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(72) Inventeurs/Inventors:
CARRILLO, RICHARD G., US;
KRAUS, JAN P., US;
MAJTAN, TOMAS, US;
NAVEH, DAVID, US

(73) Propriétaire/Owner:
THE REGENTS OF THE UNIVERSITY OF COLORADO, A BODY CORPORATE, US

(74) Agent: GOWLING WLG (CANADA) LLP

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(54) Title: PURIFICATION OF CYSTATHIONINE BETA-SYNTHASE

(57) Abrégé/Abstract:
This invention provides chromatographic methods for the purification of a cystathionine ß-Synthase (CBS) protein, particularly truncated variants thereof and compositions and pharmaceutical compositions prepared therefrom.

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(71) Applicant *(for all designated States except US)*: **THE REGENTS OF THE UNIVERSITY OF COLORADO** [US/US]; 1800 Grant Street, 8th Floor, Denver, CO 80203 (US).

(72) Inventors; and
(71) Applicants *(for US only)*: **CARRILLO, Richard, G.** [US/US]; 725 45th Street, Oakland, CA 94609 (US). **KRAUS, Jan, P.** [US/US]; 5000 Lakeshore Drive, Littleton, CO 80123 (US). **MAJTAN, Thomas** [US/US]; 100 Idalia Court, Apt. #302, Aurora, CO 80011 (US). **NAVEH, David** [US/US]; 119 Monte Avenue, Piedmont, CA 94611 (US).

(74) Agent: **BOSMAN, Joshua, D.**; Mcdonnell Boehnen Hulbert & Berghoff LLP, 300 South Wacker Drive, Suite 3100, Chicago, IL 60606 (US).

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(54) Title: PURIFICATION OF CYSTATHIONINE BETA-SYNTHASE

(57) Abstract: This invention provides chromatographic methods for the purification of a cystathionine β-Synthase (CBS) protein, particularly truncated variants thereof and compositions and pharmaceutical compositions prepared therefrom.

PURIFICATION OF CYSTATHIONINE BETA-SYNTHASE

Field of the Invention

The present invention generally relates to methods for purification of Cystathionine β-Synthase (CBS), particularly truncated variants thereof. The present invention also relates to compositions of substantially pure CBS produced through said methods of purification.

Background of the Invention

Cystathionine β-synthase (CBS) plays an essential role in homocysteine (Hcy) metabolism in eukaryotes (Mudd *et al.*, 2001, in *The Metabolic and Molecular Bases of Inherited Disease*, 8 Ed., pp. 2007-2056, McGraw-Hill, New York). The CBS enzyme catalyzes a pyridoxal 5'-phosphate (PLP; Vitamin B_6)-dependent condensation of serine and homocysteine to form cystathionine, which is then used to produce cysteine by another PLP-dependent enzyme, cystathionine γ-lyase. In mammalian cells that possess the transsulfuration pathway, CBS occupies a key regulatory position between the remethylation of Hcy to methionine or its alternative use in the biosynthesis of cysteine. The relative flux between these two competing pathways is roughly equal and is controlled by intracellular S-adenosylmethionine (AdoMet) concentrations (Finkelstein and Martin, 1984, *J. Biol. Chem.* 259:9508-13). AdoMet activates the mammalian CBS enzyme by as much as 5-fold with an apparent dissociation constant of 15μM

(Finkelstein *et al.*, 1975, *Biochem. Biophys. Res. Commun.* 66: 81-87; Roper *et al.*, 1992, *Arch. Biochem. Biophys.* 298: 514-521; Kozich *et al.*, 1992, *Hum. Mutation* 1: 113-123).

The C-terminal regulatory domain of human CBS consists of ~140 amino acid residues (Kery *et al.*, 1998, *Arch. Biochem. Biophys.* 355: 222-232). This region is required for tetramerization of the human enzyme and AdoMet activation (Kery *et al.*, 1998, *id.).* The C-terminal regulatory region also encompasses the previously defined "CBS domains" (Bateman, 1997, *Trends Biochem. Sci.* 22: 12-13). These hydrophobic sequences (CBS 1 and CBS 2), spanning amino acid residues 416-468 and 486-543 of SEQ ID NO: 1, respectively, are conserved in a wide range of otherwise unrelated proteins. Their function remains unknown, although the sharp transition of thermally induced CBS activation and the observation that mutations in this domain can constitutively activate the enzyme indicates that they play a role in the autoinhibitory function of the C-terminal region (Janosik *et al.*, 2001, *Biochemistry* 40: 10625-33; Shan *et al.*, 2001, *Hum. Mol. Genet.* 10: 635-643; Miles and Kraus, 2004, *J. Biol. Chem.* 279: 29871-4). Two well-conserved CBS domains are also present in the C-terminal region of the yeast CBS, which is of approximately the same length as the human enzyme.

In healthy normal individuals, CBS-mediated conversion of Hcy to cystathionine is the rate-limiting intermediate step of methionine (Met) metabolism to cysteine (Cys). Vitamin B_6 is an essential coenzyme for this process. In patients with certain genetic mutations in the CBS enzyme, the conversion of Hcy to cystathionine is slowed or absent, resulting in elevations in the serum concentrations of the enzymatic substrate (Hcy) and a corresponding decrease in the serum concentrations of the enzymatic product (cystathionine). The clinical condition of an elevated serum level of Hcy, and its concomitant excretion into the urine, is collectively known as homocystinuria.

Deficiency of CBS is the most common cause of inherited homocystinuria, a serious life-threatening disease that results in severely elevated homocysteine levels in plasma, tissues and urine. Estimates on the prevalence of homocystinuria vary widely. Ascertainment by newborn screening and clinical ascertainment have indicated a prevalence ranging from 1:200,000 to 1:335,000 (Mudd *et al.*, 1995, *The Metabolic and Molecular Basis of Inherited Diseases*, McGraw-Hill: New York, p. 1279). The primary health problems associated with CBS-deficient homocystinuria (CBSDH) include: cardiovascular disease with a predisposition to thrombosis, resulting in a high rate of mortality in untreated and partially treated patients; connective tissue problems affecting the ocular system with progressive myopia and lens dislocation; connective tissue problems affecting the skeleton characterized by marfanoid habitus, osteoporosis, and scoliosis; and central nervous system problems, including mental retardation and seizures. Symptoms include dislocated optic lenses, skeletal disorders, mental retardation and premature arteriosclerosis and thrombosis (Mudd *et al.*, 2001, *id.)*. Homozygous CBS deficiency is associated with a multitude of clinical symptoms, including mental retardation, osteoporosis, kyphoscoliosis, stroke, myocardial infarction, ectopia lentis, and pulmonary embolism. Cardiovascular complications of the disease, in particular arterial and venous thrombosis, are the principal contributors to early mortality.

The pathophysiology of CBS deficiency is undoubtedly complex, but there is a consensus that the fundamental instigator of end-organ injury is an extreme elevation of serum Hcy, a substrate of CBS that builds-up in tissues and blood due to the absence of its CBS-catalyzed condensation with L-serine to form cystathionine. The toxicity of profound elevations in blood and tissue concentrations of Hcy may ensue from the molecular reactivity and biological effects of Hcy *per se* or from its metabolites (*e.g.* Hcy-thiolactone) that affect a number of biological

processes (Jakubowski *et al.*, 2008, *FASEB J* 22: 4071-6). Abnormalities in chronic platelet aggregation, changes in vascular parameters, and endothelial dysfunction have all been described in patients with homocystinuria.

Currently, three treatment options exist for the treatment of CBSDH:

1) Increase of residual activity of CBS activity using pharmacologic doses of Vitamin B_6 in Vitamin B_6-responsive patients
2) Lowering of serum Hcy by a diet with a strict restriction of the intake of Met; and
3) Detoxification by betaine-mediated conversion of Hcy into Met, thus lowering serum Hcy concentration.

Each of these three therapies is aimed at lowering serum Hcy concentration. The standard treatment for individuals affected with Vitamin B_6 non-responsive CBSDH consists of a Met-restricted diet supplemented with a metabolic formula and Cys in the form of cysteine (which has become a conditionally essential amino acid in this condition). Intake of meat, dairy products, and other food high in natural protein is prohibited. Daily consumption of a poorly palatable, synthetic metabolic formula containing amino acids and micronutrients is required to prevent secondary malnutrition. Supplementation with betaine (trade name: Cystadane™, synonym: trimethylglycine) is also standard therapy, wherein betaine serves as a methyl donor for the remethylation of Hcy to Met catalyzed by betaine-homocysteine methyltransferase in the liver (Wilcken *et al.*, 1983, *N. Engl. J. Med.* 309: 448-53). Dietary compliance generally has been poor, even in those medical centers where optimal care and resources are provided, and this non-compliance has major implications on the development of life-threatening complications of homocystinuria.

To enable patients with homocystinuria enjoy a far less restrictive diet (*e.g.* daily intake

limited to 2 g protein per kg, which is easily attainable), and have a significantly decreased Hcy plasma level leading in the long-term to clinical improvement, a strategy for increasing enzyme activity provides potential for treatment as set forth in co-pending U.S. provisional patent application Serial No. 61/758,138 . The most effective therapeutic strategy is to increase enzyme activity, as is evident when Vitamin B_6-responsive homocystinuria patients are given pyridoxone. However, this strategy is not possible for Vitamin B_6 non-responsive patients due to the nature of the mutations. Enzyme replacement therapy (ERT) as a way to increase enzyme activity in these patients requires exogenous enzyme, which is not present in the art and thus raises a need in the art for improved reagents and methods for producing CBS in greater yields of sufficiently purified enzyme for therapeutic administration.

Kraus and colleagues have developed expression systems and fermentation conditions for generating active recombinant human CBS and variants thereof (US patents No. 5,635,375, 5,523,225 and 7,485,307.

These proteins were purified by processes relevant for academic purposes, including use of protein leads on the proteins which are not considered useful for preparation of pharmaceuticals.

In order to employ methods of increasing CBS enzyme activity, an efficient method of CBS enzyme purification is required. Existing methods of purification for recombinant CBS protein rely on affinity tags to facilitate purification that does not provide the desired purity and efficiency. Therefore to more efficiently obtain the necessary levels of CBS required for therapeutic use there is a need for improved downstream purification of CBS protein produced in microbial cells.

Summary of the Invention

This invention provides methods for purifying cystathionine β-Synthase (CBS), wherein said CBS protein is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof, and particularly truncated CBS produced in recombinant cells. In particular embodiments, the method comprises the steps of: (a) providing a CBS-containing solution in the presence of at least one impurity; and (b) performing chromatographic separation of said CBS-containing solution using a metal affinity chromatography (IMAC) resin. In additional particular embodiments, the method comprises the steps of: (a) providing a CBS-containing solution in the presence of at least one impurity; and (b) performing chromatographic separation of said CBS-containing solution using an ion exchange chromatography column and a metal affinity chromatography (IMAC) resin.

In certain embodiments the method further comprises performance of additional chromatographic steps (known in the art as "polishing" steps). In particular embodiments, the methods of the invention include the step of performing chromatographic separation using a Hydrophobic Interaction Chromatography (HIC) column. In other embodiments the method further comprises the step of performing chromatographic separation using a ceramic hydroxyapaptite resin.

In certain embodiments the ion exchange column is an anion exchanger, preferably a weak anion exchanger. In particular embodiments the anion exchanger is a DEAE–Sepharose FF column. In further embodiments the IMAC resin is charged with a divalent ion. In yet further embodiments the divalent metal ion is nickel, copper, cobalt or zinc. In more specific embodiments the divalent metal ion is zinc.

In certain other embodiments the method further comprises eluting CBS from the IMAC resin with an elution buffer comprising imidazole. In certain embodiments the CBS-containing

solution is a clarified CBS solution, wherein cell debris and other particulate matter is removed from a suspension comprising CBS including but not limited to supernatant after centrifugation or filtrate after filtration. In yet other embodiments the CBS-containing solution is obtained by homogenizing cells expressing a recombinant construct comprising a nucleic acid sequence encoding CBS. In certain embodiments the CBS nucleic acid sequence comprises SEQ ID NO. 1 and encodes a protein have the amino acid sequence identified as SEQ ID NO: 2. In other embodiments the nucleic acid sequence is truncated. In yet other embodiments the truncated CBS nucleic acid sequence has been truncated to an ending position of one of amino acid residues from 382-532, 382-550 or 543-550 of SEQ ID NO:2

In other certain embodiments the recombinant cells are microbial cells, particularly bacterial cells. In particular embodiments, the bacterial cells are *E. coli* cells, particularly recombinant *E. coli* cells that produce a mammalian, preferably human, CBS protein. In certain particular embodiments, said human CBS protein has an amino acid sequence as set forth in SEQ ID NO:3 or a truncated CBS nucleic acid sequence that has been truncated to an ending position of one of amino acid residues from 382-532 or 543-550 of SEQ ID NO:2. In other particular embodiments, the truncated CBS nucleic acid sequence is optimized for expression in *E. coli*, identified by SEQ ID NO: 4.

In another aspect, a substantially purified CBS solution is provided using a method comprising the steps of: a) providing a CBS-containing solution in the presence of at least one impurity, wherein said CBS protein is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof, and particularly truncated; and (b) performing chromatographic separation of said CBS-containing solution using a metal affinity chromatography (IMAC) resin. In additional particular embodiments, a substantially purified

CBS solution is provided using a method comprising the steps of: (a) providing a CBS-containing solution in the presence of at least one impurity; and (b) performing chromatographic separation of said CBS-containing solution using an ion exchange chromatography column and a metal affinity chromatography (IMAC) resin.

In certain embodiments of the invention the substantially purified CBS solution is formulated in a pharmaceutically acceptable carrier.

In another aspect, the invention provides methods for producing an enriched CBS solution, the method comprising of: (a) providing a CBS-containing solution in the presence of at least one impurity, wherein said CBS protein is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof, and particularly truncated; and (b) performing chromatographic separation of said CBS-containing solution using an immobilized metal affinity chromatography (IMAC) resin charged with a divalent metal ion.

In another aspect, an enriched CBS solution is provided using a method comprising the steps of: a) providing a CBS-containing solution in the presence of at least one impurity, wherein said CBS protein is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof, and particularly truncated; and (b) performing chromatographic separation of said CBS-containing solution using an immobilized metal affinity chromatography (IMAC) resin charged with a divalent metal ion.

It is a particular advantage of this invention that purification of recombinant, full-length or truncated CBS, particularly human CBS, can be achieved without further modification of the protein, *e.g.*, by incorporating a "tag" molecule known in the art (poly-HIS, FLAG, etc.). Use of the chromatographic methods disclosed herein advantageously makes these tags unnecessary, thus avoiding additional recombinant manipulation and any disadvantages (in immunogenicity,

in vivo half-life or biochemical activity) that might be introduced into any preparation of recombinant CBS containing such a tag.

Specific preferred embodiments of the invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of Drawings

The following detailed description of the embodiments of the present invention can be best understood when read in conjunction with the following drawings.

Fig. 1 is a purification train summary from scale-up generation runs using a multi-step chromatography method including DEAE-Sepharose-FF, Zn-IMAC and HIC chromatography.

Fig. 2 is a purification summary from purification experiments using a DEAE-Sepharose-FF column and CBS purified using the "non-optimized" bacterial expression construct. Mobile phases included 10% ethylene glycol in addition to other components as set forth in the Examples.

Figs. 3 A-D are purification train summaries from scale-up generation runs using a multi-step chromatography method including DEAE-Sepharose-FF, Zn-IMAC, ceramic hydroxyapaptite resin and HIC chromatography.

Fig. 4 is a photoimage of a SDS page gel showing the relative amounts of CBS protein and impurities for each stage of the purification step using a DEAE column.

Fig. 5 is a photoimage of a SDS page gel showing the relative amounts of CBS protein and impurities for a 3 column purification method including: a DEAE column, a Zn-IMAC column and HIC column.

Fig. 6 is a photoimage of a SDS page gel showing the relative amounts of CBS protein and impurities for a 4 column purification method including: a DEAE column, a Zn-IMAC column, a ceramic hydroxyapaptite resin and a HIC column.

Fig. 7 is chromatograms demonstrating the components of the separated mixture following purification using Zn-IMAC.

Fig. 8 is a purification summary from development runs using a Ni-IMAC column.

Fig. 9 is a summary table demonstrating the total protein following a purification method using a Ni-IMAC column.

Fig. 10 is a photoimage of a SDS page gel showing the relative amounts of CBS protein and impurities for each stage of the purification step using a Ni-IMAC column.

Fig. 11 is a purification summary from scale-up generation runs using a Cu-IMAC column.

Fig. 12 is a summary table demonstrating the total protein following a purification method using a Zn-IMAC column.

Fig. 13 is a photoimage of a SDS page gel showing the relative amounts of CBS protein and impurities for each stage of the purification step using a Zn-IMAC column.

Fig. 14 is a scheme of the purification method using multi-step chromatography purification steps.

Detailed Description of the Invention

This invention provides methods for purification of CBS protein, wherein said CBS protein is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof, and particularly a truncated protein CBS produced in recombinant cells. In particular, the invention provides methods for the purification of a CBS protein that include the steps (a) providing a CBS-containing solution in the presence of at least one

impurity; and (b) performing chromatographic separation of said CBS-containing solution using a metal affinity chromatography (IMAC) resin. In additional particular embodiments, the method comprises the steps of: (a) providing a CBS-containing solution in the presence of at least one impurity; and (b) performing chromatographic separation of said CBS-containing solution using an ion exchange chromatography column and a metal affinity chromatography (IMAC) resin.

A particular chromatographic separation step in the certain embodiments of the methods provided by this invention comprises an ion exchange chromatography column. In one embodiment, the ion exchange chromatography column is an anion exchanger, preferably a weak anion exchanger. Various types of anion exchange resins can be used, including DEAE-Sephadex, QAE-Sephadex, DEAE-Sephacel, DEAE-cellulose and DEAE-Sepharose-FF. According to one embodiment, the anion exchange resin is DEAE-Sepharose-FF.

Another particular chromatographic separation step in the certain of the methods provided by this invention comprises a metal affinity chromatography (IMAC) resin having appropriate pH and conductivity such to allow the protein to bind to the column while selective intermediate washes are used to remove weaker binding proteins and other molecular species. In certain embodiments, varying concentrations of imidazole were used to modulate the partitioning during the chromatography. Suitable metal affinity resins include immobilized metal affinity columns charged with a divalent metal ion including nickel, copper, cobalt or zinc. In certain embodiments of the methods of the invention, the metal affinity chromatography (IMAC) column is used following ion exchange chromatography. In such embodiments, the IMAC column is preferably charge with zinc as a divalent cation. In other embodiments of the

inventive methods, the IMAC column is used as an initial chromatographic step. In such embodiments, nickel or copper divalent cations are preferably used to charge the IMAC column.

Additional chromatographic steps provided in certain embodiments of the methods of this invention for purifying CBS from a CBS-containing solution include without limitation hydrophobic interaction chromatography (HIC). HIC is useful for removing impurities that have relatively closely related chromatographic properties that are eluted together with the target protein during the capture step.

Further additional chromatographic steps provided in certain embodiments of the methods of this invention for purifying CBS from a CBS-containing solution include without limitation a ceramic hydroxyapatite resin. "Ceramic hydroxyapatite" or "CHAP" refers to an insoluble hydroxylated calcium phosphate of the formula $(Ca_{10}(PO_4)_6(OH)_2)$, which has been sintered at high temperatures into a spherical, macroporous ceramic form. The methods of the invention also can be used with hydroxyapatite resin that is loose or packed in a column. The choice of column dimensions can be determined by the skilled artisan.

Chromatography matrices useful in the method of the invention are materials capable of binding biochemical compounds, preferably proteins, nucleic acids, and/or endotoxins, wherein the affinity of said biochemical compounds to said chromatography matrix is influenced by the ion composition of the surrounding solution (buffer). Controlling the ion composition of said solution allows to use the chromatography materials of the invention either in subtractive mode (CBS passes through said chromatography matrix, at least certain contaminants bind to said chromatography matrix) or, preferably, in adsorptive mode (CBS binds to the chromatography matrix).

In particular embodiments, the method for purification comprises the step of homogenizing host cells, particularly recombinant cells and in certain embodiments, recombinant cells producing mammalian, preferable human, CBS protein, wherein said recombinant construct encodes a CBS protein that is a naturally occurring truncated variant, or a genetically engineered truncate thereof, and particularly wherein said construct has been optimized for recombinant cell expression. In particular embodiments, said recombinant cells are microbial cells and particularly bacterial cells. In certain particular embodiments, the bacterial cells are *E. coli* cells and the CBS sequence has been engineered in the recombinant expression construct to be optimized for expression in said cells; a specific embodiment of such a nucleic acid sequence optimized for CBS expression in *E. coli* is set forth in SEQ ID NO: 4. In said methods, cells are harvested, *e.g.* by centrifugation, and optionally stored at -80 degree °C. Homogenization of host cells is performed by disrupting the cells host using physical, chemical or enzymatic means or by a combination thereof. Advantageously, for purification from bacterial sources homogenation is performed by disrupting the cell wall of said bacterial host by sonication. Alternatively or additionally homogenizing is performed by destabilizing the bacterial cell wall of the host by exposure to a cell wall degrading enzyme such as lysozyme.

The methods of the invention can further comprise a clarified CBS homogenate, wherein cell debris is removed from the homogenate by either filtration or centrifugation. In certain embodiments, clarifying is performed by centrifuging the homogenate at an effective rotational speed. The required centrifugation time depends *inter alia* on the volume of the homogenate, which can be determined empirically to obtain a sufficiently solid pellet. To obtain an essentially cell debris-free clarified homogenate a combination of centrifugation and filtration can be performed on the homogenate.

The term "recombinant cell" as used herein refers to suitable cells (including progeny of such cells) from any species into which has been introduced a recombinant expression construct capable of expressing a nucleic acid encoding CBS protein, preferably human CBS protein and most particularly a human CBS protein that is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof. In specific embodiments, the truncated CBS protein encoded by said recombinant expression construct has an amino acid sequence as set forth in SEQ ID NO: 3.

The term, "bacterial cell", as used herein refers to bacteria that produces a mammalian, preferably human, CBS protein *inter alia* using recombinant genetic methods including progeny of said recombinant cell, wherein said CBS protein is a naturally occurring truncated variant, or a genetically engineered truncate thereof.

The term "recombinant expression construct" as used herein refers to a nucleic acid having a nucleotide sequence of a mammalian, preferably human, CBS protein, and sequences sufficient to direct the synthesis of CBS protein in cultures of cells into which the recombinant expression construct is introduced and the progeny thereof.

As used herein, reference to CBS protein or polypeptide preferably includes a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof, or fusion proteins, or any homologue (variant, mutant) thereof, and specifically mammalian CBS and preferably human CBS. Such a CBS protein can include, but is not limited to, purified CBS protein, recombinantly produced CBS protein, soluble CBS protein, insoluble CBS protein, and isolated CBS protein associated with other proteins. In addition, a "human CBS protein" refers to a CBS protein from a human *(Homo sapiens)* preferably includes a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate thereof., As such, a

human CBS protein can include purified, partially purified, recombinant, mutated/modified and synthetic proteins. As disclosed herein and in related U.S. Patent No. 8,007,787 and 7,485,307, the CBS protein truncates are advantageously soluble CBS proteins that are produced in bacteria without the creation of insoluble inclusion bodies.

As used herein, the term "homologue" (or variant or mutant) is used to refer to a protein or peptide which differs from a naturally occurring protein or peptide (*i.e.*, the "prototype" or "wild-type" protein) by modifications to the naturally occurring protein or peptide, but which maintains the basic protein and side chain structure of the naturally occurring form. Such changes include, but are not limited to: changes in one, few, or even several amino acid side chains; changes in one, few or several amino acids, including deletions (*e.g.*, a truncated version of the protein or peptide), insertions and/or substitutions; changes in stereochemistry of one or a few atoms; and/or minor derivatizations, including but not limited to: methylation, glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation and/or addition of glycosylphosphatidyl inositol. A homologue can have enhanced, decreased, changed, or substantially similar properties as compared to the naturally occurring protein or peptide. A homologue can include an agonist of a protein or an antagonist of a protein.

Homologues can be the result of natural allelic variation or natural mutation. A naturally occurring allelic variant of a nucleic acid encoding a protein is a gene that occurs at essentially the same locus (or loci) in the genome as the gene which encodes such protein, but which, due to natural variations caused by, for example, mutation or recombination, has a similar but not identical sequence. Allelic variants typically encode proteins having similar activity to that of the protein encoded by the gene to which they are being compared. One class of allelic variants can encode the same protein but have different nucleic acid sequences due to the degeneracy of the

genetic code. Allelic variants can also comprise alterations in the 5' or 3' untranslated regions of the gene (*e.g.*, in regulatory control regions). Allelic variants are well known to those skilled in the art.

Homologues can be produced using techniques known in the art for the production of proteins including, but not limited to, direct modifications to the isolated, naturally occurring protein, direct protein synthesis, or modifications to the nucleic acid sequence encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis. CBS variants are described in U.S. Patent No. 8,007,787;

in particular and preferred embodiments, the reagents and methods of the invention set forth herein preferably include a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate of human CBS protein. Particular truncated forms of SEQ ID NO: 3 according to the present invention include N-terminal deletion variants, C-terminal deletion variants, and variants having both N-terminal and C-terminal deletions.

As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein *in vitro, ex vivo* or *in vivo*. For a protein to be useful *in vitro, ex vivo* or *in vivo*, it is preferably substantially free of contaminants, other proteins and/or chemicals that might interfere or that would interfere with its use, or that at least would be undesirable for inclusion with a CBS protein (including homologues thereof).

As used herein an enriched CBS solution is a solution subjected to one or more purification steps.

The purity of protein can be determined by calculating fold purification, *i.e.* a formula that provides a measure of how much more a purified solution is compared to a less purified solution or crude extract. Fold purification is calculated using the following formula:

Specific activity final fraction/Specific activity crude fraction.

Another measurement to assess purity is the "specific activity" which measures the purity of an enzyme. Specific activity can be measured using the following formula:

$$\frac{Units}{mL} \times \frac{mL}{mg} = \frac{Units}{mg}$$

CBS protein compositions provided by this invention are useful for regulating biological processes and particularly, processes associated with the catalysis of the pyridoxal 5'-phosphate (PLP)-dependent condensation of serine and homocysteine to form cystathionine. In particular, compositions of the present invention are useful for producing cystathionine and cysteine in vitro or for treating a patient that will benefit from increased CBS activity (*e.g.*, a patient with homocystinuria). In certain embodiments, the invention provides said compositions of CBS protein, preferably human CBS protein, wherein said CBS protein is a naturally occurring truncated variant, or a chemically cleaved or genetically engineered truncate of human CBS protein, as pharmaceutical compositions comprising said CBS protein and a pharmaceutically acceptable carrier.

As used herein, a "pharmaceutically acceptable carrier" includes pharmaceutically acceptable excipients and/or pharmaceutically acceptable delivery vehicles, suitable for use in suitable administration of the composition *in vitro*, *ex vivo* or *in vivo*. Suitable *in vitro*, *in vivo* or *ex vivo* administration preferably comprises any site where it is desirable to regulate CBS activity. Suitable pharmaceutically acceptable carriers are capable of maintaining a CBS protein

as provided by this invention in a form that, upon arrival of the protein at the target cell or tissue in a culture or in patient, the protein has its expected or desired biological activity. Examples of pharmaceutically acceptable carriers include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity. Compositions of the present invention can be sterilized by conventional methods and/or lyophilized.

The following examples are provided for the purpose of illustration and are not intended to limit the scope of the present invention.

Examples

Example 1: Production of Truncated CBS Protein in Bacteria

A truncated human CBS variant lacking specific portions of the non-conserved regions (r-hCβSΔC; SEQ ID No: 3) were constructed and over-expressed using the previously described *E. coli* based expression system (Kozich and Kraus, 1992, *supra).* In the modification of this system disclosed herein (*i.e.*, expressing the truncate rather than the full-length CBS protein), the CBS truncate encoded by SEQ ID NO: 3 was expressed without any fusion partner under the control of the *tac* promoter. Constructs encoding the truncated human CBS protein variant r-hCβSΔC (SEQ ID NO: 4) were generated by a modification of the previously described pHCS3 CBS expression construct (Kozich and Kraus, 1992, Hum. Mutat. 1,113-123) which contains the CBS full-length coding sequence (SEQ ID NO: 1) cloned into pKK388.1. In this construct, CBS

expression was governed by the IPTG inducible *lac* promoter. To generate C-terminal deletion constructs, CBS cDNA fragments spanning the desired nucleotide residues were amplified using primers incorporating Sph I and Kpn I sites to the 5' and 3' respective ends of the PCR product. All PCR products were then cut with Sph I and Kpn I and cloned by ligation into the pHCS3 vector digested with Sph I and Kpn I. An Sph I site naturally occurs in the CBS cDNA, just upstream of the antisense primer hybridization site (base pare position 1012, according to the CBS cDNA numbering, ref. 25). PCR products thus generated were then digested with Nco I and Sph I and ligated into the pHCS3 plasmid cut with the same enzymes.

pKK CBS Δ414-551

sense: CGTAGAATTCACCTTTGCCCGCATGCTGAT (SphI)(SEQ ID NO: 5)

antisense: TACGGGTACCTCAACGGAGGTGCCACCACCAGGGC (KpnI)(SEQ ID NO: 6)

Finally, the construct was transformed into *E. coli* BL21 (Stratagene). The authenticity of the construct was verified by DNA sequencing using a Thermo Sequenase Cy5.5 sequencing kit (Amersham Pharmacia Biotech) and the Visible Genetics Long-Read Tower System-V3.1 DNA sequencer according to the manufacturer's instructions.

Bacterial Expression analysis of CBS Deletion Mutants-Growth of *E. coli.* BL21 cells bearing the CBS truncation mutant construct, induction of expression and the generation of crude cell lysates were performed as described previously (Maclean *et al.*, 2002, *Hum. Mutat. 19(6),* 641-55). Briefly, bacteria were grown at 37° C aerobically in 1 L NZCYMT media (Gibco/BRL, Gaithersburg, Md.) containing 75 μg/mL ampicilin and 0.001% thiamine in the presence or absence of 0.3 mM δ-aminolevulinate (δ-ALA) until they reached turbidity of 0.5 at 600 nm. IPTG was then added to 0.5 mM and the bacteria were grown further. The insoluble fraction was prepared as follows: after the centrifugation of the sonicated homogenate, pelleted

cell debris were thoroughly washed with chilled Ix Tris-buffered saline, pH 8.0. The pellets were then resuspended in1ml of the lysis buffer (Maclean *et al.*, *ibid.)* followed by a brief sonication in order to homogenize the insoluble fraction.

CBS Activity Assay- CBS activity was determined by a previously described radioisotope assay using [^{14}C] serine as the labeled substrate (Kraus, 1987, *Methods Enzymol.* 143,388-394). Protein concentrations were determined by the Lowry procedure (Lowry *et al.*, 1951, *J. Biol. Chem.* 193, 265-275) using bovine serum albumin (BSA) as a standard. One unit of activity is defined as the amount of CBS that catalyzes the formation of 1 μmol of cystathionine in 1 h at 37°C.

Denaturing and Native Polyacrylamide Gel Electrophoresis and Western Blotting- Western blot analysis of crude cell lysates under both denaturing and native conditions was performed as described previously (Janosik, 2001, *supra)* with some modifications. Soluble fractions of *E. coli* lysates containing the expressed mutant protein were mixed with sample buffer and run on a 6% native PAGE without a stacking gel. The final composition of the sample buffer was: 50mM Tris-HCl, pH 8.9, 1mM DTT, 10% glycerol, 0.001% bromphenol blue. Detection of heme was performed using a previously described method that relies on heme peroxidase activity (Vargas *et al.*, 1993, *Anal. Biochem.* 209(2), 323-6).

Densitometric Scanning Analysis-Quantitative densitometry analysis was performed using the Imagemaster ID (version 2.0) software (Pharmacia). To construct a calibration curve, 50, 75, 100,250,500 and 1000ng of purified wild type CBS protein were run on an SDS-PAGE together with crude cell lysates of the individual mutants. Following electrophoresis, Western blot immunoanalysis was conducted using rabbit anti-CBS serum. The signals corresponding to

the experimentally observed CBS mutant subunits were all within the linear range of the calibration curve constructed with purified human CBS.

Example 2: Preparation of Crude Extraction

Crude CBS protein-containing extracts was prepared for use in downstream chromatography steps. Frozen pellets (cells) obtained from fermentation of recombinant bacteria producing human truncated CBS variant (r-hCβSΔC; SEQ ID No: 3) were lysed, wherein said bacteria expressed truncated human CBS encoded by SEQ ID NO: 4. Lysis buffer for initial isolations contained 1 mM DTT, 1% Triton X-100, and Protease Inhibitor. These components were eventually removed from the buffer. The buffer used for the final isolations that produced material for scale-up runs consisted of 20mM Sodium Phosphate, 50 mM NaCl, 0.1 mM PLP (pH 7.2), with lysozyme added to a concentration of 2 mg/mL after homogenization. Following mixing with lysozyme for 1 hr at 4°C, the homogenate was sonicated until viscosity was reduced and then subjected to centrifugation at 20,000 rpm (48,000 x *g*) for 30 min. The supernatant was collected, aliquoted, and stored at -70°C until use. Generally, the crude extract was thawed at 37°C prior to chromatographic purification.

Example 3: DEAE-Sepharose FF Chromatography

DEAE-Sepharose FF was used in this Example of the purification methods for CBS because it possesses good capacity and flow properties and has been manufactured consistently for several years. This step employed a drip/gravity column that contained approximately 6 mL of resin. The column was equilibrated in Sodium Phosphate buffer with 50 mM NaCl, pH 7.0. Loading of the crude extract was targeted at approximately 20 mg total protein/mL resin. After loading the column, the red color of the load was concentrated near the top of the column.

Following a wash with equilibration buffer, the column was washed with a buffer containing 150 mm NaCl, whereby the majority of color eluted from the column (all steps were performed at pH 7.0). Essentially all color was removed from the column with a 300mM NaCl wash. Based on these results, a column was packed that could be operated in flow mode. The conditions employed equilibration/loading at a NaCl concentration 50mM, with elution at 250mM NaCl. The final conditions required dilution of the column load with H_2O to approach the ionic strength of the equilibration/wash buffer (50mM NaCl), and elution with 137 mM NaCl (Figs. 1, 2 and 3). Samples were analyzed by SDS-PAGE to determine the relative amounts of CBS protein and impurities (Fig. 4). The following tables represent column operational parameters and data from the scale-up runs that employed them.

Table 1. Operational Parameters for DEAE Capture Step

Process Step	Column load target (total protein mg/mL)	NaCl Concentration (with 20mM Na_3PO_4 pH 7.0)	Column Volumes (mL)	Contact time Column vol./flow rate (min.)
Equilibration	N/A	50 mM	3-5	10
Load	20-25 mg/mL	Approx. 50 mM	Variable	15
Wash	N/A	50 mM	3	10
Elution	N/A	137 mM	Variable*	15
2M NaCl Strip	N/A	2 M	3	10

* Note: Eluate collection starts at approx. 0.4 AU and ends at approx. 0.55 AU. Void volume typically approx. 1 column volume.

Table 2. Data from Scale-up Runs (n=6)

Input		Output		
Column loading (per mL Resin)				
Total Protein (mg)	Units	Recovery (%)	Fold Purif. (By S.A.)	
14.5 - 19.8	3275 – 5443	79.3 – 93.0	2.5 – 3.3	*Range*
18.2	4451	86	2.8	*Average*

Example 4: IMAC Chromatography

The ability for an immobilized metal affinity column (IMAC) to separate CBS protein from impurities and other contaminants from a biological source, such as a recombinant bacterial cell homogenate, was demonstrated. Because of the desire to avoid low pH conditions (< 5, anecdotal), varying concentrations of imidazole were used to modulate partitioning during the chromatography.

Copper (Cu^{++}) was tested as a candidate species of IMAC column based on its relatively strong binding characteristics. Prior to being applied to the IMAC column, the CBS solution was adjusted to 0.4M NaCl. The results indicated that capture was near complete, with an acceptable activity recovery (70-80%). Recovery of CBS was obtained using 100mM imidazole, which resulted in significant precipitation upon thawing from storage at -70° C. (Fig. 11). In addition, there was only a small increase in purity relative to the load. Thus, experiments employing Ni^{++}IMAC were conducted as the metal of choice. In these experiments, the CBS sample was run through a G-25 column to remove dithiothreitol (DTT) prior to loading the solution onto the IMAC column. Purity enhancement remained low and selectivity was similar to Cu^{++}, as evidenced by a relatively small A_{280} peak in the high imidazole strip fraction. (Figs 8, 9 and 10).

The relatively weak binding Zn^{++} was also tested. Although capture, wash and elution conditions required significantly lower imidazole concentrations, potential for purity enhancement provided positive results due to the significant size of the A_{280} peaks in the post-load wash and high imidazole strip fractions. 0.4 M NaCl and 0.01% Triton X-100 were added to the equilibration and wash buffers to minimize non-specific binding. (Figs. 1 and 3). Samples were analyzed by SDS-PAGE to determine the relative amounts of CBS protein and impurities

(Fig. 13). The results of the IMAC experiments are presented in Fig. 7. The following tables represent column operational parameters and data from the scale-up runs that employed them.

Table 3. Operational Parameters for Zn-IMAC Step

Process Step	Column load target (total protein mg/mL)	Imidazole Concentration (with 20mM Na_3PO_4 pH 7.0)	Column Volumes (mL)	Contact time Column vol./flow rate (min.)
Equilibration	N/A	1 mM	3	10
Load	10	0	Variable	10
Wash	N/A	1 mM	3	10
Elution	N/A	11 mM	Variable*	10
Strip	N/A	100 mM	3	10

* Note: Eluate collection starts at approx. 0.25 AU and ends at approx. 0.16 AU. Void volume typically approx. 1.5 column volumes.

Table 4. Data from Scale-up Runs (n=5)

Input		Output		
Column loading (per mL Resin)				
Total Protein (mg)	Units	Recovery (%)	Fold Purif. (By S.A.)	
6.5-9.3	4414-7038	71.8 – 84.6	1.3 -1.6	*Range*
8.1	5687	80	1.4	*Average*

Example 5: HIC Chromatography

Multiple experiments were conducted to identify the parameters for HIC chromatography. Initial drip column experiments were conducted that employed a resin with a relatively strong binding ligand (phenyl) with an IMAC eluate as starting material/load. This experiment resulted in empirically complete binding at 1.3M $(NH_4)_2SO_4$. However, there was evidence of significant retention of CBS on the column even after washing with a low ionic

strength buffer. Based on these results, a resin with a weaker binding ligand (butyl) was tested. Initial experiments with this resin showed no apparent capture at 0.5M $(NH_4)_2SO_4$. The non-binding flow through of this column experiment was collected and adjusted to 1.25M $(NH_4)_2SO_4$, and reloaded on to a column equilibrated to the same concentration of $(NH_4)_2SO_4$. In this case there was evidence of significant binding to the column. A 20 column volume $(NH_4)_2SO_4$ gradient elution was performed from 1.25M to 0.25M $(NH_4)_2SO_4$ with fractions collected. SDS-PAGE analysis of the fractions indicated that there was significant potential for impurity clearance on the lower end of the gradient. Experiments utilizing step gradient washes at varying concentrations of $(NH_4)_2SO_4$ determined the final operational parameters. (Figs. 1 and 3). Those parameters and the scale-up run data are summarized in the tables below.

Table 5. Operational parameters for HIC Step (n=6)

Process Step	Column load target (total protein mg/mL)	$(NH_4)_2SO_4$ Concentration (with 20mM Na_3PO_4 pH 7.0)	Column Volumes (mL)	Contact time Column vol./flow rate (min.)
Equilibration	N/A	1.4M	3	10
Load	5-10	1.4M	Variable	10
Wash	N/A	1.4M	3	10
Elution	N/A	1.1M	Variable*	10
Strip	N/A	0.05M NaCl	3	10

* Note: Eluate collection starts at approx. 0.25 AU and ends at approx. 0.15 AU. Void volume typically approx. 1.4 column volumes.

Table 6. Data from Scale-up Runs (n=5)

Input		Output		
Column loading (per mL Resin)				
Total Protein (mg)	Units	Recovery (%)	Fold Purif. (By S.A.)	
5.1 - 7.2	5375 - 9248	77.8 – 92.7	1.0 – 1.3	*Range*
6.3	7638	85	1.2	*Average*

Example 6: CHAP Chromatography

Ceramic hydroxyapatite is a resin that has a unique, potentially mixed binding mode chemistry that was utilized in a CBS purification method. CBS displayed acidic characteristics and therefore initial investigation focused on using phosphate-modulated partitioning. The initial experiments utilized HIC eluate that was buffer exchanged into a 0.05M NaCl, 0.005M Potassium Phosphate (pH 6.8) buffer. A 5 mL ceramic hydroxyapatite (Type 1) cartridge was equilibrated in the same buffer and the conditioned HIC eluate was loaded onto the column. There was no visible breakthrough of protein (as measured by A_{280}) during the load and subsequent wash with equilibration/wash buffer. A linear gradient (5%) of 0.005M to 0.5M Potassium Phosphate was then run and fractions were collected. Based on the chromatogram, samples were analyzed by SDS-PAGE to determine the relative amounts of CBS protein and impurities. In subsequent experiments (based on analysis of the results of previous experiments), step washes with varying levels of phosphate were employed to determine optimal conditions for load, wash, and elution steps. In addition, the composition of buffer salts was transitioned from Potassium to Sodium Phosphate. (Fig. 3). The following tables represent column operational parameters and data from the scale-up runs that employed them.

Table 7. Operational Parameters for CHAP Step

Process Step	Column load target (total protein mg/mL)	Na_3PO_4 Concentration (with 50mM NaCl, pH 7.0)	Column Volumes (mL)	Contact time Column vol./flow rate (min.)
Equilibration	N/A	10 mM	3	6
Load	10-15	10 mM	Variable	6
Wash	N/A	30 mM	3	6
Elution	N/A	90 mM	Variable*	6
Strip	N/A	150 mM	3	6

* Note: Eluate collection starts at approx. 0.20 AU and ends at approx. 0.16 AU. Void volume typically approx. 1.0 column volumes.

Table 8. Data from Scale-up Runs (n=5)

Input		Output		
Column loading (per mL Resin)				
Total Protein (mg)	Units	Recovery (%)	Fold Purif. (By S.A.)	
9.9 –12.2	11205 - 12297	84.6 – 92.4	1.1 -1.2	*Range*
11.1	11751	89	1.2	*Average*

Example 7: Integrated Process Results

The particular multi-step method described in these Examples was evaluated at the scale of a 60mL capture column. All of the purification trains utilized starting material (crude extract) obtained from fermentations that were seeded with recombinant cells comprising a construct comprising a truncated variant of human CBS encoded by a nucleic acid having codons optimized for expression in *E. coli*. This construct resulted in starting material that was approximately 2-fold higher in specific activity, and significantly impacted the final purity achieved from the integrated purification method. The overall purification results using the multi-step method were measured by SDS-PAGE and Specific Activity (Figs. 5 and 6). The results demonstrated that the purity and specific activity met or exceeded that of the purified tagged truncated CBS. All Specific Activities of final column eluates obtained by the largest scale currently possible exceeded 1200 U/mg total protein. The following table summarizes the overall purification results from the scale-up runs.

Table 9. Overall Results from Scale-Up Runs

	Total Recovery (%)		Fold Purification	
	Range	Average	Range	Average

	Total Recovery (%)		Fold Purification	
3 Column Train (n=3)	57 – 60	58	5.7 – 6.2	5.9
4 Column Train (n=2)	47 – 52	50	4.6 – 5.4	5.0

Specific Activity of Final Column Eluate = 1206 -1509.

Having described the invention in detail and by reference to specific embodiments thereof, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims. More specifically, although some aspects of the present invention are identified herein as particularly advantageous, it is contemplated that the present invention is not necessarily limited to these particular aspects of the invention.

Table 10: CβS Sequences

Molecule	SEQ ID NO	Sequence
Native human CβS nucleic acid sequence	**1**	atgccttctgagacccccaggcagaagtggggcccacag gctgcccccaccgctcagggccacactcggcgaaggggag cctggagaaggggtccccagaggataaggaagccaaggag cccctgtggatccggcccgatgctccgagcaggtgcacct ggcagctgggccggcctgcctccgagtccccacatcacca cactgccccggcaaaatctccaaaaatcttgccagatatt ctgaagaaaatcggggacacccctatggtcagaatcaaca agattgggaagaagttcggcctgaagtgtgagctcttggc caagtgtgagttcttcaacgcgggcgggagcgtgaaggac cgcatcagcctgcggatgattgaggatgctgagcgcgacg ggacgctgaagcccggggacacgattatcgagccgacatc cgggaacaccgggatcgggctggccctggctgcggcagtg aggggctatcgctgcatcatcgtgatgccagagaagatga gctccgagaaggtggacgtgctgcgggcactgggggctga gattgtgaggacgcccaccaatgccaggttcgactccccg gagtcacacgtgggggtggcctggcggctgaagaacgaaa tccccaattctcacatcctagaccagtaccgcaacgccag caacccccctggctcactacgacaccaccgctgatgagatc ctgcagcagtgtgatgggaagctggacatgctggtggctt cagtgggcacgggcggcaccatcacgggcattgccaggaa gctgaaggagaagtgtcctggatgcaggatcattggggtg gatcccgaagggtccatcctcgcagagccggaggagctga accagacggagcagacaacctacgaggtggaagggatcgg ctacgacttcatccccacggtgctggacaggacggtggtg gacaagtggttcaagagcaacgatgaggaggcgttcacct

Molecule	SEQ ID NO	Sequence
		ttgcccgcatgctgatcgcgcaagaggggctgctgtgcgg tggcagtgctggcagcacggtggcggtggccgtgaaggct gcgcaggagctgcaggagggccagcgctgcgtggtcattc tgcccgactcagtgcggaactacatgaccaagttcctgag cgacaggtggatgctgcagaagggctttctgaaggaggag gacctcacggagaagaagccctggtggtggcacctccgtg ttcaggagctgggcctgtcagccccgctgaccgtgctccc gaccatcacctgtgggcacaccatcgagatcctccgggag aagggcttcgaccaggcgcccgtggtggatgaggcggggg taatcctgggaatggtgacgcttgggaacatgctctcgtc cctgcttgccgggaaggtgcagccgtcagaccaagttggc aaagtcatctacaagcagttcaaacagatccgcctcacgg acacgctgggcaggctctcgcacatcctggagatggacca cttcgccctggtggtgcacgagcagatccagtaccacagc accgggaagtccagtcagcggcagatggtgttcggggtgg tcaccgccattgacttgctgaacttcgtggccgcccagga gcgggaccagaagtga
Native human CβS polypeptide sequence	**2**	MPSETPQAEVGPTGCPHRSGPHSAKGSLEKGSPEDKEAKE PLWIRPDAPSRCTWQLGRPASESPHHHTAPAKSPKILPDI LKKIGDTPMVRINKIGKKFGLKCELLAKCEFFNAGGSVKD RISLRMIEDAERDGTLKPGDTIIEPTSGNTGIGLALAAAV RGYRCIIVMPEKMSSEKVDVLRALGAEIVRTPTNARFDSP ESHVGVAWRLKNEIPNSHILDQYRNASNPLAHYDTTADEI LQQCDGKLDMLVASVGTGGTITGIARKLKEKCPGCRIIGV DPEGSILAEPEELNQTEQTTYEVEGIGYDFIPTVLDRTVV DKWFKSNDEEAFTFARMLIAQEGLLCGGSAGSTVAVAVKA AQELQEGQRCVVILPDSVRNYMTKFLSDRWMLQKGFLKEE DLTEKKPWWWHLRVQELGLSAPLTVLPTITCGHTIEILRE KGFDQAPVVDEAGVILGMVTLGNMLSSLLAGKVQPSDQVG KVIYKQFKQIRLTDTLGRLSHILEMDHFALVVHEQIQYHS TGKSSQRQMVFGVVTAIDLLNFVAAQERDQK

Molecule	SEQ ID NO	Sequence
Truncated, Human CβS polypeptide sequence	**3**	MPSETPQAEVGPTGCPHRSGPHSAKGSLEKGSPED KEAKEPLWIRPDAPSRCTWQLGRPASESPHHHTAP AKSPKILPDILKKIGDTPMVRINKIGKKFGLKCEL LAKCEFFNAGGSVKDRISLRMIEDAERDGTLKPGD TIIEPTSGNTGIGLALAAAVRGYRCIIVMPEKMSS EKVDVLRALGAEIVRTPTNARFDSPESHVGVAWRL KNEIPNSHILDQYRNASNPLAHYDTTADEILQQCD GKLDMLVASVGTGGTITGIARKLKEKCPGCRIIGV DPEGSILAEPEELNQTEQTTYEVEGIGYDFIPTVL DRTVVDKWFKSNDEEAFTFARMLIAQEGLLCGGSA GSTVAVAVKAAQELQEGQRCVVILPDSVRNYMTKF LSDRWMLQKGFLKEEDLTEKKPWWWHLR
Truncated, Optimized Human CβS nucleic acid sequence	**4**	ATGCCGTCAGAAACCCCGCAGGCAGAAGTGGGTCCGACGG GTTGCCCGCACCGTAGCGGTCCGCATTCTGCAAAAGGCAG TCTGGAAAAAGGTTCCCCGGAAGATAAAGAAGCCAAAGAA CCGCTGTGGATTCGTCCGGACGCACCGTCACGCTGTACCT GGCAGCTGGGTCGTCCGGCAAGCGAATCTCCGCATCACCA TACGGCTCCGGCGAAAAGTCCGAAAATTCTGCCGGATATC

Molecule	SEQ ID NO	Sequence
		CTGAAGAAAATTGGTGACACCCCGATGGTTCGTATCAACA AAATCGGCAAAAAATTCGGTCTGAAATGCGAACTGCTGGC TAAATGTGAATTTTTCAATGCGGGCGGTTCCGTGAAAGAT CGTATCTCACTGCGCATGATTGAAGATGCTGAACGCGACG GCACCCTGAAACCGGGTGATACGATTATCGAACCGACCTC TGGCAACACGGGTATCGGTCTGGCACTGGCGGCGGCAGTC CGTGGTTATCGCTGCATTATCGTGATGCCGGAAAAAATGA GCTCTGAAAAAGTTGATGTCCTGCGTGCTCTGGGCGCGGA AATTGTTCGTACCCCGACGAATGCCCGCTTCGACAGTCCG GAATCCCATGTGGGTGTTGCATGGCGCCTGAAAAACGAAA TCCCGAATTCGCACATTCTGGATCAGTATCGTAACGCTAG CAATCCGCTGGCGCATTACGATACCACGGCCGACGAAATC CTGCAGCAATGTGATGGCAAACTGGACATGCTGGTCGCTT CTGTGGGTACCGGCGGTACCATTACGGGCATCGCGCGTAA ACTGAAAGAAAAATGCCCGGGCTGTCGCATTATCGGTGTG GATCCGGAAGGCAGTATTCTGGCGGAACCGGAAGAACTGA ACCAGACCGAACAAACCACGTATGAAGTTGAAGGCATCGG TTACGATTTTATTCCGACCGTCCTGGATCGCACGGTGGTT GACAAATGGTTCAAAAGCAATGACGAAGAAGCCTTTACCT TCGCACGTATGCTGATCGCTCAGGAAGGTCTGCTGTGCGG TGGTTCAGCAGGTTCGACGGTCGCAGTGGCAGTTAAAGCT GCGCAGGAACTGCAAGAAGGTCAACGTTGTGTCGTGATTC TGCCGGATTCTGTTCGCAACTACATGACCAAATTTCTGAG TGACCGTTGGATGCTGCAAAAAGGCTTCCTGAAAGAAGAA GATCTGACCGAGAAAAAACCGTGGTGGTGGCACCTGCGCT AA

Claims:

1. A method for purifying cystathionine β-Synthase (CBS) protein, wherein the CBS protein is a naturally occurring, chemically cleaved or genetically engineered truncated CBS protein with an ending position of one of amino acid residues from 382-532, 382-550 or 543-550 compared to SEQ ID NO: 2 and without a His-tag, comprising the steps of:

(a) providing a CBS-containing solution comprising one or a plurality of impurities;

(b) first performing chromatographic separation of the CBS-containing solution using an ion exchange chromatography column; and

(c) second performing chromatographic separation using a metal affinity chromatography (IMAC) resin, wherein the impurities are removed thereby.

2. The method of claim 1, further comprising a step of performing chromatographic separation using a Hydrophobic Interaction Chromatography (HIC) column.

3. The method of claims 1 or 2, further comprising a step of performing chromatographic separation using a ceramic hydroxyapatite resin.

4. The method according to any one of claims 1-3, wherein the ion exchange chromatography column is a weak anion exchanger.

5. The method of claim 4, wherein the weak anion exchanger is a DEAE-Sepharose® FF column.

6. The method according to any one of claims 1-5, wherein the metal affinity chromatography (IMAC) resin is charged with a divalent metal cation.

7. The method of claim 6, wherein the divalent metal cation is nickel, copper, cobalt or zinc.

8. The method of claim 7, wherein the divalent metal ion is zinc.

9. The method according to any one of claims 1-8, further comprising eluting CBS from the metal affinity chromatography (IMAC) resin with an elution buffer comprising imidazole.

10. The method according to any one of claims 1-9, wherein the truncated CBS protein has an amino acid sequence identified by SEQ ID NO: 3.

11. The method according to any one of claims 1-10, wherein the CBS-containing solution is a clarified CBS solution.

12. The method according to any one of claims 1-11, wherein the CBS is produced in a recombinant cell.

13. The method of claim 12, wherein the recombinant cell is a bacterial cell.

14. The method of claim 13, wherein the CBS-containing solution is obtained by homogenizing recombinant bacterial cells expressing a recombinant construct comprising a nucleic acid sequence encoding CBS.

15. The method of claim 14, wherein the nucleic acid encodes a truncated CBS protein.

16. The method of claim 15, wherein the nucleic acid sequence comprises SEQ ID NO. 4.

17. The method according to any one of claims 12-16, wherein the recombinant cells are *E. coli* cells.

18. The method of claim 15, wherein the nucleic acid sequence encoding the truncated CBS protein is optimized for expression in *E. coli* cells.

19. A method for producing an enriched CBS solution, wherein the CBS protein is a naturally occurring, chemically cleaved or genetically engineered truncate with an ending position of one of amino acid residues from 382-532 or 543-550 of SEQ ID NO: 2 and without a His-tag, the method comprising:

(a) providing a CBS-containing solution comprising one or a plurality of impurities; and

(b) performing chromatographic separation of the CBS-containing solution using an immobilized metal affinity chromatography (IMAC) resin charged with a divalent metal ion, wherein the impurities are removed thereby.

20. The method of claim 19, wherein the divalent metal ion is nickel, copper, cobalt or zinc.

21. The method of claim 20, wherein the divalent metal ion is zinc.

22. The method according to any one of claims 19-21, wherein the truncated CBS protein has an amino acid sequence identified by SEQ ID NO: 3.

23. The method according to any one of claims 19-22, wherein the CBS solution is a clarified CBS solution.

24. The method according to any one of claims 19-23, wherein the CBS is produced in a recombinant cell.

25. The method of claim 24, wherein the recombinant cell is a bacterial cell.

26. The method of claim 25, wherein the CBS solution is obtained by homogenizing recombinant bacterial cells expressing a recombinant construct comprising a nucleic acid sequence encoding CBS.

27. The method of claim 26, wherein the nucleic acid encodes a truncated CBS protein.

28. The method of claim 27, wherein the CBS nucleic acid sequence comprises SEQ ID NO. 4.

29. The method according to any one of claims 25-28, wherein the bacterial cells are *E. coli* cells.

30. The method according to any one of claims 26-29, wherein the nucleic acid sequence encoding the truncated CBS protein is optimized for expression in *E. coli* cells.

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Figure 1A

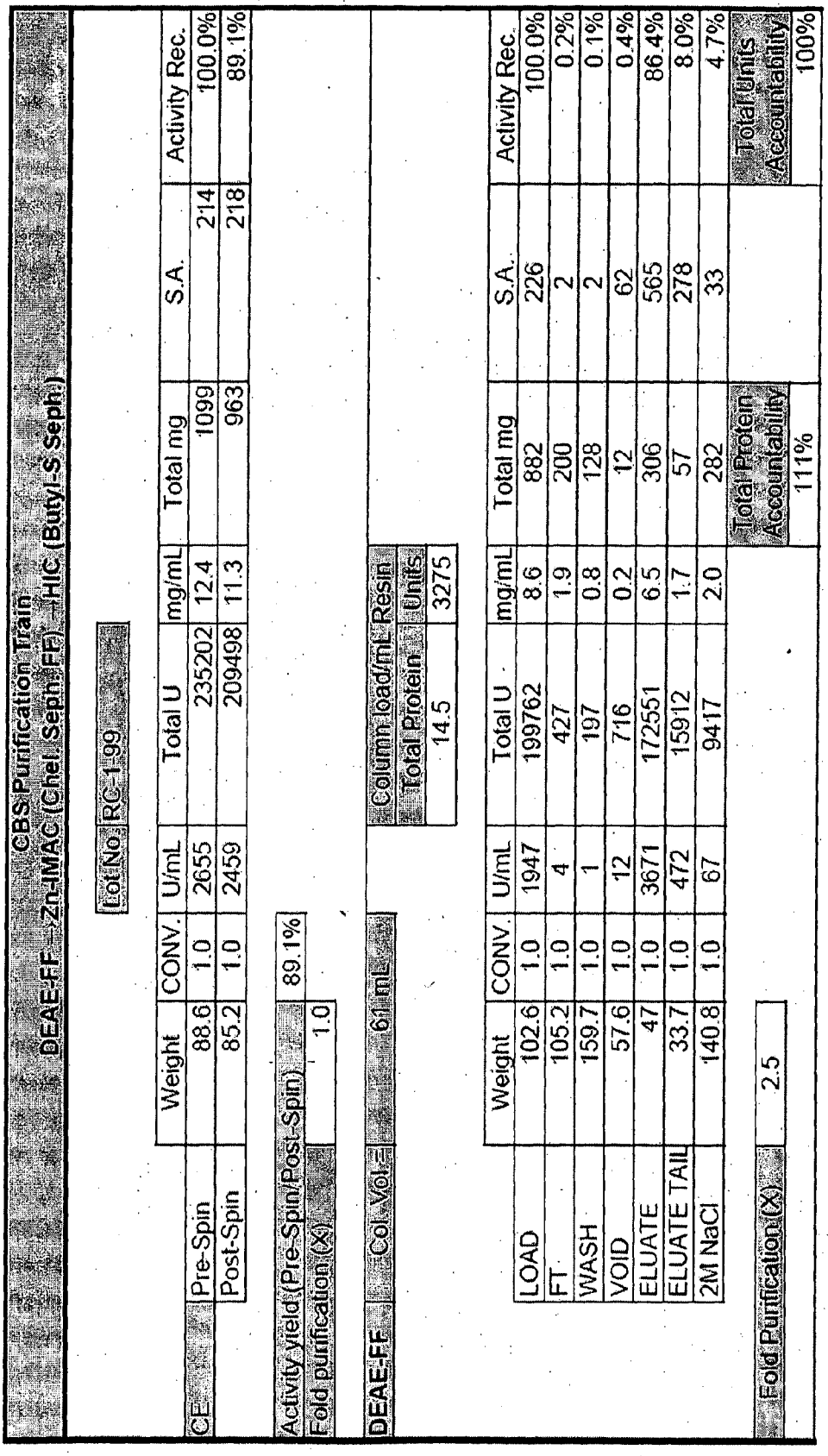
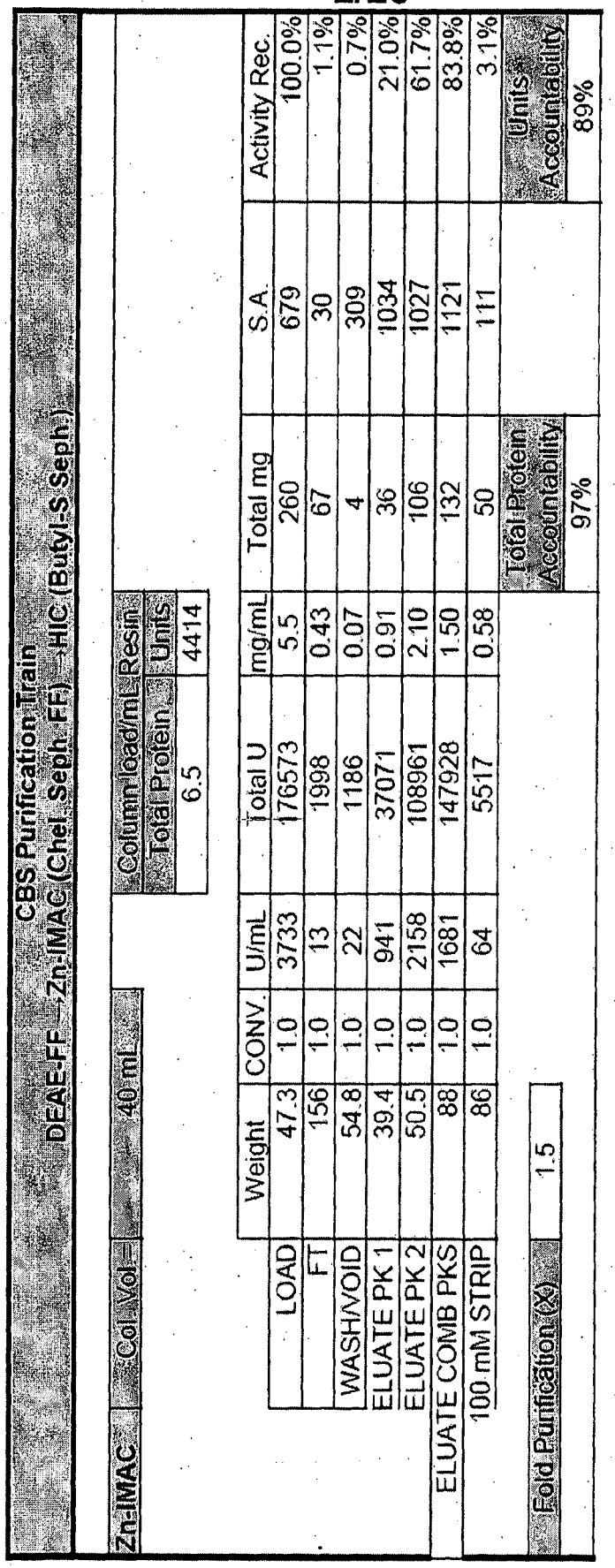


Figure 1B



CBS Purification Train

DEAE-FF → Zn-IMAC (Chel. Seph. FF) → HIC (Butyl-S Seph.)

Zn-IMAC	Col. Vol =	40 mL	Column load/mL Resin	
			Total Protein	Units
			6.5	4414

	Weight	CONV.	U/mL	Total U	mg/mL	Total mg	S.A.	Activity Rec.
LOAD	47.3	1.0	3733	176573	5.5	260	679	100.0%
FT	156	1.0	13	1998	0.43	67	30	1.1%
WASH/VOID	54.8	1.0	22	1186	0.07	4	309	0.7%
ELUATE PK 1	39.4	1.0	941	37071	0.91	36	1034	21.0%
ELUATE PK 2	50.5	1.0	2158	108961	2.10	106	1027	61.7%
ELUATE COMB PKS	88	1.0	1681	147928	1.50	132	1121	83.8%
100 mM STRIP	86	1.0	64	5517	0.58	50	111	3.1%
					Total Protein Accountability	97%	Units* Accountability	89%

Fold Purification (X)	1.5

Figure 1C

CBS Purification Train

DEAE-FF →Zn-IMAC (Chel. Seph. FF) →HIC (Butyl-S Seph.)

HIC	Col. Vol.=	23 mL

Column load/mL Resin	
Total Protein	Units
6.5	6745

	Weight	CONV.	U/mL	Total U	mg/mL	Total mg	S.A.	Activity Rec.
LOAD	103.9	1.1	1357	155143	1.58	149	1040	100.0%
FT/WASH	85	1.1	26	2470	0.080	6	400	1.6%
VOID	30.5	1.1	34	1133	0.060	2	681	0.7%
ELUATE	41.1	1.1	2671	120762	2.50	93	1293	77.8%
STRIP	43.8	1.0	282	13591	0.87	38	357	8.8%
						Total Protein Accountability		Units Accountability
						93%		89%

Fold Purification (X)	1.2

Recovery Total Protein From First Column Load
10.6%

Recovery Total Units From First Column Load
60%

Total Fold Purification (X)
5.7

Figure 2

RC-1-64 DEAE FF (DEAE-IMAC-HIC Process Train)

Date	04/21/11
Column ID	RC-3-10

Exp No. RC-1-64

Col Vol =	30
Total protein/mL resin loaded	26 mg/mL

Fraction ID	Weight or Volume (g or mL)	CONV.	Protein (mg/mL)	Total Protein in fraction (mg)	Cystathionine from assay (nmol.mL.hr)	Dilution factor from assay	Total Units in fraction	S.A. Fraction	% Rec. Units (%)	% Rec. Total Protein (%)
CE Pre-Spin	37.6	1.0	20.9	784.3	46423	40	69820	89.0	100.0%	100.0%
Ce Post-Spin	36.3	1.0	20.7	752.9	52427	40	76124	101.1	109.0%	96.0%
DEAE Load	49.8	1.0	15.4	766.8	36740	40	73186	95.4	96.1%	101.8%
DEAE FT	49.7	1.0	3.0	151.0	3403	1	169	1.1	0.2%	20.1%
DEAE Void	31	1.0	0.3	8.8	10298	1	319	36.4	0.4%	1.2%
DEAE Eluate	24.9	1.0	5.2	130.0	63422	40	63168	485.8	83.0%	17.3%
DEAE Eluate Tail	18.9	1.0	1.9	35.16	69389	5	6557	186.5	8.6%	4.7%
DEAE Wash	89.3	1.0	1.6	143.16	5519	1	493	3.4	0.6%	19.0%
DEAE 2M NaCl	76	1.0	3.2	241.44	13935	5	5295	21.9	7.0%	32.1%

Mass Balance	
Units	99.8%

Mass Balance Units (from load)	Mass Balance Protein
103.8%	94.2%

Recovery units (from load)
86.3%

Buffers:

Equilibration	0.02 M Na3PO4, 0.05 M NaCl, 10% Ethylene Glycol, pH 7.0
Wash	0.02 M Na3PO4, 0.05 M NaCl, 10% Ethylene Glycol, pH 7.0
Elution	0.02 M Na3PO4, 0.137 M NaCl, 10% Ethylene Glycol, pH 7.0
Strip	2 M NaCl
Sanitze	1.0 M NaOH

Figure 3A

CBS Purification Train
DEAE-FF →Zn-IMAC (Chel. Seph. FF) →HAP (Ceramic Type 1)→HIC (Butyl-S Seph.)

Lot No. RC-1-103

CE		Weight	CONV.	U/mL	Total U	mg/mL	Total mg	S.A.	Activity Rec.
	Pre-Spin	87.1	1.0	3369	293422	13.9	1211	242	100.0%
	Post-Spin	100.4	1.0	3050	306270	11.7	1175	261	104.4%

Activity yield (Pre-Spin/Post-Spin) **104.4%**
Fold purification (X) 1.1

DEAE-FF Col. Vol.= 60 mL

	Column load/mL Resin	
	Total Protein	Units
	19.6	5105

	Weight	CONV.	U/mL	Total U	mg/mL	Total mg	S.A.	Activity Rec.
LOAD*	100.4	1.0	3050	306270	11.7	1175	261	100.0%
FT	102.6	1.0	6	585	2.3	236	2	0.2%
WASH	164.6	1.0	2	410	1.0	165	2	0.1%
VOID	56.3	1.0	11	644	0.3	17	38	0.2%
ELUATE	52.6	1.0	4976	261744	7.2	379	691	85.5%
ELUATE TAIL	30.6	1.0	441	13488	1.3	40	339	4.4%
2M NaCl	115.4	1.0	75	8635	3.0	346	25	2.8%
						Total Protein Accountability		Total Units Accountability
						101%		93%

Fold Purification (X) 2.7

**Load same as Post-Spin;*
Dilution occurred during balancing of tubes for centrifugation

Figure 3B

CBS Purification Train
DEAE-FF →Zn-IMAC (Chel. Seph. FF) →HAP (Ceramic Type 1)→HIC (Butyl-S Seph.)

Zn-IMAC | **Col. Vol.=** 40 mL

Column load/mL Resin	
Total Protein	Units
9.3	6337

	Weight	CONV.	U/mL	Total U	mg/mL	Total mg	S.A.	Activity Rec.
LOAD	52.9	1.0	4792	253492	7.0	370	685	100.0%
FT/WASH	144.9	1.0	23	3388	0.65	94	36	1.3%
VOID	52.3	1.0	64	3355	0.16	8	401	1.3%
ELUATE COMB PKS	82.6	1.0	2457	202919	2.67	221	920	**80.0%**
100 mM STRIP*	89	1.0	19	1689	0.15	13	127	0.7%
						Total Protein Accountability		**Units Accountability**
Fold Purification (X)	**1.3**					**91%**		**83%**

Precipitation observed in strip sample.

HAP | **Col. Vol.=** 18 mL

Column load/mL Resin	
Total Protein	Units
12.2	11205

	Weight	CONV.	U/mL	Total U	mg/mL	Total mg	S.A.	Activity Rec.
LOAD	82.1	1.0	2457	201691	2.67	219	920	100.0%
FT/WASH	132.2	1.0	26	3383	0.08	11	320	1.7%
ELUATE	37.5	1.0	4552	170694	4.34	163	1049	**84.6%**
STRIP	34.5	1.0	348	12013	0.55	19	633	6.0%
						Total Protein Accountability		**Units Accountability**
Fold Purification (X)	**1.1**					**88%**		**92%**

Figure 3C

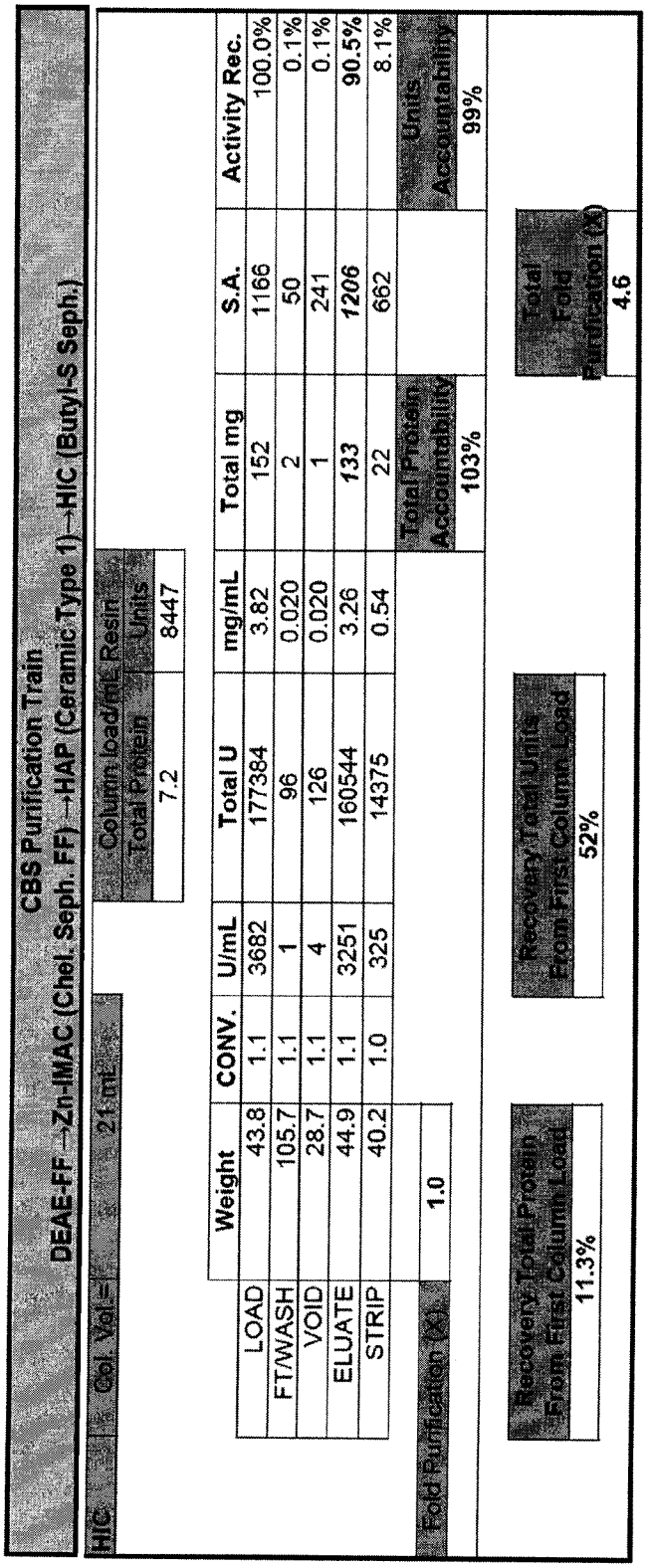
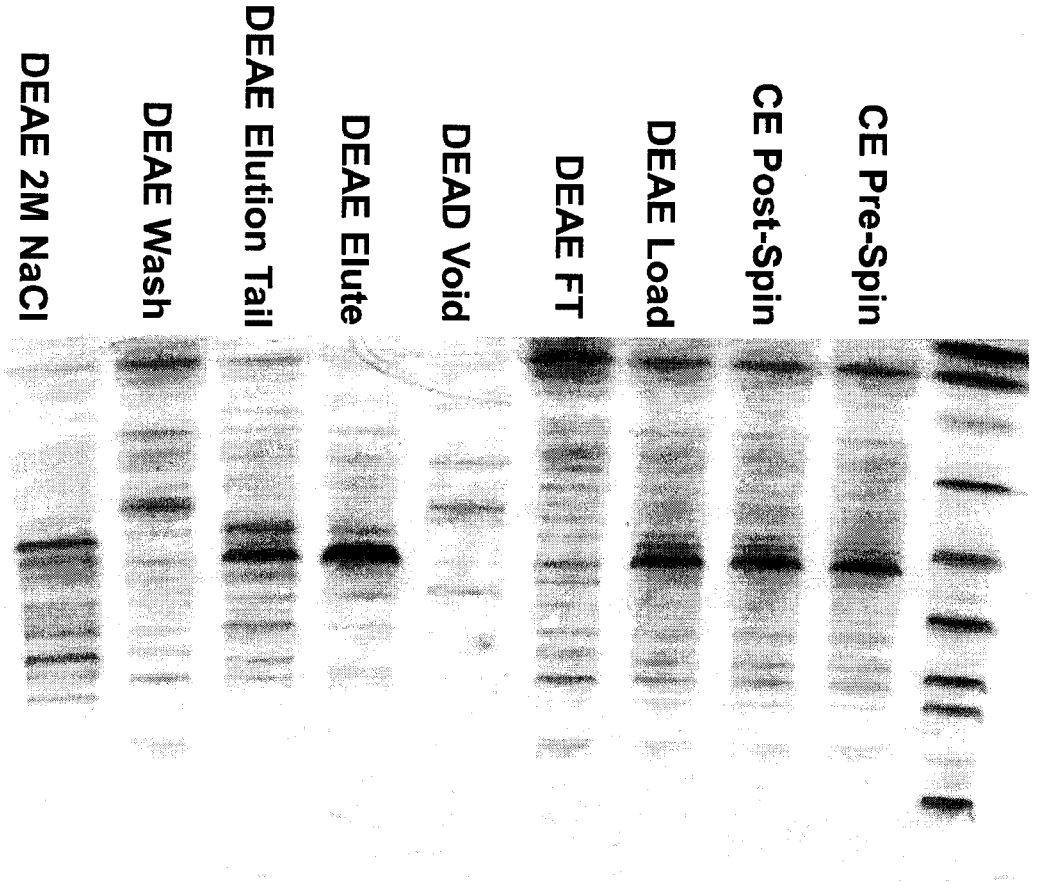

CBS Purification Train
DEAE-FF →Zn-IMAC (Chel. Seph. FF) →HAP (Ceramic Type 1)→HIC (Butyl-S Seph.)
HIC
Col. Vol.=
21 mL
Column load/mL Resin
Total Protein
7.2
Units
8447
Weight
CONV.
U/mL
Total U
mg/mL
Total mg
S.A.
Activity Rec.
LOAD
43.8
1.1
3682
177384
3.82
152
1166
100.0%
FT/WASH
105.7
1.1
1
96
0.020
2
50
0.1%
VOID
28.7
1.1
4
126
0.020
1
241
0.1%
ELUATE
44.9
1.1
3251
160544
3.26
133
1206
90.5%
STRIP
40.2
1.0
325
14375
0.54
22
662
8.1%
Total Protein Accountability
103%
Units Accountability
99%
Fold Purification (A)
1.0
Recovery Total Protein From First Column Load
11.3%
Recovery Total Units From First Column Load
52%
Total Fold Purification (A)
4.6

Figure 4



RC-1-90

Figure 5

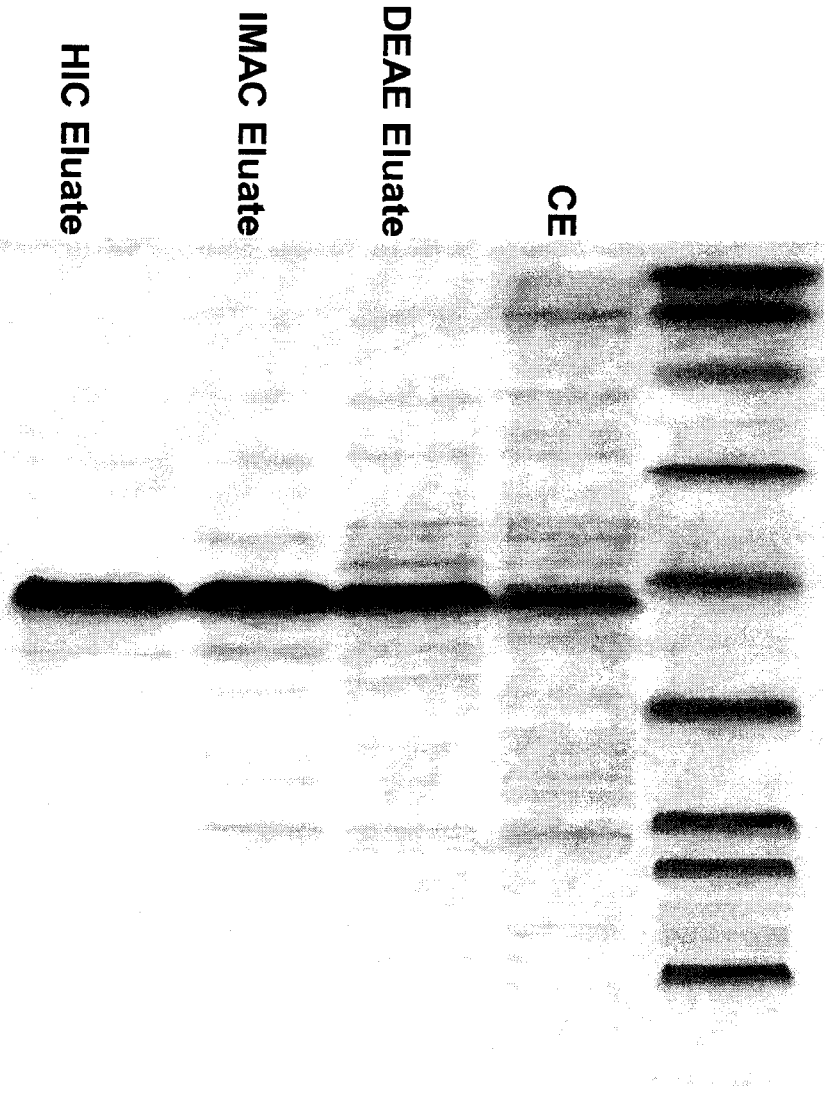


Figure 6

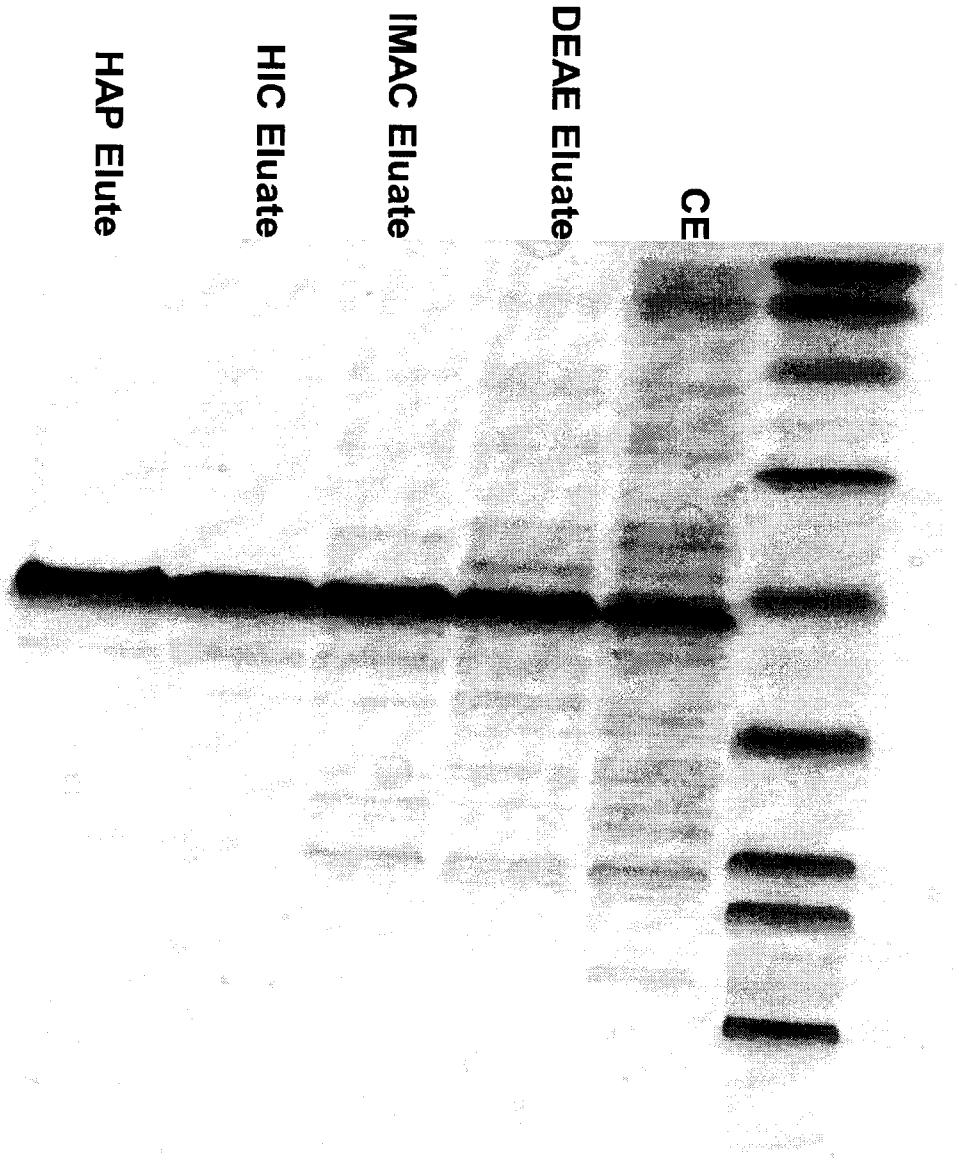


Figure 7A

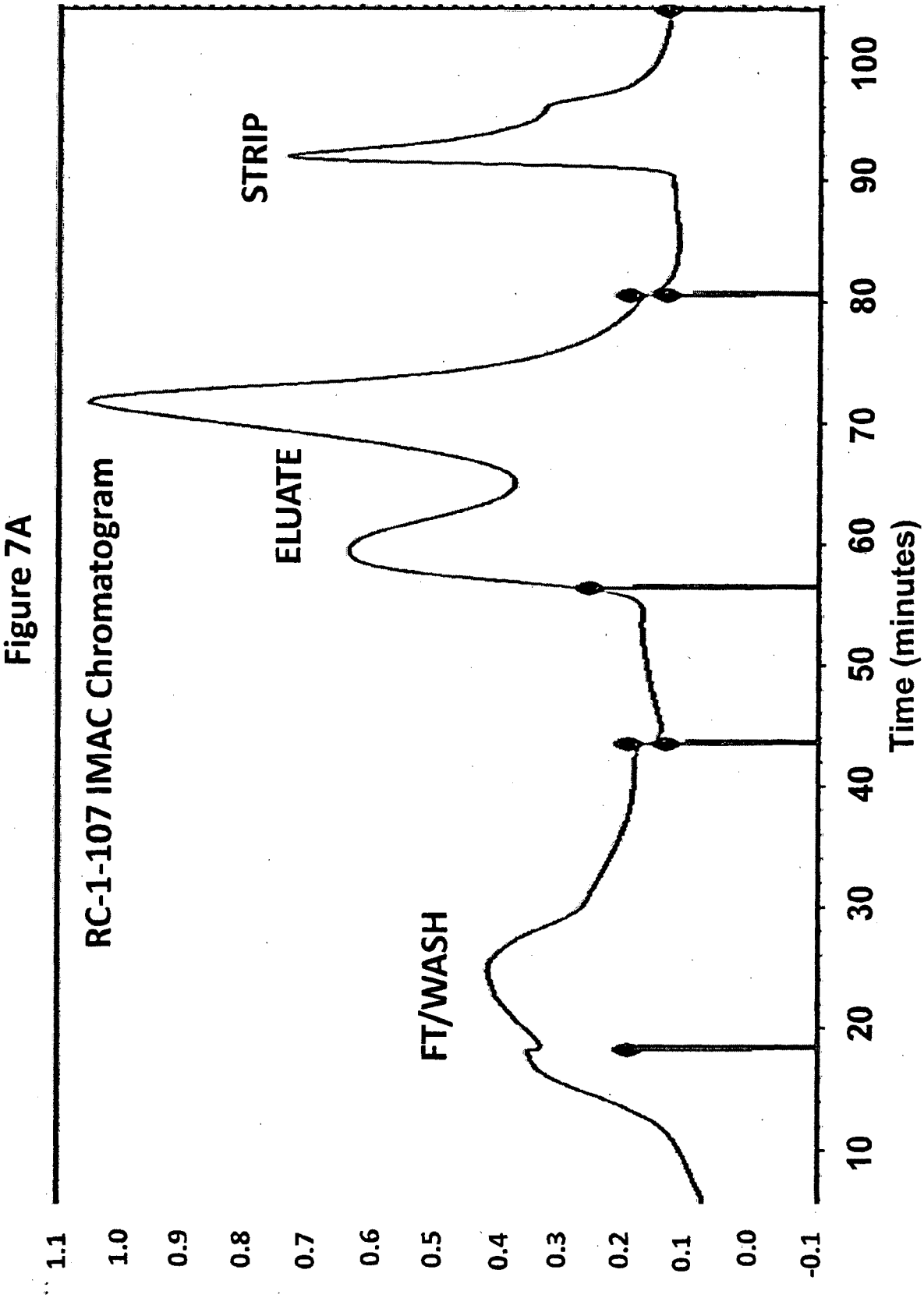

RC-1-107 IMAC Chromatogram
STRIP
ELUATE
FT/WASH
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
10
20
30
40
50
60
70
80
90
100
Time (minutes)

Figure 7B

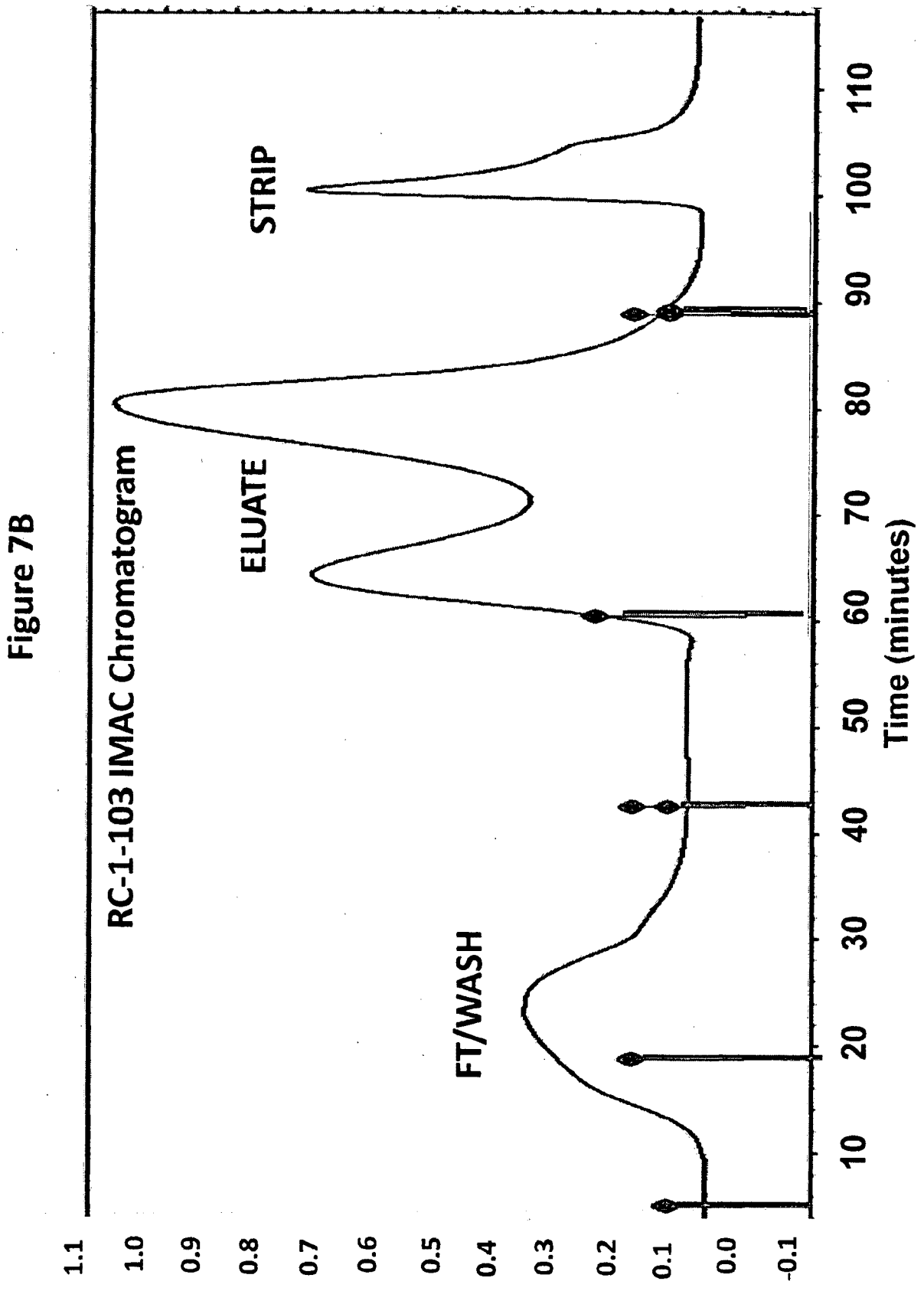

RC-1-103 IMAC Chromatogram
STRIP
ELUATE
FT/WASH
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
10
20
30
40
50
60
70
80
90
100
110
Time (minutes)

Figure 8

Date	04/06/11
Column ID	RC-3-

Exp. No.	RC-1-57 IMAC

Col Vol=	9	
Total protein/mL resin loaded	29	mg/mL

Fraction ID	Weight or Volume (g or mL)	CONV.	Protein (mg/mL)	Total Protein in fraction (mg)	Cystathionine from assay (nmol.mL.hr)	Dilution factor from assay	Total Units in fraction	S.A. Fraction	% Rec. Units (%)	% Rec. Total Protein (%)
CE	16	1.0	15.3	244.1	76039	20	24332	100	100.0%	100.0%
IMAC Load	34.7	1.0	7.6	264.7	40808	20	28321	107	116.4%	108.5%
IMAC FT	34.3	1.0	2.3	77.9	50	20	34	0	0.1%	31.9%
IMAC Equil Wash	32.6	1.0	2.8	91.6	4111	1	134	1	0.5%	37.5%
IMAC 5mM Imid.	34.3	1.0	0.6	19.2	9211	1	316	16	1.1%	7.9%
IMAC Void	6.9	1.0	0.4	3.0	3813	1	264	89	0.9%	1.2%
IMAC Eluate Front	14	1.0	0.7	9.8	77625	1	1087	110	3.8%	4.0%
IMAC Eluate	16.6	1.0	4.6	77.2	75941	20	25212	327	89.0%	31.6%
IMAC Eluate Tail	6.7	1.0	1.4	9.3	40388	5	1353	145	4.8%	3.8%
IMAC 100mM Imid.	38	1.0	0.4	16.7	29197	1	1109	66	3.9%	6.8%

Mass Balance Units (from load)	Mass Balance Protein
104.2%	115.1%

Buffers:

Charge	50 mM NiSO4
Equilibration	0.02 M Na3PO4, 0.4 M NaCl, 0.002 M Imidazole, 0.01%Triton, pH 7.0
Wash	0.02 M Na3PO4, 0.4 M NaCl, 0.005 M Imidazole, 0.01%Triton, pH 7.0
Elution	0.02 M Na3PO4, 0.4 M NaCl, 0.050 M Imidazole, 0.01%Triton, pH 7.0
Strip	100 mM Imidazole
Sanitize	1 M NaOH
Storage	0.001 M NaOH

Figure 9

Bradford Assay (Total Protein) **RC-1-57 IMAC**

Date: 4/7/2011

Standard Curve: BSA Stock: 2000 ug/mL

Well#	ug/mL BSA	BSA (uL)	Water (uL)
1	0	0	40
2	50	1	39
3	125	2.5	37.5
4	250	5	35
5	500	10	30
6	750	15	25
7	1000	20	20

Samples:

Number of samples Don't include triplicates): 10

Amount of Bradford Reagent Needed (mL): 19

For 10uL per standard, run in triplicate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17
B	Std 2	Std 2	Std 2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
C	Std 3	Std 3	Std 3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
D	Std 4	Std 4	Std 4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
E	Std 5	Std 5	Std 5	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
F	Std 6	Std 6	Std 6	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
G	Std 7	Std 7	Std 7	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
H				Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24

1 Remove appropriate amount of reagent and let it come to RT
2 Add 10uL Standard or sample to each well
3 Add 300uL Reagent to each well using multichannel pipettor
4 Incubate at RT 10min
5 Read on VersaMax

Sample #		Dilution	Protein (uL)	Water (uL)	Bradford (ug/mL)	ug/uL
1	CE	100	2	198	**15255.9**	15.2559
2	IMAC Load	50	4	196	**7629.2**	7.6292
3	IMAC FT	50	4	196	**2270.7**	2.2707
4	IMAC Equil Wash	25	8	192	**2810.3**	2.8103
5	AC 5mM Imidizole Wash	10	20	180	**559.3**	0.5593
6	IMAC Void	10	20	180	**432.3**	0.4323
7	IMAC Eluate Front	10	20	180	**702.7**	0.7027
8	IMAC Eluate	50	4	196	**4649.2**	4.6492
9	IMAC Eluate Tail	25	8	192	**1389**	1.389
10	IMAC 100mM Imidizole	10	20	180	**439.3**	0.4393

Figure 10A

Gel 1

Lane	Sample	TP Conc. (ug/ul)	Conc. for gel (ug)	Dilution (X)	Sample buffer (ul)	Protein (for 10ug/25uL)	ddH2O (ul)	
1	BIORAD Standard	--	--	1	12.5	1.00	11.50	Only 7ug loaded
2	CE	15.256	10	1		0.66	11.84	
3	IMAC Load	7.6292	10	1		1.31	11.19	
4	IMAC FT	2.2707	10	1		4.40	8.10	
5	IMAC Equil Wash	2.8103	10	1		3.56	8.94	
6	IMAC 5mM Imidizole Wash	0.5593	10	1		12.50	0.00	

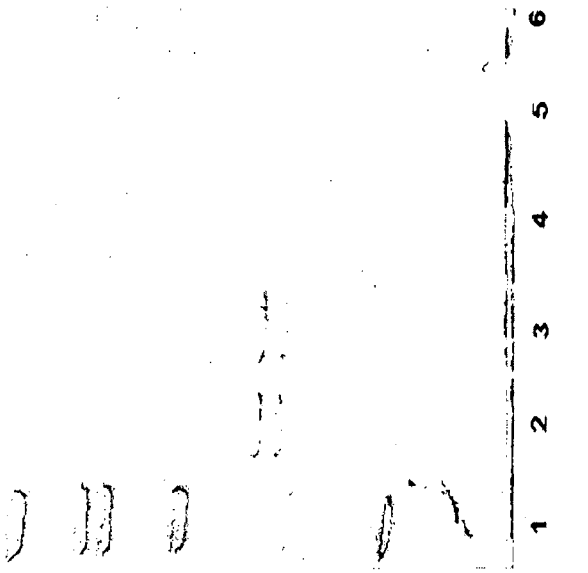


Figure 10B

Gel 1:

Lane	Sample	TP Conc. (ug/ul)	Conc for gel (ug)	Dilution (X)	Sample buffer (ul)	Protein (for 10ug/25uL)	ddH2O (ul)	
1	BIORAD Standard	--	--	1	12.5	1.00	11.5	
2	IMAC Void	0.4323	10	1		12.50	0.00	Only 5.4ug loaded
3	IMAC Eluate Front	0.7027	10	1		12.50	0.00	Only 8.78ug loaded
4	IMAC Eluate	4.6492	10	1		2.15	10.35	
5	IMAC Eluate Tail	1.389	10	1		7.20	5.3	
6	IMAC 100mM Imidizole	0.4393	10	1		12.50	0.00	Only 5.49ug loaded

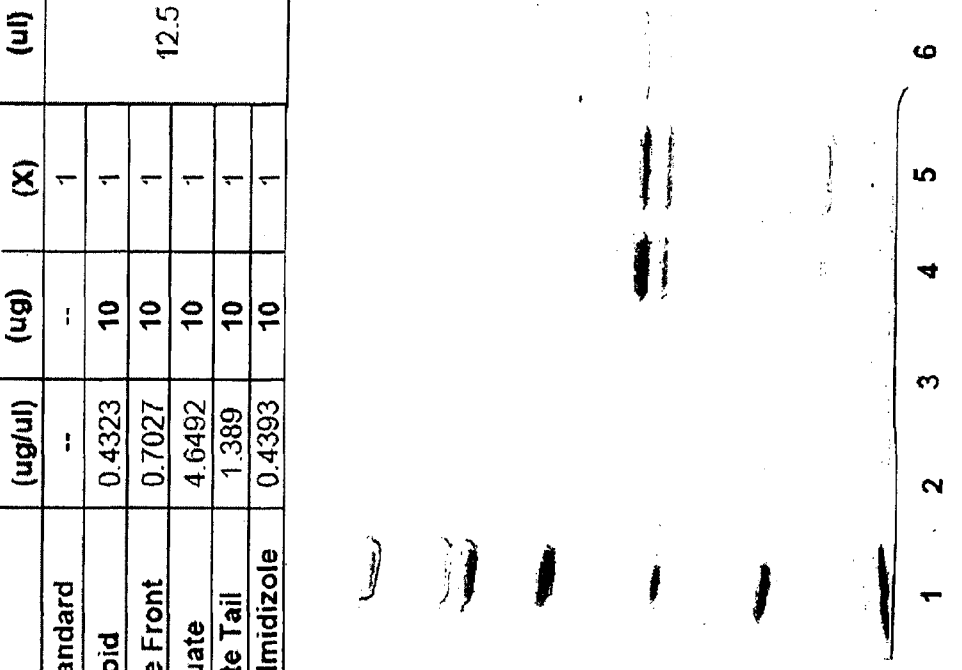


Figure 11

CBS Crude Extract on Chelating Sepharose FF

Date	02/18/11	Exp. No.	RC-1-39
Column ID	RC-3-2 Chelating Seph. FF		

Col Vol.= 6.2 mL | Note: column charged to 6.2 mL, 6.5cm

Total protein/mL resin loaded 14 mg/mL

Fraction ID	Weight or Volume (g or mL)	CONV.	Protein (mg/mL)	Total Protein in fraction (mg)	Cystathionine from assay (nmol.mL.hr)	Dilution factor from assay	Total Units in fraction	S.A. Fraction	% Rec. Units (%)	% Rec. Total Protein (%)
CE	11	1.0	8.0	88.0	74067	10	8147	92.6	100.0%	100.0%
Load	11.4	1.0	8.1	92.3	82223	10	9373	101.5	115.0%	104.9%
Void	16	1.0	0.3	4.8	7	1	0	0.0	0.0%	5.5%
FT/Wash	62	1.0	1.2	74.4	5939	1	368	4.9	4.5%	84.5%
Eluate	25	1.0	2.1	52.5	56851	5	7106	135.4	87.2%	59.7%
500 mM Imid. Strip	36	1.0	0.2	7.2	1152	1	41	5.8	0.5%	8.2%

Recovery in Eluate (from load) 75.8%

Mass Balance Units (from CE) 92.3%	Mass Balance Protein 152.4%

Mass Balance Units (from load) 80.2%

Buffers:

Equilibration	0.02 Phosphate, 0.002 M imidazole, 0.4 M NaCl, pH 7.0
Wash	0.02 Phosphate, 0.002 M imidazole, 0.4 M NaCl, pH 7.0
Elution	0.02 Phosphate, 0.1 M imidazole, 0.4 M NaCl, pH 7.0
Strip	0.5 M Imidazole
Sanitize	1.0 M NaOH
Storage	0.001 M NaOH

Figure 12

Bradford Assay (Total Protein)						RC-1-63												
Date: 4/18/2011																		
Standard Curve:		BSA Stock:	2000	ug/mL		Samples:												
	Well#	ug/mL BSA	BSA (uL)	Water (uL)		Number of samples (Don't include triplicates):	6											
	1	0	0	40														
	2	50	1	39		Amount of Bradford Reagent Needed (mL):	15.4											
	3	125	2.5	37.5														
	4	250	5	35														
	5	500	10	30														
	6	750	15	25														
	7	1000	20	20														
For 10uL per standard, run in triplicate																		
	1	2	3	4	5	6	7	8	9	10	11	12						
A	Std 1	Std 1	Std 1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17						
B	Std 2	Std 2	Std 2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18						
C	Std 3	Std 3	Std 3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19						
D	Std 4	Std 4	Std 4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20						
E	Std 5	Std 5	Std 5	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21						
F	Std 6	Std 6	Std 6	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22						
G	Std 7	Std 7	Std 7	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23						
H				Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24						
1	Remove appropriate amount of reagent and let it come to RT																	
2	Add 10uL Standard or sample to each well																	
3	Add 300uL Reagent to each well using multichannel pipettor																	
4	Incubate at RT 10min																	
5	Read on VersaMax																	

Sample #		Dilution	Protein (uL)	Water (uL)	Bradford (ug/mL)	ug/uL
1	Post Spin CE Load	100	2	198	32187.209	32.187209
2	Equil Wash	20	10	190	11830.182	11.830182
3	FT	10	20	180	3310.385	3.310385
4	5mM Wash	10	20	180	252.568	0.252568
5	8mM Wash	10	20	180	670.278	0.670278
6	20mM Wash	10	20	180	524.895	0.524895

Figure 13

Lane	Sample	TP Conc. (ug/ul)	Conc for gel (ug)	Dilution (X)	Sample buffer (ul)	Protein (for 10ug/25uL)	ddH2O (ul)	
1	BIORAD Standard	--	--	1	12.5	1.00	11.50	
2	Equil Wash	11.83	10	1		0.85	11.65	3.16 ug protein loaded
3	5mM Wash	0.2526	10	1		12.50	0.00	8.38 ug protein loaded
4	8mM Wash	0.6703	10	1		12.50	0.00	6.56 ug protein loaded
5	20mM Wash	0.5249	10	1		12.50	0.00	

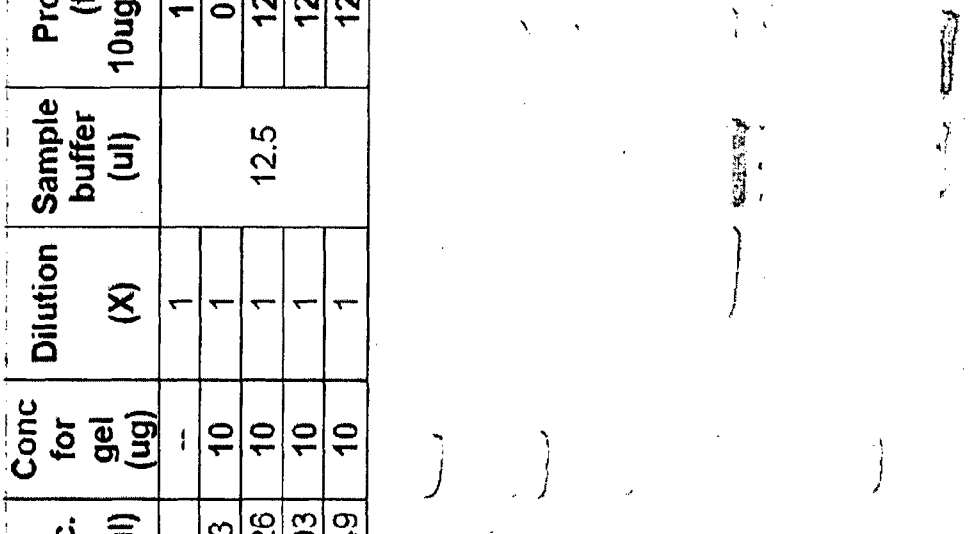
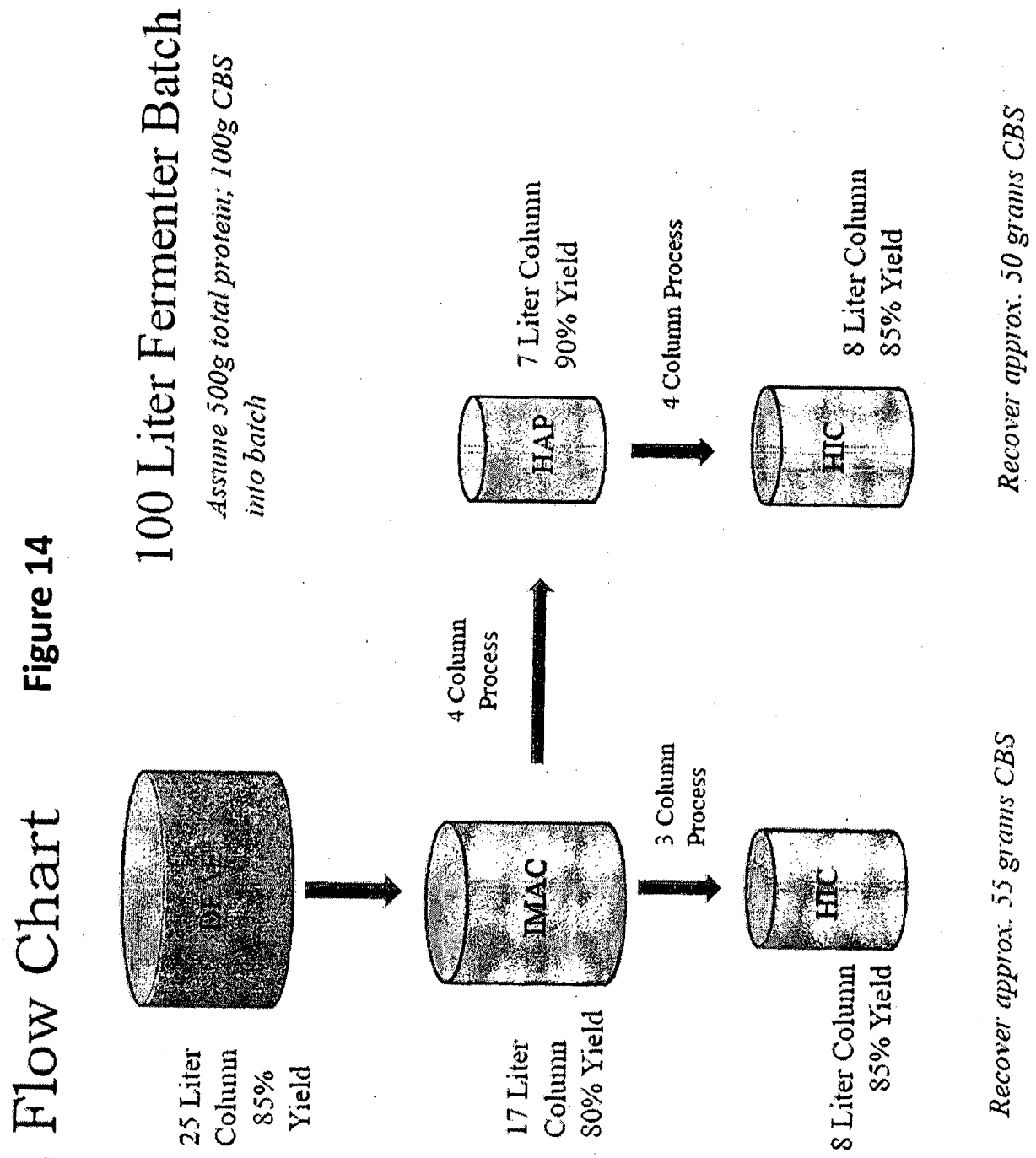


Figure 14


Process Flow Chart
100 Liter Fermenter Batch
Assume 500g total protein; 100g CBS into batch
25 Liter Column 85% Yield
17 Liter Column 80% Yield
8 Liter Column 85% Yield
4 Column Process
3 Column Process
7 Liter Column 90% Yield
8 Liter Column 85% Yield
4 Column Process
HAP
HIC
HIC
Recover approx. 55 grams CBS
Recover approx. 50 grams CBS