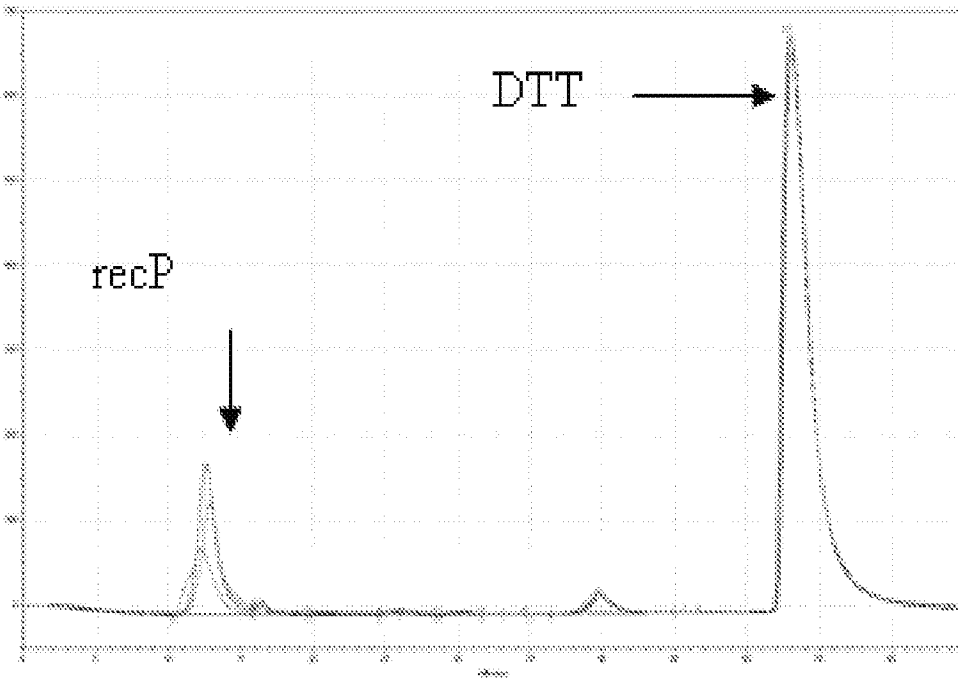


FIG.15B

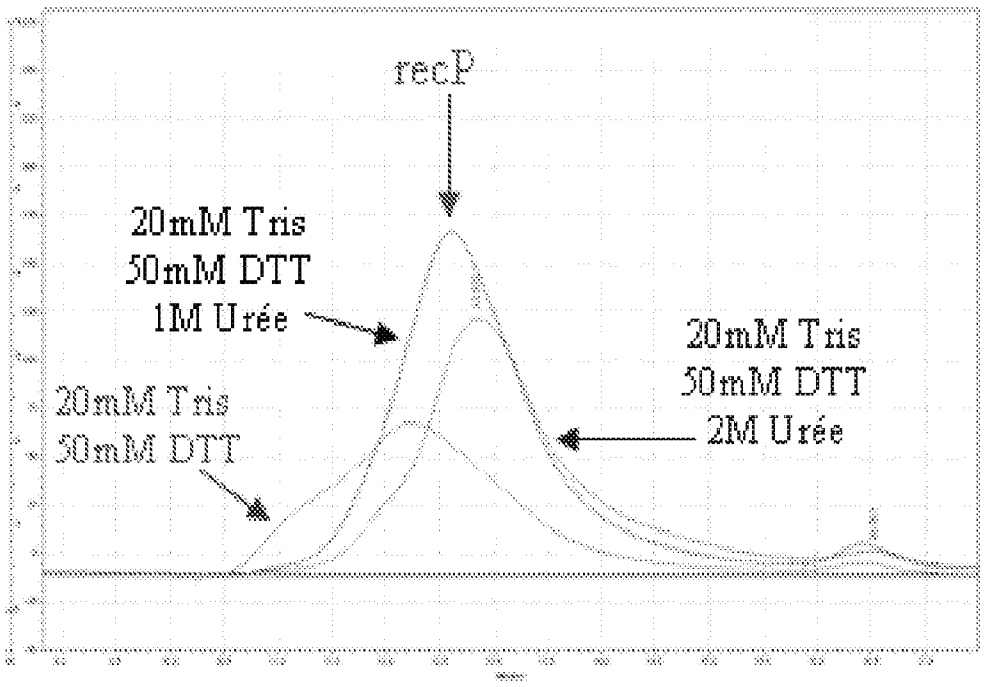


Figure 16

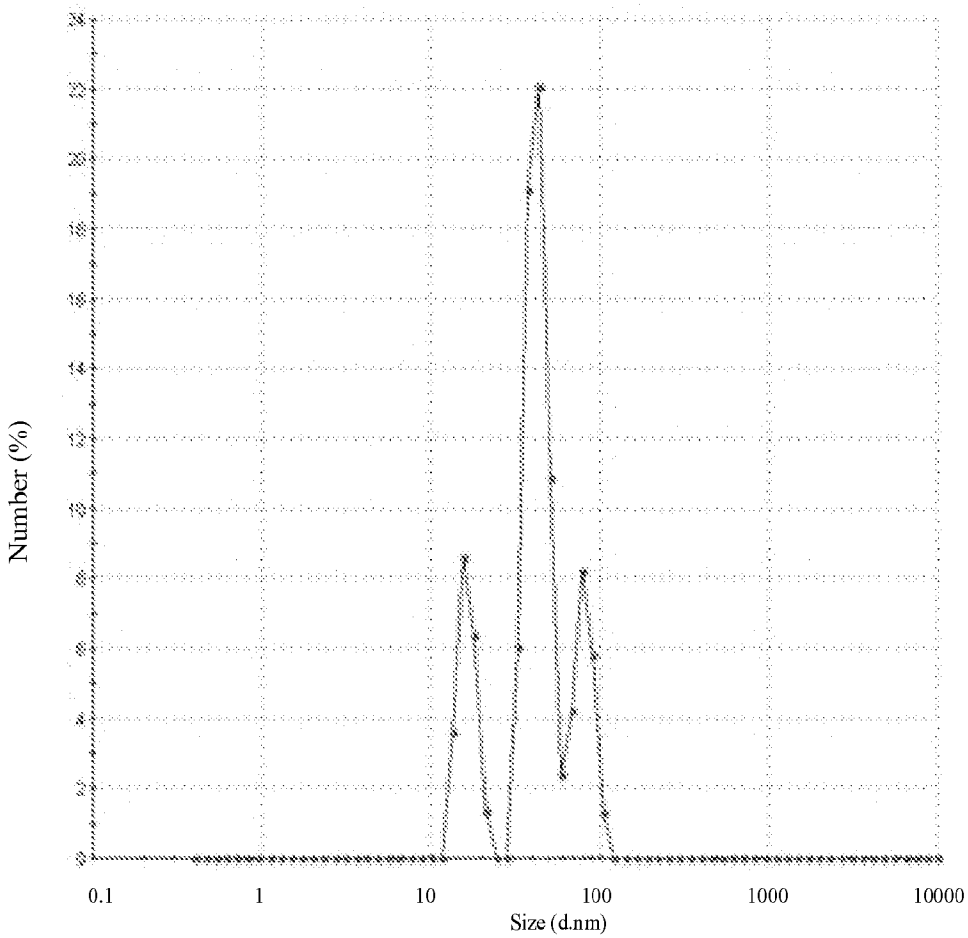


Figure 17

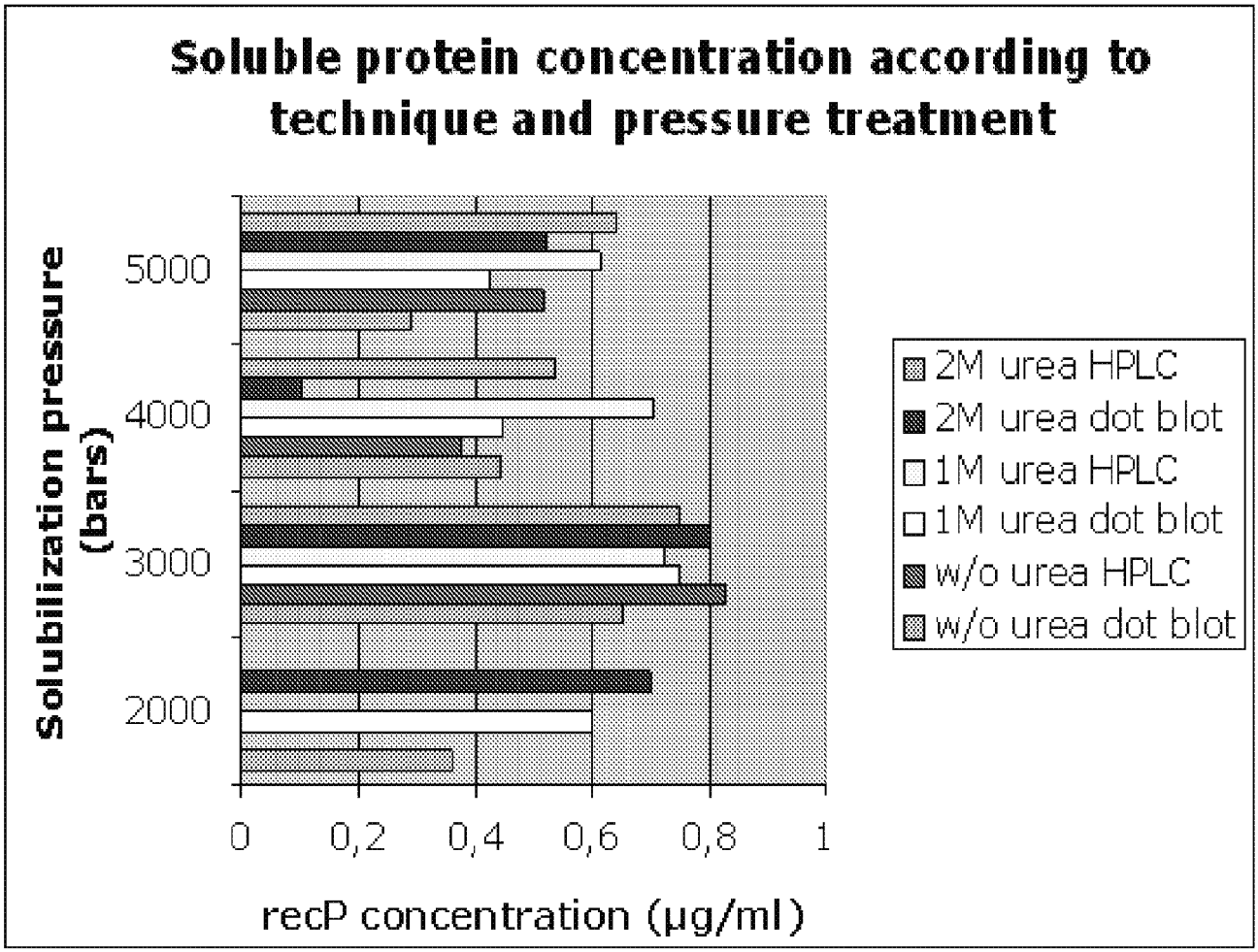
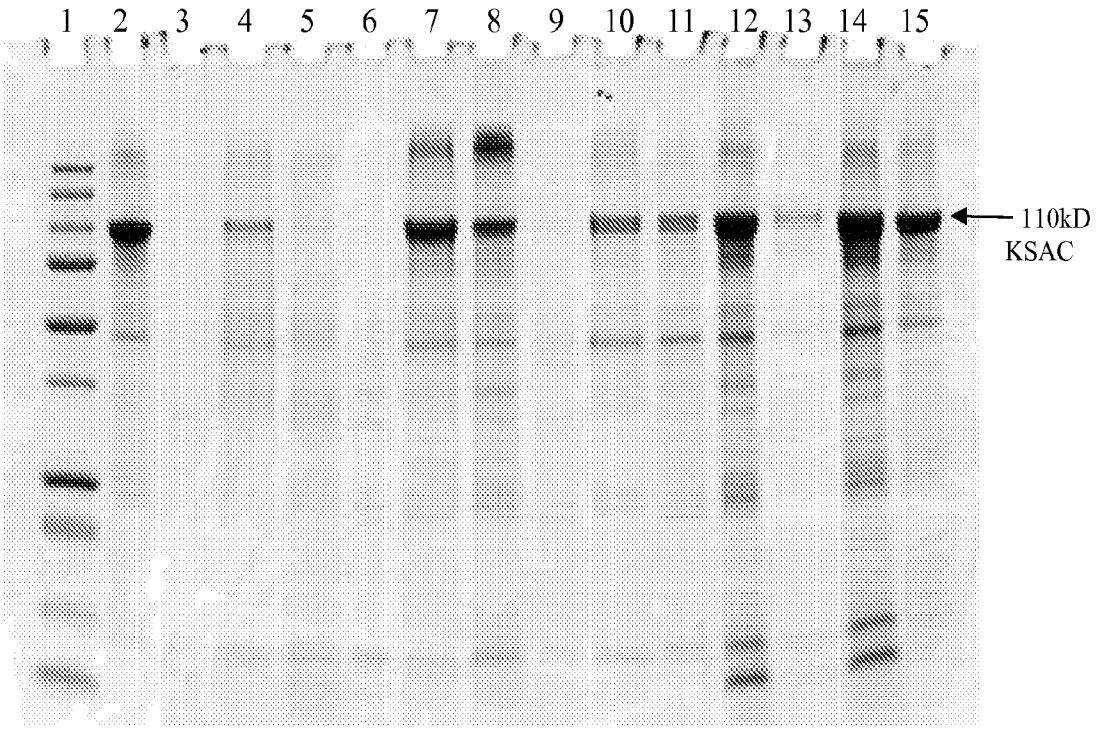

Soluble protein concentration according to technique and pressure treatment
Solubilization pressure (bars)
5000
4000
3000
2000
0
0,2
0,4
0,6
0,8
1
recP concentration (µg/ml)
2M urea HPLC
2M urea dot blot
1M urea HPLC
1M urea dot blot
w/o urea HPLC
w/o urea dot blot

Figure 18A

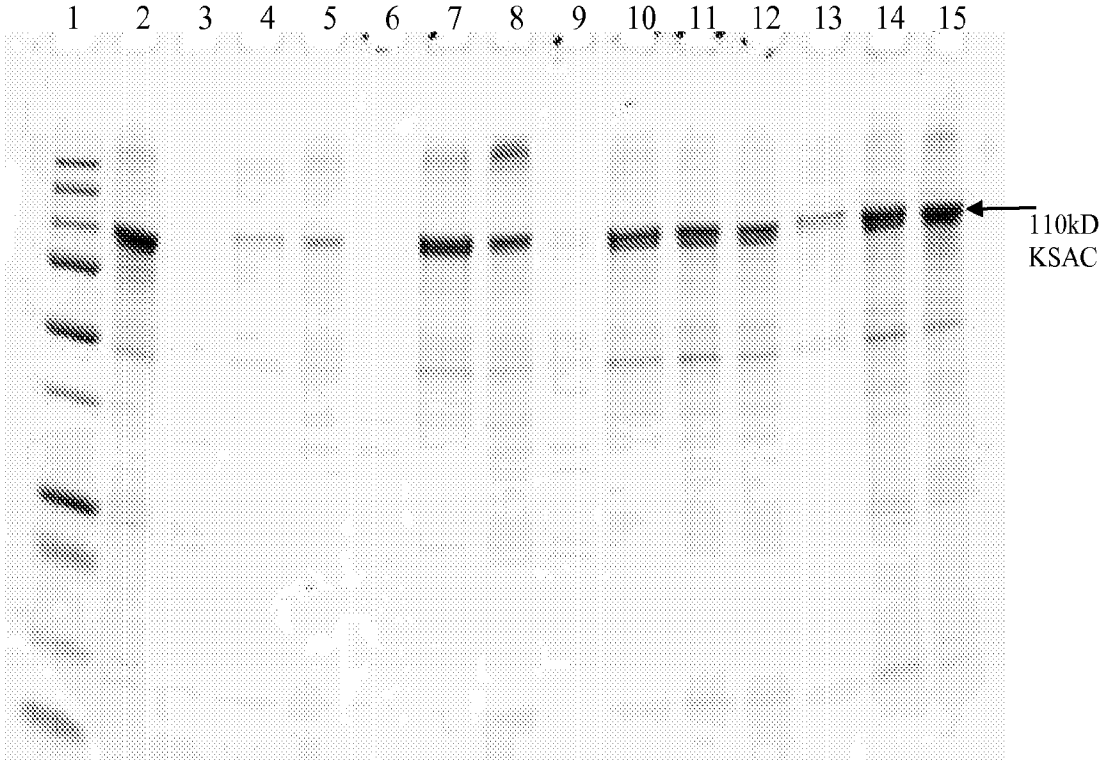
SDS Page Analysis of KSAC protein by process A



Lane 1: marker
Lane 2: KSAC inclusion bodies control
Lane 3: sample 3 supernatant
Lane 4: sample 3 pellet
Lane 5: sample 3 before centrifugation
Lane 6: sample 1 supernatant
Lane 7: sample 1 pellet
Lane 8: sample 1 before centrifugation
Lane 9: sample 4 supernatant
Lane 10: sample 4 pellet
Lane 11: sample 4 before centrifugation
Lane 12: sample 2 supernatant
Lane 13: sample 2 pellet
Lane 14: sample 2 before centrifugation
Lane 15: KSAC inclusion bodies control

Figure 18B

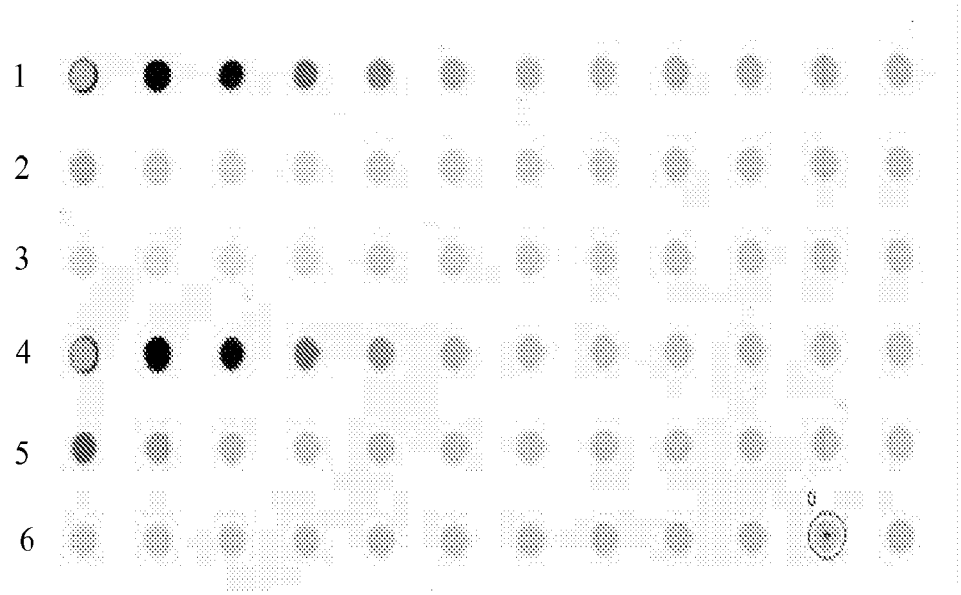
SDS Page Analysis of KSAC protein by process B



Lane 1: marker
Lane 2: KSAC inclusion bodies control
Lane 3: sample 7 supernatant
Lane 4: sample 7 pellet
Lane 5: sample 7 before centrifugation
Lane 6: sample 5 supernatant
Lane 7: sample 5 pellet
Lane 8: sample 5 before centrifugation
Lane 9: sample 8 supernatant
Lane 10: sample 8 pellet
Lane 11: sample 8 before centrifugation
Lane 12: sample 6 supernatant
Lane 13: sample 6 pellet
Lane 14: sample 6 before centrifugation
Lane 15: KSAC inclusion bodies control

Figure 19A

QDot Blott analysis of KSAC protein by process A



1: KSAC reference
2: process A without DTT (sample 1)
3: control without DTT (sample 3)
4: process A with DTT (sample 2)
5: control with DTT (sample 4)
6: 20 mM Tris buffer

Figure 19B

QDot Blott analysis of KSAC protein by process B

1: KSAC reference
2: process B without DTT (sample 5)
3: control without DTT (sample 7)
4: process B with DTT (sample 6)
5: control with DTT (sample 8)
6: 20 mM Tris buffer

Figure 19C

KSAC protein quantification from QDot Blott

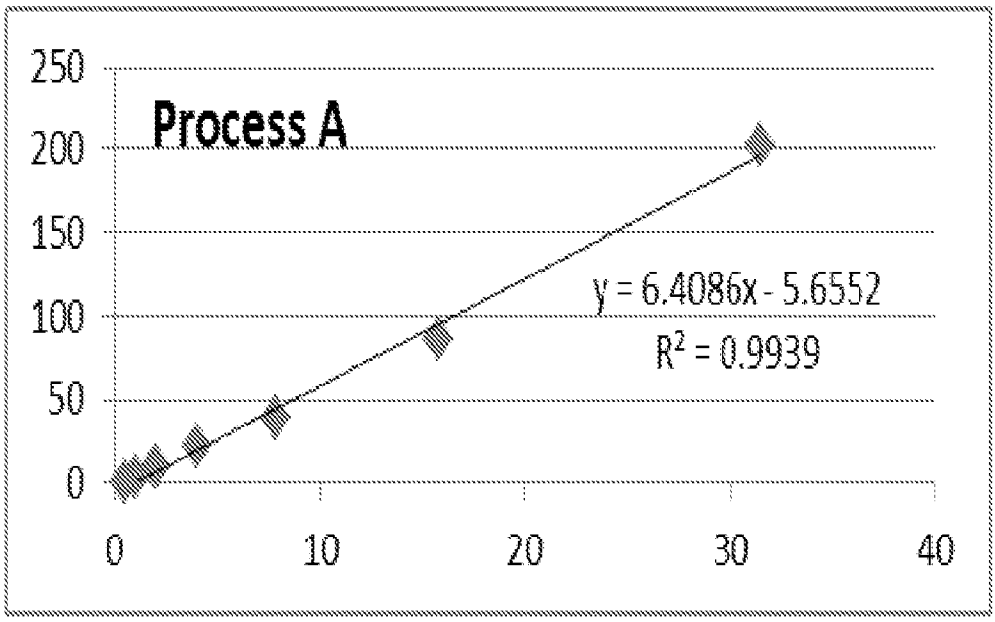


Figure 19D

KSAC protein quantification from QDot Blott

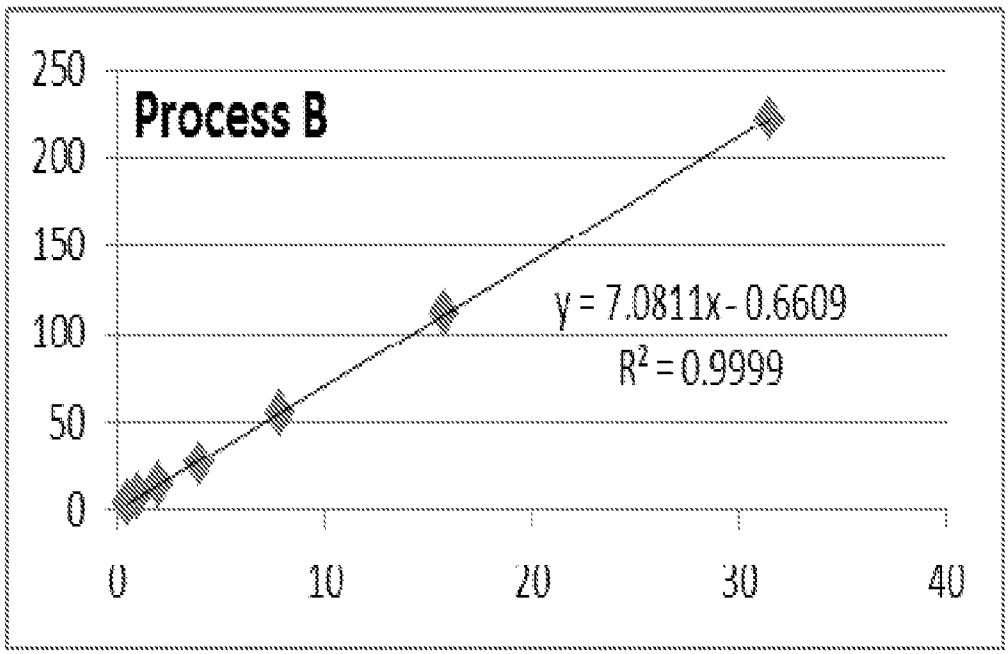


Figure 20

HPLC analysis

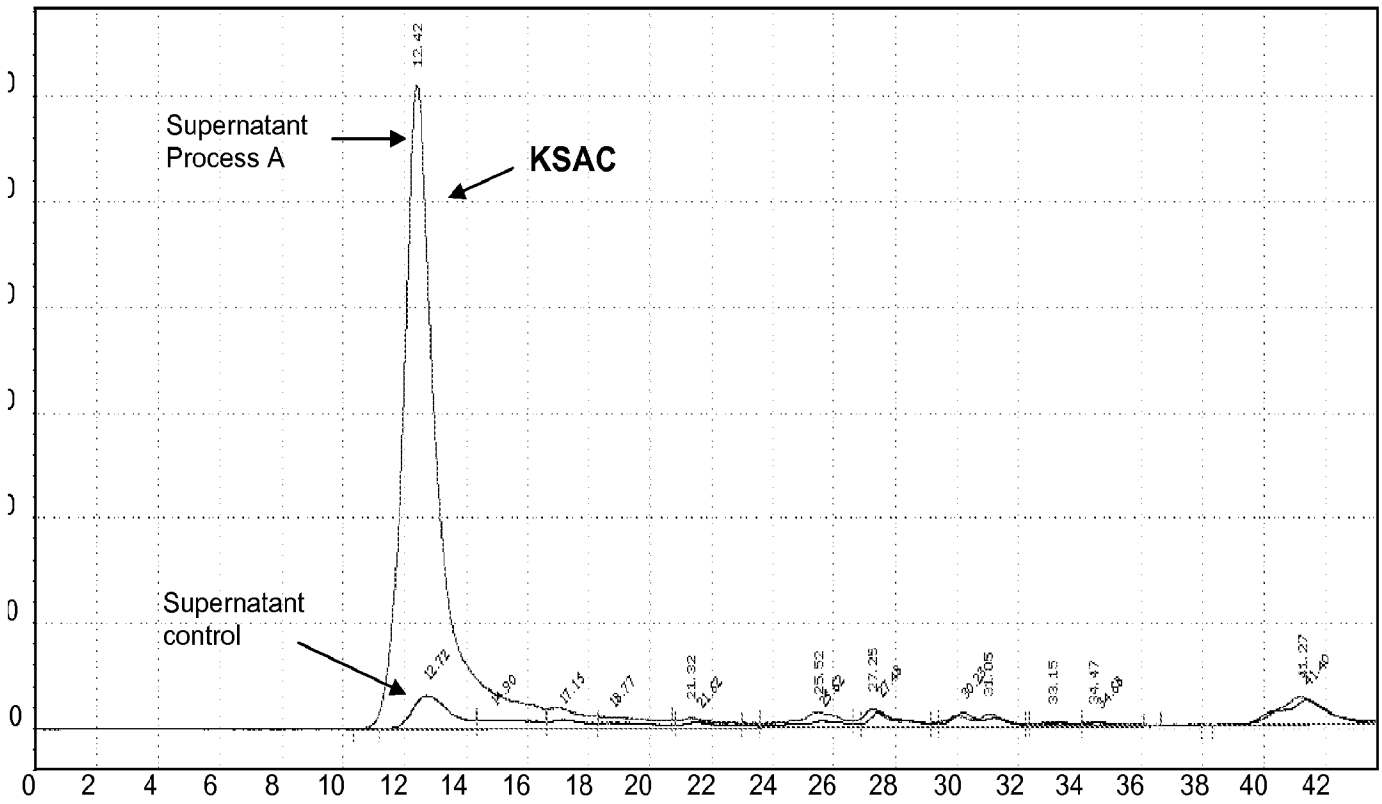


Figure 21

HPLC analysis

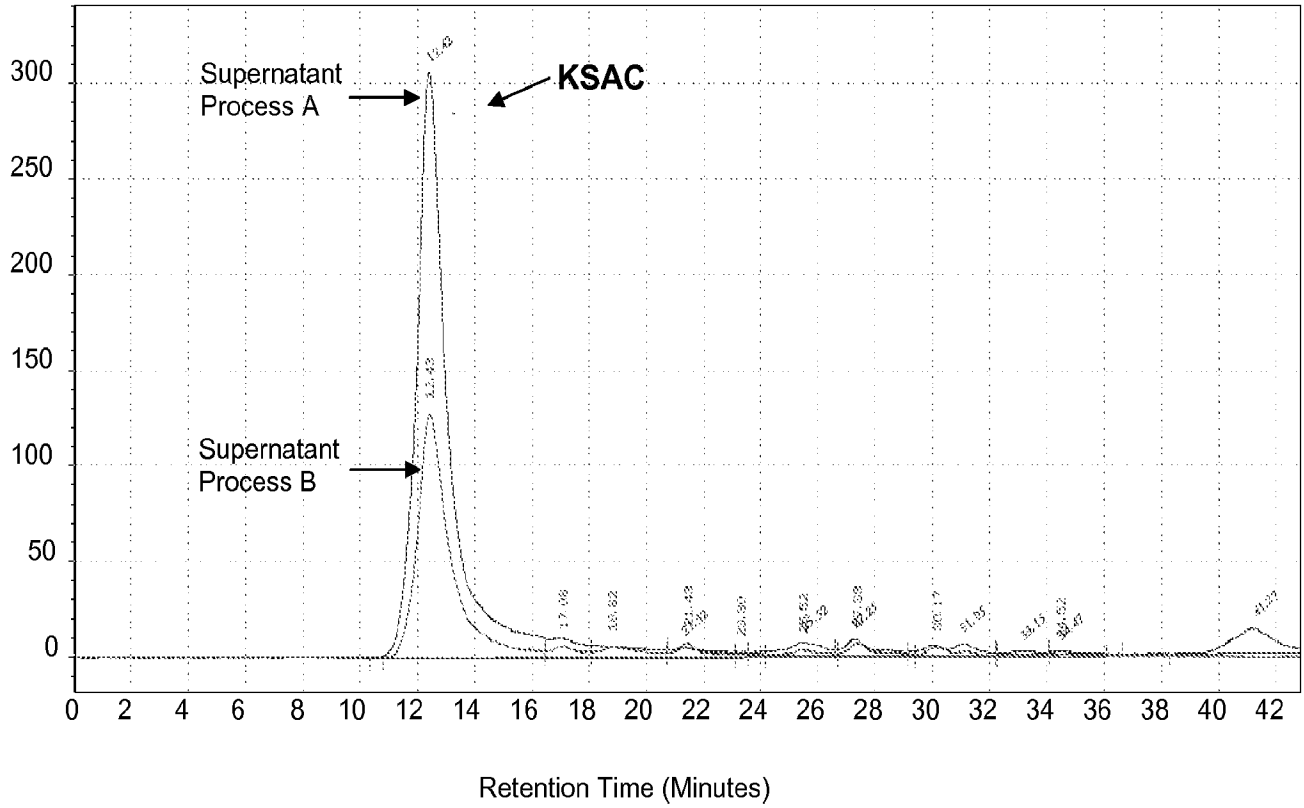
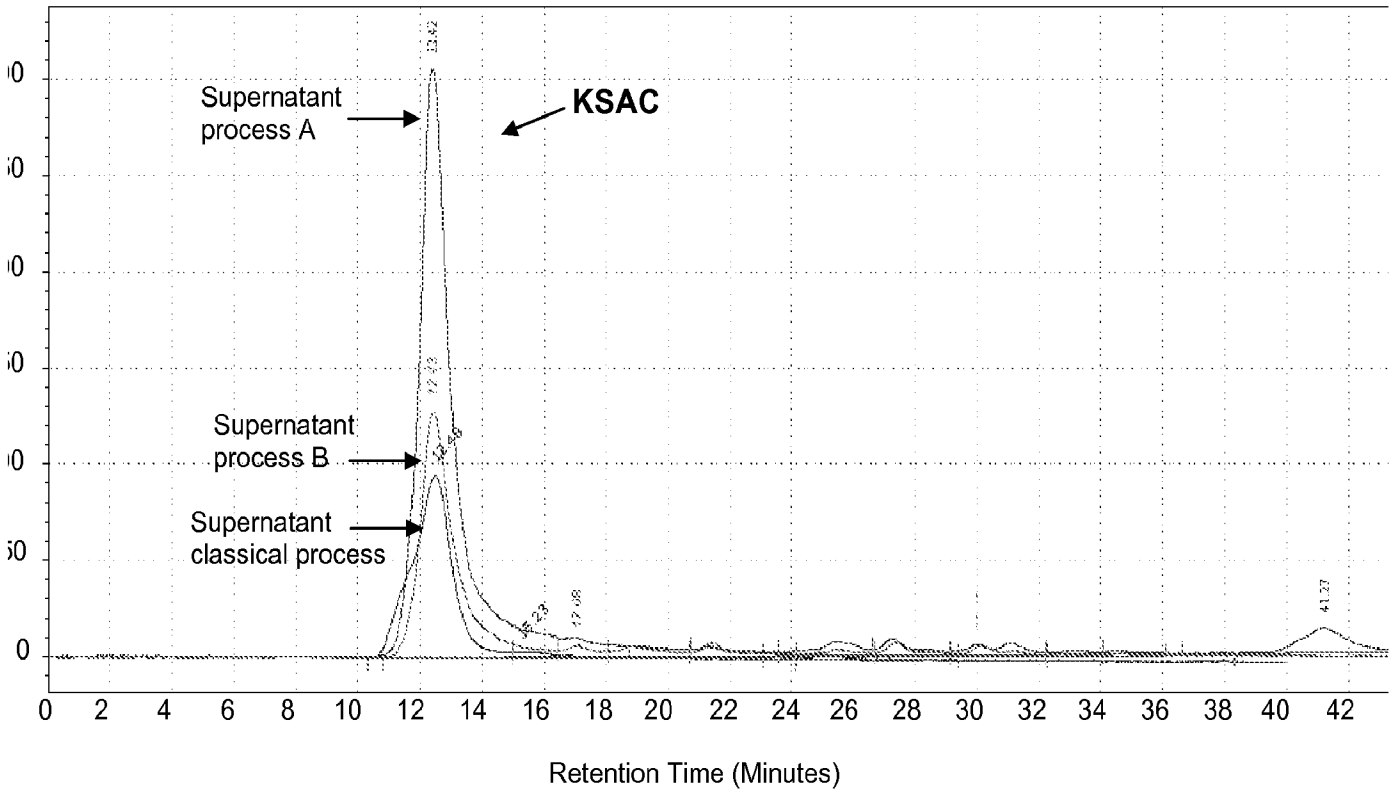


Figure 22

HPLC analysis



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<110> Merial Limited
Fischer, Laurent
Carboulec, Nicolas
Lux, Fabien

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tcgcccgaga tgaaggagca ctacgagaag ttcgagcgca tgatcaagga acacacagag 180

aagttcaaca agaagatgca cgagcactcg gagcacttca agcagaagtt cgccgagctg 240

ctcgagcagc agaaggctgc gcagtacccg tccaagacta gttccgccgg tggccgtgag 300

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tggggccaga acttccattt cgcgccgcgc tacgccggcg agaccttctt cgagtccctc 540

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20 25 30

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35 40 45

Glu Lys Phe Glu Arg Met Ile Lys Glu His Thr Glu Lys Phe Asn Lys
50 55 60

Lys Met His Glu His Ser Glu His Phe Lys Gln Lys Phe Ala Glu Leu
65 70 75 80

Leu Glu Gln Gln Lys Ala Ala Gln Tyr Pro Ser Lys Thr Ser Ser Ala
85 90 95

Gly Gly Arg Glu Thr Ala Pro Thr Asn Leu Ile Arg Arg Arg Asn Lys
100 105 110

Asp Glu Thr Asn Gly Asp Val Ser Ala Ala Ala Asp Arg Phe Arg Asp
115 120 125

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    130                 135                 140

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145                 150                 155                 160

Trp Gly Gln Asn Phe His Phe Ala Pro Arg Tyr Ala Gly Glu Thr Phe
                165                 170                 175

Phe Glu Ser Leu Ala Arg His Glu Tyr Phe Leu Ala Ala Arg Gly Gly
            180                 185                 190

Phe Met Glu Gly Asp His Ile Val Asp Val Gly Cys Gly Val Gly Gly
        195                 200                 205

Pro Ala Arg Asn Met Val Arg Leu Thr Arg Cys Asn Val Ile Gly Val
    210                 215                 220

Asn Asn Asn Asp Tyr Gln Ile Ser Arg Ala Arg Arg His Asp Ala Leu
225                 230                 235                 240

Ala Gly Met Ser Ser Lys Ile Asp Tyr Val Lys Thr Asp Phe Cys Asn
                245                 250                 255

Met Ser Leu Ala Asp Asn Thr Phe Asp Gly Ala Tyr Ala Ile Glu Ala
            260                 265                 270

Thr Cys His Ala Lys Asp Lys Val Lys Cys Tyr Ser Glu Val Phe Arg
        275                 280                 285

Val Ile Lys Pro Gly Thr Cys Phe Val Leu Tyr Glu Trp Cys Met Thr
    290                 295                 300

Asp Lys Tyr Asn Pro Asn Asp Glu Tyr His Arg Thr Ile Lys His Arg
305                 310                 315                 320

Ile Glu Leu Gly Asp Gly Leu Pro Glu Met Glu Thr Cys Lys Gln Val
                325                 330                 335

Ile Glu Tyr Met Lys Gln Ala Gly Phe Val Val Glu Glu Ala Ile Asp
            340                 345                 350

Val Ile Ser Gln Phe Glu Ser Ser Pro Ile Lys Ser Ile Pro Trp Tyr
        355                 360                 365

Gln Pro Leu Val Gly Asp Tyr Ser Ser Leu Gln Gly Leu Arg Ser Thr
    370                 375                 380

Pro Ile Gly Arg Ile Leu Thr Asn Val Met Cys Arg Val Leu Glu Phe
385                 390                 395                 400

Val Arg Leu Ala Pro Lys Gly Thr Tyr Lys Ala Thr Glu Ile Leu Glu
                405                 410                 415

Glu Ala Ala Glu Ser Leu Val Val Gly Gly Gln Leu Gly Ile Phe Thr
            420                 425                 430

Pro Ser Phe Tyr Ile Arg Ala Arg Lys Pro Ser Lys Gln Ala Gly Ser
        435                 440                 445

Lys Ile Arg Ser Val Arg Pro Leu Val Val Leu Leu Val Cys Val Ala
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        515                 520                 525

Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Leu Ser Val Gly Pro
    530                 535                 540

Gln Ser Val Gly Pro Leu Ser Val Gly Ser Gln Ser Val Gly Pro Leu
545                 550                 555                 560

Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ala
                565                 570                 575

Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val
            580                 585                 590

Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly
        595                 600                 605

Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Ser
    610                 615                 620

Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu
625                 630                 635                 640

Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser
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Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val
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Gly Pro Gln Ser Val Asp Val Ser Pro Val Ser Gly Ser Glu Phe Asp
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Ala Val Asp Trp Arg Glu Lys Gly Ala Val Thr Pro Val Lys Asn Gln
    690                 695                 700

Gly Ala Cys Gly Ser Cys Trp Ala Phe Ser Ala Val Gly Asn Ile Glu
705                 710                 715                 720

Ser Gln Trp Ala Arg Ala Gly His Gly Leu Val Ser Leu Ser Glu Gln
                725                 730                 735

Gln Leu Val Ser Cys Asp Asp Lys Asp Asn Gly Cys Asn Gly Gly Leu
            740                 745                 750

Met Leu Gln Ala Phe Glu Trp Leu Leu Arg His Met Tyr Gly Ile Val
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Glu Cys Leu Asn Ser Ser Lys Leu Val Pro Gly Ala Gln Ile Asp Gly
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Glu Asn Gly Pro Ile Ala Ile Ala Val Asp Ala Ser Ser Phe Met Ser
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Val Ile Lys Asn Ser Trp Gly Glu Asp Trp Gly Glu Lys Gly Tyr Val
865                 870                 875                 880

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Ser Ala His Val Pro Arg Ser Leu Thr Pro Gly Pro Gly Thr Glu Ser
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Glu Glu Arg Ala Pro Lys Arg Val Thr Val Glu Gln Met Met Cys Thr
        915                 920                 925

Asp Met Tyr Cys Arg Glu Gly Cys Lys Lys Ser Leu Leu Thr Ala Asn
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Val Cys Tyr Lys Asn Gly Gly Gly Gly Ser Ser Met Thr Lys Cys Gly
945                 950                 955                 960

Pro Gln Lys Val Leu Met Cys Ser Tyr Ser Asn Pro His Cys Phe Gly
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Asp Lys Pro Asp Glu Ser Thr Leu Ser Pro Glu Met Lys Glu His Tyr
        35                  40                  45

Glu Lys Phe Glu Arg Met Ile Lys Glu His Thr Glu Lys Phe Asn Lys
    50                  55                  60

Lys Met His Glu His Ser Glu His Phe Lys Gln Lys Phe Ala Glu Leu
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Thr Thr Met Val Asn Glu Tyr Tyr Asp Leu Val Thr Asp Phe Tyr Glu
    50                  55                  60

Tyr Gly Trp Gly Gln Asn Phe His Phe Ala Pro Arg Tyr Ala Gly Glu
65                  70                  75                  80

Thr Phe Phe Glu Ser Leu Ala Arg His Glu Tyr Phe Leu Ala Ala Arg
                85                  90                  95

Gly Gly Phe Met Glu Gly Asp His Ile Val Asp Val Gly Cys Gly Val
            100                 105                 110

Gly Gly Pro Ala Arg Asn Met Val Arg Leu Thr Arg Cys Asn Val Ile
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Gly Val Asn Asn Asn Asp Tyr Gln Ile Ser Arg Ala Arg Arg His Asp
    130                 135                 140

Ala Leu Ala Gly Met Ser Ser Lys Ile Asp Tyr Val Lys Thr Asp Phe
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Cys Asn Met Ser Leu Ala Asp Asn Thr Phe Asp Gly Ala Tyr Ala Ile
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Glu Ala Thr Cys His Ala Lys Asp Lys Val Lys Cys Tyr Ser Glu Val
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Asp Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser
        35                  40                  45


Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val
    50                  55                  60


Gly Pro Leu Ser Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly
65                  70                  75                  80


Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Leu Ser Val Gly Pro
                85                  90                  95


Gln Ser Val Gly Pro Leu Ser Val Gly Ser Gln Ser Val Gly Pro Leu
            100                 105                 110


Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ala
        115                 120                 125


Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val
    130                 135                 140


Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly
145                 150                 155                 160


Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Ser
                165                 170                 175


Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu
            180                 185                 190


Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser
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Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val
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Gly Pro Gln Ser Val Asp Val Ser Pro Val Ser
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Asp Ala Val Asp Trp Arg Glu Lys Gly Ala Val Thr Pro Val Lys Asn
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Gln Gly Ala Cys Gly Ser Cys Trp Ala Phe Ser Ala Val Gly Asn Ile
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Glu Ser Gln Trp Ala Arg Ala Gly His Gly Leu Val Ser Leu Ser Glu
        35                  40                  45

Gln Gln Leu Val Ser Cys Asp Asp Lys Asp Asn Gly Cys Asn Gly Gly
    50                  55                  60

Leu Met Leu Gln Ala Phe Glu Trp Leu Leu Arg His Met Tyr Gly Ile
65                  70                  75                  80

Val Phe Thr Glu Lys Ser Tyr Pro Tyr Thr Ser Gly Asn Gly Asp Val
                85                  90                  95

Ala Glu Cys Leu Asn Ser Ser Lys Leu Val Pro Gly Ala Gln Ile Asp
            100                 105                 110

Gly Tyr Val Met Ile Pro Ser Asn Glu Thr Val Met Ala Ala Trp Leu
        115                 120                 125

Ala Glu Asn Gly Pro Ile Ala Ile Ala Val Asp Ala Ser Ser Phe Met
    130                 135                 140

Ser Tyr Gln Ser Gly Val Leu Thr Ser Cys Ala Gly Asp Ala Leu Asn
145                 150                 155                 160

His Gly Val Leu Leu Val Gly Tyr Asn Lys Thr Gly Gly Val Pro Tyr
                165                 170                 175

Trp Val Ile Lys Asn Ser Trp Gly Glu Asp Trp Gly Glu Lys Gly Tyr
            180                 185                 190

Val Arg Val Val Met Gly Leu Asn Ala Cys Leu Leu Ser Glu Tyr Pro
        195                 200                 205

Val Ser Ala His Val Pro Arg Ser Leu Thr Pro Gly Pro Gly Thr Glu
    210                 215                 220

Ser Glu Glu Arg Ala Pro Lys Arg Val Thr Val Glu Gln Met Met Cys
225                 230                 235                 240

Thr Asp Met Tyr Cys Arg Glu Gly Cys Lys Lys Ser Leu Leu Thr Ala
                245                 250                 255

Asn Val Cys Tyr Lys Asn Gly Gly Gly Gly Ser Ser Met Thr Lys Cys
            260                 265                 270

Gly Pro Gln Lys Val Leu Met Cys Ser Tyr Ser Asn Pro His Cys Phe
        275                 280                 285

Gly Pro Gly Leu Cys Leu Glu Thr Pro Asp Gly Lys Cys Ala Pro Tyr
    290                 295                 300

Phe Leu Gly Ser Ile Met Asn Thr Cys Gln Tyr Thr
305                 310                 315
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