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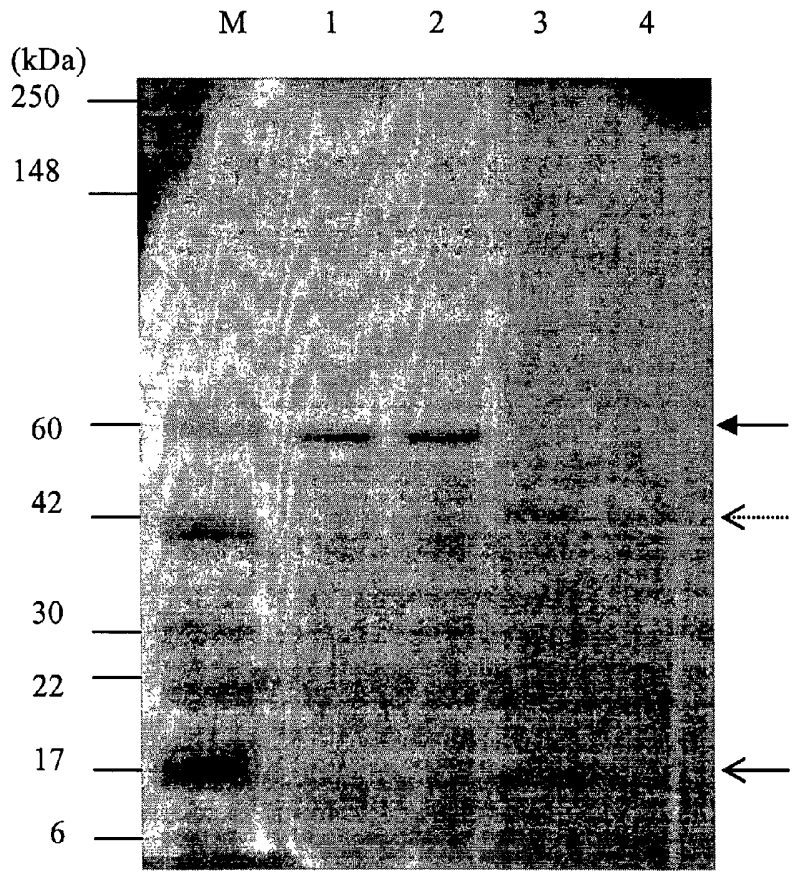
(54) Title: CLOSTRIPAIN ACTIVATION PROCESS



(57) Abstract: A process for activating clostripain comprising: solubilizing in a urea solution clostripain inclusion bodies which have been harvest from a fermentation mixture comprising recombinant host cells which have expressed clostripain, and thereafter diluting said urea solution into a calcium-containing activation buffer to a final urea concentration of less than about 3 M. Activation by dilution to moderately high urea concentrations leads a surprising increase in clostripain activation and product solution purity.

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CLOSTRIPAIN ACTIVATION PROCESS

FIELD OF THE INVENTION

The invention relates to an improved method for activating recombinantly produced clostripain. The invention provides clostripain of a high purity, high specific activity, and high concentration.

BACKGROUND OF THE INVENTION

Clostripain is a two-chain proteinase that can be isolated from *Clostridium histolyticum*. It is highly specific for the carboxyl peptide bond of arginine. To maintain the catalytic activity of clostripain, its sulfhydryl groups must be reduced, which can be achieved by reducing reagents such as dithiothreitol and cysteine. The presence of calcium ions is essential for such activity. The enzyme is inhibited by oxidizing agents, sulfhydryl-modifying agents, and by Co^{2+}, Cu^{2+}, Cd^{2+}, and other metal ions. Citrate, borate, and Tris anions are weakly inhibitory. Clostripain is assayed using a method that measures reaction velocity as an increase in absorbance at 253 nm resulting from the hydrolysis of N-benzoyl-L-arginine ethyl ester.

Clostripain may be expressed recombinantly in *E. coli* as a chimeric protein in the form of an inclusion body. Proteins isolated from *E. coli* expressed inclusion bodies may be contaminated with varying amounts of bacterial cells, bacterial cell products including pigments, nucleic acids, *E. coli* derived proteins and lipids.

Witte, et al. *"., Heterologous Expression of the Clostripain Gene from Clostridium histolyticum in Escherichia coli and Bacillus subtilis: maturation of the Clostripain precursor is coupled with self-activation"*, Microbiology, 140: *1175-82 (1994)* (Witte et al. 1994), ("Witte et al."), disclose the expression of Clostripain in *E. coli* and *B. Subtilis*. Witte, et al. indicate that the major portion of the protein from E. coli is degraded during activation. Consequently, Witte, et al. chose *B. subtilis* as an alterative expression organism for production. In this instance, the signal sequence associated with the expressed clostripain caused secretion of soluble prepro enzyme from *B. subtilis*.

Inclusion bodies form convenient tools for peptide and protein purification. They are believed to be aggregates of partially or incorrectly folded proteins, and such states of proteins are generally accepted to be influenced by solvent structure, and thereby by the nature and concentrations of denaturants, chaotropes, detergents, and the like. When solubilized in a strong denaturant (chaotrope), the aggregates are dissociated and dissolved. The denaturing agent is typically removed, or its concentration lowered, to allow for the correct folding of the protein.

In the Witte et al. situation, however, the inclusion body technique proved to be a failure. Witte et al. treated their pelletized fraction from *E. coli* expression with 4M urea. Witte et al. found that only a minor portion of the core protein was converted into the correctly processed form. They explained that a major portion of the core protein was degraded due to the proteolytic action of the processed clostripain. Consequently, Witte et al. indicate that *B. subtilis* is the organism of choice for clostripain expression. That expression involves soluble clostripain.

In sum, there is no known method for producing gram-scale quantities of active recombinant clostripain through use of inclusion body techniques. Nor is there a clostripain activation-through-dilution process which produces activated enzyme that does not require further processing such as freezing, precipitation or lyophilization, and which can be stored in solution under standard refrigeration.

OBJECTS OF THE INVENTION

It is an object of the instant invention to provide a process that will produce, activate, and purify gram-scale amounts of clostripain.

It is an additional object of the instant invention to provide a process that will produce, activate and purify gram-scale amounts of clostripain to high specific activity, high purity and at high concentration.

It is a still further object of the instant invention to provide a clostripain that does not require the use of anti-aggregation agents and that can be stored in solution under standard refrigeration conditions.

SUMMARY OF THE INVENTION

In accordance with the above stated objects, the instant invention provides a process for producing gram scale amounts of clostripain having high specific activity, high purity and high concentration. The process is highly cost-effective, robust, environmentally safe, and provides product for immediate or long-term consumption.

Specifically, in the instant invention, highly active clostripain is produced by (a) solubilizing in a urea solution clostripain inclusion bodies which have been harvested from a fermentation mixture comprising recombinant host cells which have expressed clostripain; and (b) activating the clostripain by diluting the urea solution of step (a) into a final urea concentration of less than 3 M. Calcium and a reducing agent are preferred as the activation buffer in the urea solution to develop highly preferred activity. The specific activity, purity and concentration of the activated clostripain can be increased in accordance with the instant invention by (a) fractionating the clostripain in the diluted urea solution over an equilibrated anion exchange resin to form a purified clostripain solution; and (b) further purifying and concentrating the clostripain by diafiltration.

Solubilization of clostripain in dilute urea concentrations of less than 3 M in accordance with the instant invention produces clostripain solutions that have a higher activity and higher yields than those otherwise obtained by activating clostripain through solubilization in more concentrated urea solutions. Without intending any limitation to the scope of the invention disclosed and claimed herein, it is theorized that this substantial improvement in clostripain activation and yield results in part from stabilized character of the clostripain at the lower urea concentration. The fact that diluting the concentration of urea in the activation buffer actually increases the activation yield is surprising in that minimal dilution of the chaotrope is required to effect activation, yet self-purification without autodegradation of activated clostripain can be obtained, even in the generally denaturing environment of 1-4 M urea. The clostripain activated in this manner can be frozen, precipitated or lyophilized, or stored as an active solution.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates optimum pH values for the urea dilution reaction mixture used in the instant invention.

FIGURE 2A-2C shows the open reading frame of the cloned prepro-clostripain (1-526; SEQ ID NO:12 and SEQ ID NO:13). The pre-sequence, pro-sequence, light-chain, linker, and heavy-chain are indicated by bracketed lines. Restriction enzyme recognition sites are indicated by enzyme name. The stop codon is indicated by a star.

FIGURE 3 shows the N-terminal sequence of the cloned T7tag-clostripain (51-526; SEQ ID NO:14 and SEQ ID NO:15). The T7tag, light-chain, linker, and portion of a heavy-chain are indicated by a bracket line. Restriction enzyme recognition sites are indicated by enzyme name.

FIGURE 4 illustrates an SDS-PAGE (4-20 % Tris-Glycine gel) analysis of recombinant clostripain core proteins. The clostripain position is indicated by an arrow.

FIGURE 5 shows a plasmid map of a representative expression construct encoding the mature clostripain.

FIGURE 6 illustrates an SDS-PAGE (4-20 % Tris-Glycine gel) analysis of *in vitro* processing of clostripain core protein T7tag-clost(51-526). The position of clostripain, and clostripain subunits is indicated by arrows.

FIGURE 7 shows a comparison of *in vitro* processing of clostripain proenzyme, core protein, and core protein mutant containing linker mutations. The positions of clostripain subunits is indicated by arrows.

FIGURE 8 illustrates HPLC analysis of the clostripain-containing protein solution before and after DEAE column filtration.

FIGURE 9 illustrates HPLC analysis of the clostripain-containing protein solution after DEAE column filtration.

FIGURE 10 illustrates HPLC analysis of the retentate from diafiltration of the clostripain-containing protein solution.

FIGURE 11 illustrates HPLC analysis of the permeate from diafiltration of the clostripain-containing protein solution.

FIGURE 12 illustrates SDS PAGE analysis of the activation of T7tag-Clost(51-526) and MRI inclusion bodies in various urea concentrations. The

positions of the pre-pro-clostripain, the clostripain heavy chain, and the clostripain light chain are indicated by arrows.

FIGURE 13 illustrates SDS PAGE analysis of the activation of MRI inclusion bodies in various urea concentrations. The positions of the pre-pro-clostripain, the clostripain heavy chain, and the clostripain light chain are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

The present invention achieves production of shelf stable, active clostripain through high yield expression from a microbial host such as *E. coli*. The yield and activity are surprising in light of earlier teaching that microbial expression in a host such as *E coli* is disfavored because yields are low and activity is poor or practically non-existent. The present invention achieves these results through astute use of processing conditions and attention to the self digestion aspects of clostripain.

Clostripain chimeric proteins employed in the instant invention may be expressed in a microbial host cell using known techniques of recombinant DNA production. *E. coli* is a preferred host cell. The host cell contains an expression vector, which encodes the chimeric protein under the control of a regulatory sequence that is capable of directing its expression in the host, as well as an origin of replication that is functional in the host cell. The vector may contain other DNA sequences conventionally employed in recombinant DNA technology such as sequences encoding selectable markers. Methods for expressing a foreign gene in a host organism also are well known in the art (see, e.g., Maniatis et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2nd ed., 1989).

The gene encoding a particular polypeptide can be constructed by chemically synthesizing the entire nucleotide sequence, by amplification, such as by the polymerase chain reaction (PCR), or by cloning the gene of interest. The gene is then subcloned into an appropriate expression vector. Cloning vectors, expression vectors, plasmids, and viral vectors are well known in the art Maniatis et al., supra, and (see, e.g., Goedell, Methods in Enzymology, Vol. 185 (Academic Press 1990)). Examples 1-7 provide a detailed description of the

preparation of a T7-based expression system useful for high-level expression of mammalian proteins in *E. coli.*

The host cell containing the expression vector is grown and the chimeric protein expressed under appropriate conditions. The conditions for growth of the host cell and expression of the chimeric protein will vary depending on various factors such as the host cell employed, the promoter, and the particular chimeric protein being expressed. Those skilled in the art are capable of determining the appropriate conditions for the particular host/vector system employed. Methods for expressing a foreign gene in a host organism also are well known in the art (see, e.g., Maniatis et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2nd ed., 1989). The gene encoding a particular polypeptide can be constructed by chemically synthesizing the entire nucleotide sequence, by amplification, such as by the polymerase chain reaction (PCR), or by cloning the gene of interest. The gene is then subcloned into an appropriate expression vector. Cloning vectors, expression vectors, plasmids, and viral vectors are well known in the art (see, e.g., Maniatis et al., *supra*, and Goedell, *supra*). Examples 1-7 provide a detailed description of the preparation of a T7-based expression system useful for high-level expression of clostripain in *E. coli.*

According to the invention, the clostripain may be conveniently packaged and isolated by inclusion bodies from *E. coli.* The inclusion bodies may be treated with a high concentration of urea solution and then activated by dilution to at least slightly less than 3M urea. This dilution factor provides the appropriate balance between denaturization, proper refolding and autocatalytic cleavage to produce stable active clostripain in solution.

In one embodiment of the instant invention, clostripain may be recombinantly expressed in *E. coli* host cells and recovered from such cells in the form of inclusion bodies ("IB's"). For example, the host cells may be harvested and opened to separate the IP's, such as by centrifugation, resuspension in a lysis medium, and treated to lyse, such as by mechanical lysing at 12,000 psi. The IB's can be collected to obtain isolated IB's sucha s by washing with water and collecting by centrifugation to form an IB paste. The collected IB's may be solubilized in a concentrated urea solution to obtain

solubilized inactive clostripain. For example, an IB paste may be solubilized by adding 8 M urea solution at a ratio of about 50 mL per g of IB, followed by centrifugation (for example at 6-8 °C at 15,000-17,000 x g) to pelletize cell debris. The centrifugation supernatant will contain solubilized inactive clostripain.

The inactive clostripain may be activated by dilution treatment according to the invention. Accordingly, the inactive solubilized clostripain, concentrated urea solution may be diluted to at least slightly less than 3M urea to activate the clostripain. Preferably, the medium with the inactive solubilized clostripain may also contain a concentration of metal ion and oxidation inhibitor, preferably a combination thereof. For example, 100-325 mg of urea-solubilized inactive clostripain solution may be clarified by centrifugation or by filtration through 0.45 micron filter membranes. The clarified, solubilized clostripain can be activated by dilution into reaction mixtures containing a calcium buffer at a concentration of 0.1 mM -5 mM to a final urea concentration of 1 to less than 3 M urea. In practice, about 850 000 to 880 000 units of the solubilized clostripain can be activated in a bench-top activation process with little or no subsequent need to stabilize or repurify.

In a preferred embodiment, the diluted reaction mixture is composed of from about 1 to about less than 3 M urea and 0.1 to 5 mM of an alkali or alkaline earth metal or transition metal ion as the activation buffer. A calcium ion activation buffer is preferred. While activation can be performed over a wide pH range, a pH range of 7 to 8 is preferred, as described in detail hereinafter in Example 8, and as illustrated in Figure 1. A variety of buffers such as Tris, HEPES, MES, or phosphate can also be employed to control the pH during activation. An activation buffer comprised of 25 mM HEPES and 5 mM $CaCl_2$ at pH 7.6 is a particularly preferred buffer. This buffer does not inhibit clostripain or form insoluble complexes with calcium. In the preferred embodiment illustrated herein, NaCl was added to the activation reaction subsequent to activation to facilitate fractionation, as explained hereinafter.

As optimum activation of clostripain occurs when the enzyme is fully reduced, an oxidation inhibitor can optionally be added to the activated clostripain medium. Any oxidation inhibitor that is compatible with proteases and proteins may be used in this capacity. Preferably, mercaptans containing SH

groups such as mercaptoethanol (0.001-5 M), dithiothreitol (DTT) (0.0001-0.25 M) or cysteine (0.001-0.25 M) can be added to ensure full reduction of the clostripain. In a preferred embodiment, 0.001-0.025 M DTT is added to the dilution mixture. A comparison of the activity values listed in Tables 1 and 2 (Example 8) shows the increase in specific activity achieved by use of DTT. Activation can occur over a wide temperature range; at room temperature, maximum activity is usually reached within one to two hours. In terms of enzyme units ('BAEE'-units, as described below in Example 11, method 2) the yield of active clostripain was in the range of 50 to 120 Units per mL of solution (i.e. 100 to 240 Units per mg of protein).

The solubilized protein is diluted into an activation buffer at a final concentration of 0.05-5 mg/mL in the diluted solution. In a preferred embodiment the final concentration of protein will be 0.4-0.6 mg/mL. The final concentration of urea is less than 3 M. In a preferred embodiment, the final concentration of urea is between about 1-3 M. The increase in enzyme activity is monitored by dilution into the standard BAEE kinetic assay as described in Example 11. The activity reaches a maximum level after about 1 to 2 hours at room temperature.

Thus, dilution of the solubilized clostripain into dilution reaction mixtures providing a final concentration of less than 3 M urea increased clostripain specific activity by as much as 475 fold over the specific activity of the solubilized clostripain diluted to a final 4 M urea concentration as illustrated in Table 1, Example 8.

The activated clostripain solution can be adjusted to a concentration of 0.05-0.15 M NaCl and filtered through an anion exchange resin to separate pigment, DNA, and other compounds from the clostripain. In a preferred embodiment, the activated clostripain solution can be adjusted to a concentration of 0.1 M NaCl and filtered through DEAE-Sepharose FF. Reducing agent (such as DTT (5 - 25 mM)) can be added to maintain activity during storage as illustrated in Example 8. Other stabilizing agents such as glycerol can be employed. Alternatively, clostripain may be precipitated by agents such as, but not limited to, ammonium sulfate, ethanol, methanol, or sodium sulfate, with or without subsequent lyophilization. In a preferred embodiment, precipitation was

achieved with about 60 % ethanol. An ethanol slurry prepared in this way was stable for greater than three months at
-20°C, and lyophilized ethanol powder for greater than 4 months.

Diafiltration can be employed to concentrate the enzyme solution and remove small molecular weight impurities. In a preferred embodiment of the instant invention, diafiltration can be employed in order to reduce the volume, and purify the DEAE-breakthrough fraction while maintaining a sufficient quantity of clostripain to prepare a commercial-size batch of recombinant glucagon-like polypeptide-1 (e.g., 100-130 g). The resulting activated, purified clostripain solutions have been maintained under refrigeration at 5°C for 50 days without any measurable loss in activity (90% –110%). "Diafiltration" is a mode of operating an ultrafiltration system in which the retentate (that portion of a sample that does not pass through the ultrafiltration membrane) is continuously recycled and diluted with fresh wash solution to replace that removed as permeate. "Ultrafiltration" may be defined as separation of particles (e.g., macromolecules) by filtration through membranes based on molecular weight.

Ultrafiltration membranes typically have a molecular weight cut-off (MWCO) in the range of 1,000 to 1,000,000 daltons. The MWCO typically is defined as the molecular weight of the globular solute which is 90 % retained by that membrane. See Filtron Catalog, 1995/96, p.5. The actual molecular weight of particles that pass through or are retained by a membrane will depend on the size as well as the conformation and charge of a given molecule. The native clostripain molecule is a heterodimer consisting of a lighter chain of 12 kDa and a heavier chain of 43 kDa (Gilles, Imhoff, and Keil, Journal of Biological Chemistry, 254: 1462-1468 (1979)); clostripain was found to be retained by a Filtron 10K membrane. While regenerated cellulose membranes are a preferred diafiltration membrane, membranes made of other materials that otherwise meet the retention criteria specified above may be used.

The invention is described further by the following examples, which are illustrative and not limiting.

EXAMPLE 1

E. coli High Yield Expression Vectors

The vectors pBN115 and pBN121 are *E. coli* high yield nucleic acid constructs constructed through use of a high copy number vector that is stably maintained within a host cell. The vectors contain an expression cassette having a strong promoter that is operably linked to an open reading frame that encodes clostripain. These vectors were constructed through use of the larger DNA fragment produced from restriction enzyme digestion of pGEX2T (Amersham Pharmacia Biotech, Piscataway, NJ) with *Fsp*I-*Sma*I. This fragment contains the replication origin of pMB1 for high copy number maintenance, the LacIq gene for promoter suppression, the GST terminator for transcription termination, and the *bla* gene for ampicillin resistance. A strong promoter, modified Tac, was amplified from the pGEX2T plasmid with restriction enzyme sites at both ends using the following primers:

Primer 1: 5' TGC ATT TCT AGA ATT GTG AAT TGT TAT CCG CTC A 3' (SEQ ID NO: 1).

Primer 2: 5' TCA AAG ATC TTA TCG ACT GCA CGG 3' (SEQ ID NO: 2).

PCR amplification produced the following product:

TCAAAGATCTTATCGACTGCACGGTGCACCAATGCTTCTGGCG TCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTAAATC ACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGT TTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGC TGTTGACAATTAATCATCGGCTCGTATAA[T]GTGTGGAATTGTGAGC GGATAACAATTCACA]ATTCTAGAAATGCA (SEQ ID NO:3).

The upstream *Bgl*II restriction endonuclease binding site sequence (A/GATCT) and the downstream *Xba*I (T/CTAGA) binding site sequence are underlined with a single line, the -35 and -10 promoter consensus sequences are bolded and underlined with dots, and the downstream transcriptional start A residue (within the *lac* operator gene sequence) is bolded and underlined with a solid line. The *lac* operator sequence is enclosed within brackets. The PCR product of the Tac promoter fragment was ligated into the larger *Fsp*I-*Sma*I fragment from pGEX2T.

The ligation mixture was transformed into high efficiency *E. coli* competent cells by heat shock at 42°C for 45 seconds and streaked on LB + 50 μg/mL Ampicillin Agar plates. Vectors from cultures of single colonies were prepared. A correct vector was identified by restriction enzyme digestion. The *Xba*I-*Xho*I fragment from pET23a plasmid (Novagen, Madison, WI), which contained the T7 gene 10 ribosome binding site and the T7tag initiation sequence, was inserted into the correct vector identified above at the *Xba*I-*Sma*I site of the nucleic acid construct. The resulting vector was named pBN115(Tac).

To introduce a kanamycin selection marker, the plasmid pBN115(Tac) was digested with modified *Aat*II-*Fsp*I to remove the 0.7 kb ampicillin resistance gene. A 1.1 kb PCR product, containing the aminophosphotransferase gene that encodes kanamycin resistance, was then cloned into the pBN115(Tac) vector at the *Aat*II-*Fsp*I sites. The kanamycin resistance gene was produced through selective PCR amplification of the pCT-Blunt plasmid (Invitrogen, Carlsbad, CA) using the following primers:

KANXY1: (5'-CCT GAC GTC CCG GAT GAA TGT CAG CTA CTG GGC-3') (*Aat*II site underlined) (SEQ ID NO:4),

KANXY2: (5'-GGC TGC GCA AAG GAG AAA ATA CCG CAT CAG GAA-3') (*Fsp*I site underlined) (SEQ ID NO:5).

The resultant plasmid was designated as pBN121(Tac), and *E. coli* that were transformed with this plasmid could be selected in LB + 25 μg/mL kanamycin media. Like plasmid pBN115(Tac), pBN121 contains unique *Nhe*I and *Xho*I restriction sites for inserting the foreign gene sequence to be expressed, such as the gene sequence that encodes clostripain.

EXAMPLE 2

Cloning of the Prepro-clostripain(1-526)

The prepro-Clost (1-526) gene was PCR amplified from the *C. histolyticum* genome by using Pfu DNA polymerase (Stratagene, La Jolla, CA) and the following primers:

0925CLA: 5'-AGA GCT CAT ATG TTA AGA AGA AAA GTA TCA ACA CTA TTA ATG-3' (*Nde*I site underlined) (SEQ ID NO:6) and

0925CLB: 5'-TTG CTC GAG TTA CCA TTG GTA ATG ATT AAC TCC TCC AGT-3' (*Xho*I site underlined) (SEQ ID NO:7).

The genomic DNA template was prepared by repeated phenol-chloroform extraction and ethanol precipitation of *C. histolyticum* collagenase (Worthington, Lakewood, NJ). The PCR product was blunt-end inserted into linearized pCR-Blunt vector using the Zero Blunt cloning kit (Invitrogen), and produced the pCR-Blunt-preproClost(1-526) plasmid. The cloned clostripain gene was confirmed by DNA sequencing. Figure 2 provides the open reading frame of the cloned prepro-Clost(1-526) gene with some restriction enzyme sites indicated.

The *Nde*I-*Xho*I fragment of the prepro-Clost(1-526) gene from the above pCR-Blunt-preproClost(1-526) plasmid was cloned into the pET23a expresson vector (Novagen) at *Nde*I-*Xho*I sites to produce a nucleic acid construct. The resulting nucleic acid construct pET23a-preproClost(1-526) was transformed into *E. coli* BL21(DE3) cells, and the colonies were grown in LB + 50 μg/mL ampicillin media. The correct nucleic acid construct was identified by restriction enzyme digestion and DNA sequencing. Glycerol stocks of the *E. coli* host cells harboring the construct were stored at –80 °C or below with 15 % glycerol.

EXAMPLE 3

Cloning of the Pro-clostripain (28-526)

A DNA fragment containing pro-clostripain (28-526) was amplified by PCR using the pCR-Blunt-preproClost(1-526) plasmid as template and the following primers

CLOSPRO (5'-TAT ACA TAT GAA TCC AGT AAC TAA ATC CAA GGA TAA TAA C-3'; *Nde*I site underlined) (SEQ ID NO:8) and

CLOSPRIM2 (5'-CCT AGG ATC CCC CAT GTT AGC TTC ATA TTT ACT-3'; *Bam*HI site underlined) (SEQ ID NO: 9).

The amplified fragment had the ATG start codon at the N-terminus of the pro-clostripain (28-526). The PCR product was cleaved with the restriction enzymes *Nde*I-*Bam*HI and inserted using the same enzymes into the pET23a-preproClost(1-526) nucleic acid construct (Example 2) to produce the pET23a-proClost(28-526) nucleic acid construct. The nucleic acid construct was transformed into *E. coli* HM174(DE3) and BL21(DE3). The correct nucleic acid construct was selected in LB + 50 μg/mL ampicillin media. Glycerol stocks of the construct were stored as in Example 2.

EXAMPLE 4

Cloning of clostripain (51-526)

To prepare an clostripain nucleic acid construct lacking the sequence encoding the pre-propeptide, a PCR amplification reaction was carried out as in Example 3 using the pCR-Blunt-preproClost(1-526) plasmid as the template and the following primers:

CLOSPRIM7 (5'-TAT ACA TAT GAA CAA AAA TCA AAA AGT AAC TAT TAT G-3'; *Nde*I site underlined) (SEQ ID NO:10) and

CLOSPRIM2 (5'-CCT AGG ATC CCC CAT GTT AGC TTC ATA TTT ACT-3'; *Bam*HI site underlined) (SEQ ID NO:9).

The amplified fragment contained the ATG start codon at the N-terminus of clostripain (51-526). The PCR product was cleaved with the restriction enzymes *Nde*I-*BamH*I and inserted using the same enzymes into the pET23a-preproClost(1-526) nucleic acid construct (Example 2) to produce the pET23a-Clost(51-526) nucleic acid construct. The pET23a-Clost(51-526) nucleic acid construct was transformed into *E. coli* BL21(DE3) or BL21(DE3)pLysS cells. The correct construct was selected in LB + 50 μg/mL ampicillin media. Glycerol stocks of the construct were stored as in Example 2.

The *Nde*I-*Xho*I fragment from pET23a-Clost(51-526), which contained the clostripain (51-526) gene, was inserted into the pET24a vector (Novagen) at the *Nde*I-*Xho*I sites to produce the pET24a-Clost(51-526) nucleic acid construct. The pET24a-Clost(51-526) nucleic acid construct was transformed into *E. coli* BL21(DE3) or BL21(DE3)pLysS cells. The correct construct was selected in LB + 25 μg/mL kanamycin media and glycerol stocks of the construct were stored, as in Example 2.

The *Nde*I-*Xho*I fragment from pET23a-Clost(51-526) was also inserted into the pBN121(Tac) plasmid (Example 1) at the *Nde*I-*Xho*I site to produce the pBN121(Tac)-Clost(51-526) nucleic acid construct. This construct was transformed into *E. coli* BL21 cells. The correct construct was selected in LB + 25 μg/mL kanamycin media and glycerol stocks of the construct were stored as in Example 2.

EXAMPLE 5

Cloning of the T7tag-clostripain (51-526)

To achieve a higher expression level of clostripain in *E. coli*, a short (8 aa) peptide (T7tag) carrying a strong translation initiation signal from a very efficiently expressed gene, T7 gene 10, was fused to clostripain (51-526) at its N-terminus to optimize clostripain translation. The DNA fragment coding the T7tag-clostripain (51-526) was PCR-amplified using the pCR-Blunt-preproClost(1-526) plasmid as the template and the following primers:

CLOSPRIM2 (5'-CCT AGG ATC CCC CAT GTT AGC TTC ATA TTT ACT-3'; BamHI site underlined) (SEQ ID NO:9). (same as in Example 3) and

CLOSPRIM1 (5'-ATA CAT ATG GCT AGC ATG ACT GGT GGA CAG AAC AAA AAT CAA AAA GTA ACT ATT ATG-3'; (SEQ ID NO:11); *Nde*I and *Nhe*I sites underlined).

The amplified fragment contained the T7tag sequence at the N-terminus of clostripain (51-526) light chain (Figure 3). The PCR product was cleaved with restriction enzymes *Nde*I-*BamH*I and inserted using the same enzymes into pET24a-Clost(51-526) nucleic acid construct (Example 4) to produce the pET24a-T7tag-Clost(51-526) nucleic acid construct. This construct was transformed into *E. coli* BL21(DE3) cells. Transformants were selected in LB + 25 μg/mL kanamycin media and glycerol stocks of cells containing the correct construct were stored as in Example 2.

The *Nde*I-*Xho*I fragment from pET24a-T7tag-Clost(51-526), which contained the T7tag-clostripain (51-526) gene, was inserted into the pBN115(Tac) nucleic acid construct (Example 1) at the *Nde*I-*Xho*I site to produce the pBN115(Tac)-T7tag-Clost(51-526) nucleic acid construct. This construct was transformed into *E. coli* BL21 cells. Transformants were selected in LB + 50 μg/mL ampicillin media and glycerol stocks of cells containing the correct construct were stored as in Example 2.

In order to introduce a kanamycin selection feature into pBN115/tac-T7tag-Clost(51-526), the construct was further modified by the method described in Example 1. The resulting construct was designated pBN121(Tac)-T7tag-Clost(51-526) (Figure 3). This construct was transformed into *E. coli* BL21 cells. Transformants were selected in LB + 25 μg/mL kanamycin media

and glycerol stocks of cells containing the correct construct were stored as in Example 2.

EXAMPLE 6

E. coli Shaking Culture Expression of Various Constructs

LBA media (LB + ampicillin) were used when expressing the pET23a or pBN115 derived nucleic acid constructs. LBK media (LB + kanamycin) were used when expressing the pET24a or pBN121(Tac) derived constructs. Shaking flask cultures of 5 mL LBA or LBK media were started from single colonies of the transformed cells. Shake flask cultures in 5 mL to 500 mL LBA or LBK media (inoculated by 100 µL to 10 mL overnight culture) were grown at 37 °C and 220 rpm to an A_{600} of 0.5-1.0. Polypeptide expression was induced by addition of IPTG (1 mM final concentration). Cultures were induced for 2 to 8 hours. Samples were taken from cells having the same A_{600} of pre- and post-induced cells. Cells were pelleted and then lysed in distilled water or 10 mM Tris, pH 8, by sonication. The lysate was then centrifuged to separate insoluble and soluble proteins.

The supernatant (soluble protein) from the cell lysate was mixed 1:1 with 2× SDS-PAGE sample buffer. The pellets were resuspended directly in 1× SDS-PAGE sample buffer. These samples were resolved by SDS-PAGE (Invitrogen) according to the manufacturer's instructions and stained with Coomassie Brilliant Blue.

E. coli BL21 cells transformed with pBN121(Tac)-T7tag-Clost(51-526) produced an insoluble protein of 60 kDa upon IPTG induction, corresponding to the calculated size of the 484 amino acid encoded by the T7tag-Clost(51-526) construct. BL21 cells transformed with pBN121(Tac)-Clost(51-526) also produced an insoluble protein that was 59 kDa. This protein corresponded in size to the 476 amino acid clostripain (51-526) encoded by the construct. The T7tag sequence markedly increased the expression yield of the recombinant clostripain (51-526) (Figure 4). Figure 4 illustrates an SDS-PAGE (4-20 % Tris-Glycine gel) analysis of recombinant clostripain core proteins T7tag-Clost(51-526) and Clost(51-526) expressed in *E. coli* strain BL21. Bacterial cells harboring pBN121(Tac)-T7-Clost(51-526) or pBN121(Tac)-Clost(51-526) were induced and harvested 4 hr after induction. Cells were lysed and the inclusion

bodies, isolated by centrifugation, were boiled in SDS sample buffer. Lane M: molecular weight markers, as indicated (kDa). Lanes 1, 2: clost(51-526) from two different isolates. Lane 3: T7tag-clost(51-526). The gel was stained with Coomassie Brilliant Blue. Figure 5 is a plasmid map of the pBN121(Tac)-T7tag-Clost(51-526) expression vector. pBR322ori = pBR322 replication origin; lacIq = lac repressor gene; kan = kanamycin resistance gene; tac = tac promoter; clostp = T7tag-clostripain gene. Such a plasmid could be used to express other forms of clostripain.

EXAMPLE 7

E. coli Fermentation Production of T7tag-clostripain (51-526)

Expression of the T7tag-clostripain by *E. coli* BL21 cells transformed with the pBN121(Tac)-T7tag-Clost(51-526) nucleic acid construct was evaluated in 5 L or larger fermentations. A 100 μL glycerol stock of the construct was used to inoculate 200 mL LB + 25 μg/mL Kanamycin media in a shaking flask. The shake culture was grown in a rotary shaker at 37°C until the A_{540} reached 1.5 ± 0.5. The contents of the shaking flask culture were then used to inoculate a 5 L fermentation tank containing a defined minimal media. Glucose served as the carbon source and was maintained between 1-8 g/L. About 10 μg/mL kanamycin was used in the fermentation. Dissolved oxygen was controlled at 40% by cascading agitation and aeration with additional oxygen. Ammonium hydroxide solution was fed to control the pH at about 6.9 and to supply additional nitrogen. After the A_{540} reached 50-75, the cells were induced with IPTG at a final concentration of 0.1-1 mM for 4-10 hours. After the induction was complete, the cells were cooled and harvested by centrifugation. The cell sediments were stored at a temperature below –20°C or were lysed immediately for use. Cells, after thawing if they were frozen, were resuspended in lysis buffer, 50 mM Tris, 2.5 mM EDTA, pH 7-8, then lysed by mechanical homogenization with a Rannie homogenizer. The lysate was centrifuged to pellet inclusion bodies (IBs) of the expressed polypeptide. IBs were washed with water until the conductivity dropped to below 100 μS/cm. Washed IBs were centrifuged to form a paste for storage at -20°C. The polypeptide sediments were dissolved in 8 M urea for further treatment. Figure 6 illustrates a

SDS-PAGE (4-20 % Tris-Glycine gel) analysis of *in vitro* processing of clostripain core protein T7tag-Clost(51-526). Lane M: molecular weight markers, as indicated (kDa). Lanes 1, 2: T7tag-Clost(51-526) before activation in 2 M urea. Lanes 3, 4: T7tag-Clost(51-526) after activation in activation buffer containing 2 M urea at room temperature for 1 hr. The gel was stained with Coomassie Brilliant Blue. Figure 7 shows a comparison of *in vitro* processing of clostripain proenzyme, core protein, and core protein mutant containing linker mutations. Inclusion bodies were extracted from 0.15 OD_{600} of IPTG-induced cells. Lane M: molecular weight marker. Lanes 1-3: clostripain proenzyme from pET23a-proClost(28-526)/HMS174DE3. Lanes 4-6: clostripain core protein from pET23a-Clost(51-526)/HMS174(DE3). After solubilization in 8 M urea, proteins were activated in activation buffer containing 2 M urea for 0 min (lanes 1, 4), 20 min (lanes 2, 5), or 60 min (lanes 3, 6). Proteins were then loaded on to a 4-20 % Tris-Glycine SDS gel for electrophoresis. The protein bands were detected by Coomassie Brilliant Blue staining. Banding positions of clostripain subunits are indicated by arrows: (←) heavy chain, (←) light chain from Clost(28- 526), (←) light chain from Clost(51-526). Protein molecular weight markers are indicated in kDa (lane M).

EXAMPLE 8

Effect of urea and DTT on clostripain activation

Clostripain was recombinantly expressed in *E. coli* host cells and recovered from such cells in the form of inclusion bodies (IBs) in accordance with the preceding Examples. The host cells were harvested by centrifugation and mechanically lysed in 50 mM Tris, 2.5 mM EDTA, pH 7.8, at 12, 000 psi. The IB's were washed with water and collected by centrifugation at 15,000-17,000 x g to form an IB paste. This paste was solubilized in 8 M urea, containing 25 mM HEPES at pH 7.6, at a ratio of about 50 mL of urea solution/g IB followed by centrifugation at 6-8 °C at 15,000-17,000 x g to pelletize cell debris. The centrifugation supernatant contained solubilized inactive clostripain.

The solubilized 8 M urea IB mixture was adjusted with 8 M urea, 25 mM HEPES at pH 7.6 to a concentration of 4 mg/mL of IB, and diluted to various concentrations of urea (1, 2, 3 and 4 M) in an activation buffer comprised of HEPES and $CaCl_2$ (e.g., 25 mM HEPES, 2 - 5 mM $CaCl_2$, pH 7.6), to yield a

final clostripain concentration of about 0.5 mg/mL, as reflected in Table 1. In a separate experiment dithiothreitol (DTT) was added at a concentration of 10 mM to the activation buffer to increase activity, and the effect of such addition is reflected in Table 2.

Table 1 reflects the specific activities noted for each of the urea concentration levels of the dilution reaction mixtures. Kinetic activity was measured by Method 2, Example 11. Enzyme purity was assessed by HPLC assay Method 3, Example 11.

Table 1 Effect of Urea on clostripain Activation

Urea concentration after dilution (M)	Maximum specific activity (U/mg)	Purity by HPLC assay (% of area under peak for sum of light and heavy chains)
1	167	22
2	190	27
3	155	15
4	0.4	14

As reflected in Table 2, the addition of dithiothreitol to the dilution reaction mixtures further increased the maximum IB activity.

Table 2 Effect of 10 mM dithiothreitol on clostripain Activations

Urea concentration after dilution (M)	Maximum specific activity (U/mg)	Purity by HPLC assay (% of area under peak for sum of light and heavy chains)
1	126	28
2	256	43
3	120	11
4	0.95	32

The maximum activity at the optimum urea concentration of 2 M obtained in the presence of 10 mM DTT was found to be 35 % higher than that obtained in the absence of DTT. In addition, the areas of the clostripain light and heavy chain peaks in HPLC chromatograms were on average 50 % greater for samples

prepared in the presence of 10 mM DTT, suggesting greater homogeneity of the activated clostripain.

EXAMPLE 9

DEAE Purification of clostripain

Solubilized clostripain, prepared by homogenizing clostripain inclusion bodies isolated as in Example 8, was activated by dilution into an activation buffer comprised of 25 mM HEPES and 5 mM $CaCl_2$ at pH 7.6, in a way that resulted in a urea concentration of 1 to 3 M.

Sodium chloride was added to the activated clostripain solution to a concentration of 0.1 M prior to loading on a DEAE column. A substantial portion of clostripain was recovered in the fractionation breakthrough (unbound fraction). For example, fractionation of the activated clostripain solution by column chromatography using a DEAE-Sepharose Fast Flow (Pharmacia) resin resulted in the recovery of over 90 % of the clostripain in the fractionation breakthrough, while contaminants such as bacterial pigments and nucleic acid remained bound to the resin and were subsequently eluted at a pH of 7.6 using a high ionic strength salt wash comprised of 25 mM Hepes, 5 mM calcium chloride and 2 M NaCl. Spectral analysis of the high salt eluate indicated a UV absorbance ratio at $A_{260\,nm}/A_{280\,nm}$ of 1.6, indicative of DNA present. Figure 8 illustrates the removal of a contaminant from clostripain as assessed by HPLC Method 1, Example 11. In the absence of the added sodium chloride, a substantial portion of the clostripain binds to the ion exchange resin.

EXAMPLE 10

Diafiltration/Concentration of clostripain

Approximately 1,200 milliliters of the activated clostripain dilution reaction mixture breakthrough fraction from DEAE-Sepharose FF, having 33024 units of BAEE activity, and prepared according to Example 12, was concentrated to a volume of 7.5 mL and a concentration of 9.1 mg/mL using a Mini Pellicon 50 cm^2 membrane (Millipore PXC010C50). Only around 12 % of the total clostripain activity units present in the solution prior to diafiltration was lost, providing a specific activity of 426 Units/mg of clostripain. Concentrations in excess of 20 mg/mL have been achieved after diafiltration. Diafiltration is preferably performed with a buffer composed of 25 mM HEPES, 5 mM $CaCl_2$ and 1 M urea, at pH 7.6, to maintain solubility and stability of the enzyme during the process.

An amount of clostripain suitable for commercial-scale transpeptidation in the preparation of GLP-1, has been obtained in accordance with this procedure, using appropriately scaled diafiltration equipment. Following the DEAE step, DTT could be added if not already present during the activation. Approximately 32 liters of impure clostripain solution have been purified at a rate of approximately 400-100 mL/min permeate flow and concentrated to 1 L in around 2.55 hours using a 1 ft^2, 10 K regenerated cellulose (RC) membrane (Pall).

Figures 9, 10 and 11 illustrate the typical HPLC profiles for the starting material, retentate and permeates after diafiltration using a 10 K Regenerated Cellulose membrane in accordance with the process of the instant invention. The starting purity for freshly activated clostripain frequently ranges from approximately 20-40 %. Analysis of the permeate (Method 3, Example 11) shows that the smaller hydrophilic compounds (peptides and degradation products of clostripain and cellular proteins) have passed through the membrane (Figure 11), while that of the retentate shows the enrichment in clostripain as its light and heavy chains, the peaks at 11 and 20 minutes respectively, in Figure 10.

Enzyme degradation products, although mostly small enough to pass through the membrane, can still be large enough to be retained by the 10 K membrane. The purity of clostripain, as calculated by the combined heavy and light chain % areas monitored at 280 nm, does not directly indicate the kinetic

activity of the enzyme. Thus, whereas the HPLC method gives an indication of the impurities present, the activity is determined by the BAEE kinetic assay.

EXAMPLE 11

Analytical methods

HPLC Method 1: A Hewlett-Packard HP 1100 system was used, a Vydac C4 column (5 μ particle size, 4.6 mm X 150 mm) and the following gradient program using solvent A: 5 % acetonitrile: 95 % water: 0.1 % TFA; solvent B: 95 % acetonitrile: 5 % water: 0.1 % TFA: The gradient was 15-50 % B (10 min), 50-100 % B (0.5 min) 100 % B (1 min), 100-15 % B (0.1 min), and 15 % B (3.4 min). Flow rate was 1.5 mL/min with detection at 280 nm. This HPLC assay was used to monitor the purity of clostripain as defined by the sum of the peak areas of its heavy and light chains relative to the other species present.

Method 2: BAEE kinetic assay: the substrate N-Benzoyl-L-Arginine ethyl ester (BAEE) was used for the kinetic assay at a temperature of 25°C. The assay buffer was 25 mM HEPES, pH 7.6, 2.5 mM DTT, 0.25 mM BAEE, 5 mM $CaCl_2$. After checking the blank reaction, the assay was started by the addition of enzyme. Enzyme was diluted as needed to ensure a linear rate of 0.02-0.04 absorbance units per minute. This assay served as a marker of enzyme activity. Enzyme that would hydrolyze BAEE would also amidate peptides in a transpeptidation reaction. Clostripain BAEE-activity units are defined as that amount of enzyme which will hydrolyze one micromole of BAEE per minute under the conditions specified here.

Method 3: HPLC assay: a Beckman system Gold System was use with a Vydac C4 column (10 μm particle size, 4.6 x 250 mm). Elution was at a flow rate of 1.5 mL/min, at a temperature of 45 °C, and with detection at 280 nm. The gradient was 10-30 % B (3 min), 30-45 % B (20 min), 45-100 % B(3min), 100 % B (0.5 min), 100-10 % B (1 min). A was 5 % acetonitrile, 0.1 % TFA, B was 95 % acetonitrile, 0.1 % TFA.

EXAMPLE 12

Large scale DEAE Sepharose Fractionation of clostripain

Two preparations of activated clostripain, prepared as in example 9, without adding sodium chloride subsequent to activation, were loaded on DEAE-Sepharose and showed partitioning of activity between the bound fraction and material eluting in the unbound fraction. Salt washes of 0.15 and 0.2 M NaCl, respectively, in HEPES/$CaCl_2$ buffer at pH 7.6, eluted the bound fractions. Thus, of 422,152 units loaded on the DEAE column in lot 1, 171,032 units broke through, and 158,850 units were eluted with 0.2 M salt. For lot 2 (253,700 units) 100,491 units broke through, and 135,000 units were eluted with 0.15 M NaCl. Amidation studies using these fractions indicated little difference in the ability of either bound or unbound fractions to catalyze the transamidation reaction of rGLP-1-(7-36)-AFAHse to rGLP-1-(7-36)amide. Thus the conversion yields were from 67 to 76 %.

Three batches of activated clostripain were prepared as in Example 9. These batches were adjusted to 0.1 M NaCl after activation, in addition to the 25 mM HEPES and 5 mM calcium chloride already present at pH 7.6. They were individually fractionated over individual DEAE-Sepharose FF (Pharmacia) fractionation columns (500 mL bed volume). The clostripain fractions were analyzed by RP-HPLC (Method 1) and the kinetic assay (Method 2) in Example 11. The first batch with 310,000 Units retained all enzyme activity after DEAE purification (330,000 units recovered). Two production scale batches were also prepared with full retention of enzyme activity. Thus lots of 855,400 and 880,000 units loaded, were recovered as 830,960 and 908,000 units, respectively. These two batches of activated clostripain were used separately in the amidation of production- scale (e.g., 100-130 g) amounts of GLP-1 (7-36)-AFAHse. DTT (25 mM) was added to the clostripain prior to storage at 4-5°C.

High rGLP-1 amidation yields (69%, in both instances; assessed by HPLC analysis of the amidation mixtures) was achieved using these DEAE purified clostripain lots.

EXAMPLE 13

SDS PAGE analysis of the activation of T7tag-Clost(51-526) and MRI inclusion bodies in various urea concentrations.

SDS PAGE analysis of the activation of T7tag-Clost(51-526) and MRI inclusion bodies was done in various urea concentrations. The following urea concentrations were used: 2.0, 2.5, 3.0, 3.5 and 4.0 M. In a first experiment, T7tag-Clost(51-526) and MRI inclusion bodies were solubilized in 8 M urea and then activated at a concentration of 2 and 4 M urea for either 1 hour at room temperature or 18 hours at room temperature (Figure 12). Figures 12 and 13 indicate lanes for the solubilized constructs and molecular weight markers. The arrows depicted in the figures indicate where the heavy and light chains of the activated clostripain migrated and also where the solubilized constructs migrated. In Figure 12 the lanes are marked : 1= Molecular weight Markers; 2 = Clost(51-526), 2 M urea, 1 hr; 3 = MRI, 2 M urea, 1 hr; 4 = Clost(51-526), 4 M urea, 1 hr; 5 = MRI, 4 M urea, 1 hr; 6 = Clost(51-526), 2 M urea, 18 hr; 7 = MRI, 2 M urea, 18 hr; 8 = Clost(51-526), 4 M urea, 18 hr; 9 = MRI, 4 M urea, 18 hr.

Referring to Figure 12, it was determined that both constructs activated at 2 M urea but not at 4 M urea. In the second experiment, MRI inclusion bodies were solubilized in 8 M urea and were then activated at a concentration of 2.0, 2.5, 3.0, 3.5 and 4 M urea for 1 hour at room temperature (Figure 13). In Figure 13 the lanes are marked: 1= Molecular weight Markers; 2,7 = 2.0 M urea; 3 = 2.5 M urea; 4,8 = 3.0 M urea; 5,9 = 3.5 M urea; 6,10 = 4.0 M urea.

Referring to Figure 13, it was determined that the amount of MRI increased as urea concentration decreased.

EXAMPLE 14

Effect of pH on the activation of clostripain

Washed inclusion bodies of clostripain were prepared as in Example 8, as a solution of 2 mg/mL in 8 M urea. Aliquots of 2 mL were diluted with 6 mL of buffers at different pH values to a final protein concentration of 0.5 mg/mL. The clostripain activity of each dilution was followed for about 3 hours by Method 2, Example 11. The buffers were: from pH 6 to 7.7 : 0.1 M NaCl, 0.1 M HEPES,

0.1 M MES, 5 mM $CaCl_2$; from pH 7.7 to 9.4: 0.1 M NaCl, 0.1 M HEPES, 0.1 M ammonia, 5 mM $CaCl_2$. The diluent solutions were adjusted to the following pH values prior to adding the 8 M urea solution of clostripain inclusion bodies: 6.09, 6.32, 7.05, 7.35, 7.65, 7.70, 8.06, 8.32, 8.98, 9.38. Maximum activities, attained within 2 to 3 hours, were normalized to the values at pH 7.7 (100%) and are reported in Figure 1. It was thereby established that maximum activation was obtained in a pH range around 7 to 8.5.

All publications, patents and patent applications listed herein, as well as priority patent application serial no. 60/399,202 filed on July 29, 2002, are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

What is claimed is:

1. A process for activating clostripain comprising:
solubilizing in a urea solution clostripain inclusion bodies which have been harvested from a fermentation mixture comprising recombinant host cells which have expressed clostripain, and thereafter diluting said urea solution to a final urea concentration of less than 3 M.

2. The process of claim 1, wherein the diluting step includes addition of an activation buffer comprises at least an alkaline, alkaline earth or transition metal salt.

3. The process of claim 1, wherein a reducing agent is added to the activation buffer.

4. The process of claim 3, wherein the reducing agent is a mercaptan.

5. The process of claim 4, wherein the mercaptan is mercaptoethanol, dithiothreitol or cysteine.

6. A process for producing an activated clostripain solution comprising:
(a) solubilizing in a urea solution of 7 to 8 M urea clostripain inclusion bodies which have been harvested from a fermentation mixture comprising recombinant *E. coli* host cells which have expressed clostripain to form solubilized clostripain; and
(b) activating the solubilized clostripain by diluting that solution to a final urea concentration of no more than about 3 M in a metal ion containing buffer.

7. The process of claim 6, wherein the clostripain inclusion bodies are solubilized in using a low speed homogenizer.

8. The process of claim 6, wherein upon solubilization of the clostripain inclusion bodies, the solution is filtered or centrifuged and the solubilized clostripain is thereafter recovered in the centrifugation supernatant or filtrate.

9. The process of claim 6, wherein:
(a) the clostripain inclusion bodies have been harvested from the fermentation mixture by lysing the host cells in lysis buffer and thereafter centrifuging the lysed cells; and
(b) prior to solubilization, the harvested inclusion bodies were washed with water until their conductivity was less than 100 μS/cm, to form an clostripain inclusion body paste.

10. The process of claim 6, wherein the clostripain inclusion bodies are solubilized in step (a) using a mechanical homogenizer set on the lowest possible setting.

11. A process for purifying and concentrating a solution containing activated clostripain which has been obtained by (i) solubilizing in a urea solution clostripain inclusion bodies which have been harvested from a fermentation mixture comprising recombinant host cells which have expressed clostripain, said urea solution having a urea concentration of 7 M or more, and (ii) thereafter activating the clostripain which has been solubilized in the urea solution by diluting that solution to a final concentration of no more than about 3 M urea in a metal ion containing activation buffer, comprising:
(a) fractionating the clostripain in the activation buffer over an equilibrated anion exchange resin to form a purified clostripain solution ; and
(b) concentrating the purified clostripain solution by diafiltration.

12. The process of claims 11, wherein diafiltration is achieved using a 10K regenerated cellulose membrane.

13. The process of claim 11, wherein the DEAE-Sepharose resin is equilibrated to a pH of between about 7 to 9 with buffer.

14. The process of claim 11, wherein sodium chloride is added to the solution containing clostripain to a concentration of 0.1 M to 0.15 M prior to fractionation.

15. The process of claim 11, wherein the solution of activated clostripain was obtained by (i) solubilizing in a urea mixture clostripain inclusion bodies which have been harvested from a fermentation mixture comprising recombinant *E. coli* host cells which have expressed clostripain, said urea mixture having a urea concentration of 7 M or more and (ii) thereafter activating the clostripain by dilution to a final concentration of no more than about 3M urea in an activation buffer containing a calcium salt and a reducing agent.

16. A process for producing activated clostripain comprising:
(a) solubilizing in a urea solution clostripain inclusion bodies which have been harvested from a fermentation mixture comprising recombinant host cells which have expressed clostripain said urea solution having a urea concentration of 7 M or more;
(b) activating the clostripain which has been solubilized in the solution of step (a) by diluting that mixture to a final concentration to less than 3 M urea in a calcium-containing urea dilution solution;
(c) fractionating the clostripain in the urea dilution solution over an equilibrated anion exchange resin to form a purified clostripain solution ; and
(d) concentrating the purified clostripain solution by diafiltration, using a urea-containing buffer.

17. The process of claim 16, wherein the urea dilution reaction mixture contains a calcium-containing activation buffer and a salt.

18. A process for producing activated clostripain comprising:
(a) solubilizing in a urea solution clostripain inclusion bodies which have been harvested from a fermentation mixture of recombinant host cells which have expressed clostripain, said urea solution having a urea concentration of 7 M or more to produce solublized colstripain; and
(b) activating the solubilized clostripain by dilution of the urea solution to a final concentration of less than 3 M urea.

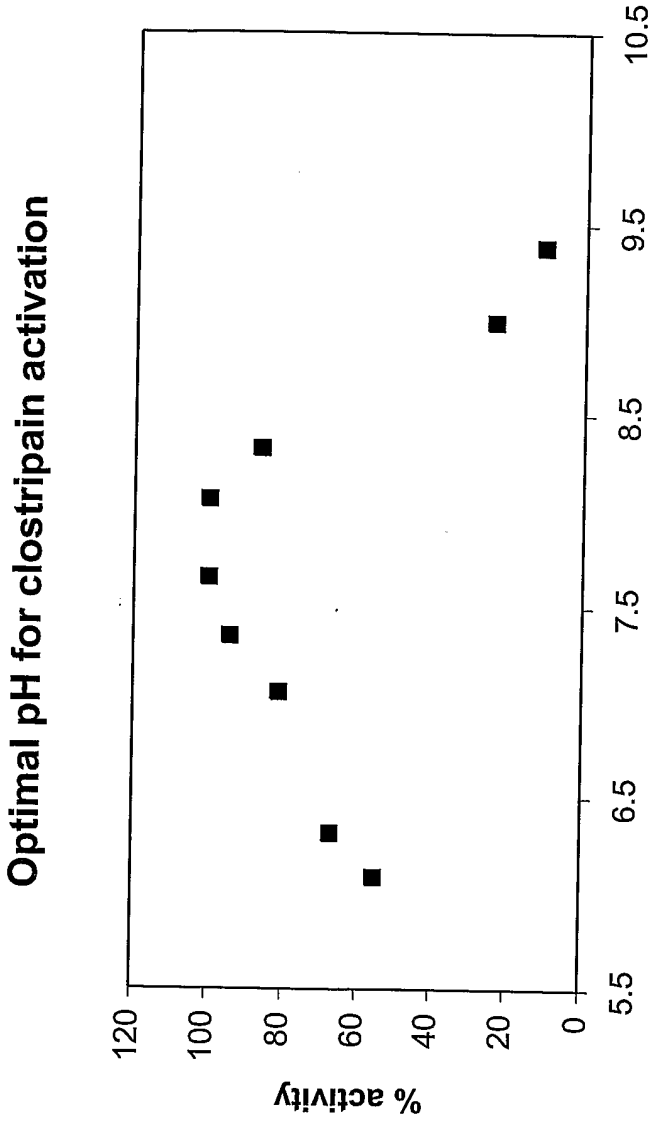
Optimal pH for clostripain activation
% activity
120
100
80
60
40
20
0
5.5
6.5
7.5
8.5
9.5
10.5
pH

Fig. 1

```
    |Pre sequence ->
   ATG TTA AGA AGA AAA GTA TCA ACA CTA TTA ATG ACA GCT TTG ATA ACT ACT TCA
 1  M   L   R   R   K   V   S   T   L   L   M   T   A   L   I   T   T   S
                                    <-| |Pro sequence ->
   TTT TTA AAT TCC AAA CCC GTA TAT GCA AAT CCA GTA ACT AAA TCC AAG GAT AAT
19  F   L   N   S   K   P   V   Y   A   N   P   V   T   K   S   K   D   N
                                                <-| |Light- chain ->
   AAC TTA AAA GAA GTA CAA CAA GTT ACA AGC AAG AGT AAT AAA AAC AAA AAT CAA
37  N   L   K   E   V   Q   Q   V   T   S   K   S   N   K   N   K   N   Q

   AAA GTA ACT ATT ATG TAC TAT TGC GAC GCA GAT AAT AAC TTG GAA GGA AGT CTA
55  K   V   T   I   M   Y   Y   C   D   A   D   N   N   L   E   G   S   L
           EcoRV
   TTA AAT GAT ATC GAG GAA ATG AAA ACA GGA TAT AAG GAT AGT CCT AAT TTA AAT
73  L   N   D   I   E   E   M   K   T   G   Y   K   D   S   P   N   L   N

   TTA ATT GCT CTT GTA GAC AGA TCC CCA AGA TAT AGC AGT GAC GAA AAA GTT TTA
91  L   I   A   L   V   D   R   S   P   R   Y   S   S   D   E   K   V   L

   GGT GAA GAT TTT AGT GAT ACA CGT CTT TAT AAG ATT GAA CAC AAT AAG GCA AAT
109 G   E   D   F   S   D   T   R   L   Y   K   I   E   H   N   K   A   N

   AGA TTA GAC GGT AAA AAT GAA TTT CCA GAA ATA AGT ACT ACT AGT AAA TAT GAA
127 R   L   D   G   K   N   E   F   P   E   I   S   T   T   S   K   Y   E
                   BamHI
   GCT AAC ATG GGG GAT CCT GAA GTT CTT AAA AAA TTT ATT GAT TAT TGT AAA TCT
145 A   N   M   G   D   P   E   V   L   K   K   F   I   D   Y   C   K   S

   AAT TAT GAG GCT GAT AAA TAT GTG CTT ATA ATG GCT AAT CAT GGT GGT GGT GCA
163 N   Y   E   A   D   K   Y   V   L   I   M   A   N   H   G   G   G   A
```

Fig. 2A

```
     |  |Linker ->                                  <-|  |Heavy chain ->
    AGG GAA AAA TCA AAT CCA AGA TTA AAT AGA GCA ATT TGC TGG GAT GAT AGT AAC
181  R   E   K   S   N   P   R   L   N   R   A   I   C   W   D   D   S   N

    CTT GAT AAA AAT GGT GAA GCA GAC TGC CTT TAT ATG GGT GAA ATT TCA GAT CAT
199  L   D   K   N   G   E   A   D   C   L   Y   M   G   E   I   S   D   H

    TTA ACA GAA AAA CAA TCA GTT GAT TTA CTT GCC TTT GAT GCG TGC CTT ATG GGA
217  L   T   E   K   Q   S   V   D   L   L   A   F   D   A   C   L   M   G
      PstI
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    ACT TTA GTT GCT TCA AGC CCA GTA GTT TGG GGT CCT GGA TTC AAA TAT GAT AAG
253  T   L   V   A   S   S   P   V   V   W   G   P   G   F   K   Y   D   K

    ATT TTC GAT AGG ATA AAA GCT GGT GGA GGA ACT AAT AAT GAG GAT GAT TTA ACT
271  I   F   D   R   I   K   A   G   G   G   T   N   N   E   D   D   L   T

    TTA GGT GGT AAA GAA CAA AAC TTT GAT CCT GCA ACC ATT ACC AAT GAG CAA TTA
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    GGT GCA TTA TTT GTA GAA GAG CAA AGA GAC TCA ACA CAT GCC AAT GGT CGC TAT
307  G   A   L   F   V   E   E   Q   R   D   S   T   H   A   N   G   R   Y
                HindIII
    GAT CAA CAC TTA AGC TTT TAT GAT TTA AAG AAA GCT GAA TCA GTA AAA AGA GCC
325  D   Q   H   L   S   F   Y   D   L   K   K   A   E   S   V   K   R   A

    ATA GAT AAT TTA GCT GTT AAT CTA AGT AAT GAA AAC AAA AAA TCT GAA ATT GAA
343  I   D   N   L   A   V   N   L   S   N   E   N   K   K   S   E   I   E
```

Fig. 2B

```
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361  K   L   R   G   S   G   I   H   T   D   L   M   H   Y   F   D   E   Y

    TCT GAA GGA GAA TGG GTT GAA TAT CCT TAT TTT GAC GTG TAT GAT TTA TGT GAA
379  S   E   G   E   W   V   E   Y   P   Y   F   D   V   Y   D   L   C   E

    AAA ATA AAT AAA AGT GAA AAT TTT AGT AGT AAA ACT AAA GAT TTA GCT TCA AAT
397  K   I   N   K   S   E   N   F   S   S   K   T   K   D   L   A   S   N

    GCT ATG AAT AAA TTA AAT GAA ATG ATA GTT TAT TCT TTT GGA GAC CCT AGT AAT
415  A   M   N   K   L   N   E   M   I   V   Y   S   F   G   D   P   S   N

    AAT TTT AAA GAA GGA AAA AAT GGA TTG AGT ATA TTC TTA CCT AAT GGA GAT AAA
433  N   F   K   E   G   K   N   G   L   S   I   F   L   P   N   G   D   K

    AAA TAT TCA ACT TAT TAT ACA TCA ACC AAG ATA CCT CAT TGG ACT ATG CAA AGT
451  K   Y   S   T   Y   Y   T   S   T   K   I   P   H   W   T   M   Q   S

    TGG TAT AAT TCA ATA GAT ACA GTT AAA TAT GGA TTG AAT CCT TAC GGA AAA TTA
469  W   Y   N   S   I   D   T   V   K   Y   G   L   N   P   Y   G   K   L

    AGT TGG TGT AAA GAT GGA CAA GAT CCT GAA ATA AAT AAA GTT GGA AAT TGG TTT
487  S   W   C   K   D   G   Q   D   P   E   I   N   K   V   G   N   W   F
            XbaI
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505  E   L   L   D   S   W   F   D   K   T   N   D   V   T   G   G   V   N
                <-|       XhoI
    CAT TAC CAA TGG TAA CTC GAG 3'
523  H   Y   Q   W   *
```

Fig.2C

```
                           |T7tag ->                  <-| |Light- chain ->
                           ATG GCT AGC ATG ACT GGT GGA CAG AAC AAA AAT CAA
                            M   A   S   M   T   G   G   Q   N   K   N   Q
                                                            51
    AAA GTA ACT ATT ATG TAC TAT TGC GAC GCA GAT AAT AAC TTG GAA GGA AGT CTA
 55  K   V   T   I   M   Y   Y   C   D   A   D   N   N   L   E   G   S   L
            EcoRV
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    TTA ATT GCT CTT GTA GAC AGA TCC CCA AGA TAT AGC AGT GAC GAA AAA GTT TTA
 91  L   I   A   L   V   D   R   S   P   R   Y   S   S   D   E   K   V   L

    GGT GAA GAT TTT AGT GAT ACA CGT CTT TAT AAG ATT GAA CAC AAT AAG GCA AAT
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                    BamHI
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145  A   N   M   G   D   P   E   V   L   K   K   F   I   D   Y   C   K   S

    AAT TAT GAG GCT GAT AAA TAT GTG CTT ATA ATG GCT AAT CAT GGT GGT GGT GCA
163  N   Y   E   A   D   K   Y   V   L   I   M   A   N   H   G   G   G   A
      |  |Linker ->                       <-|  |Heavy chain ->
    AGG GAA AAA TCA AAT CCA AGA TTA AAT AGA GCA ATT TGC TGG GAT GAT AGT AAC
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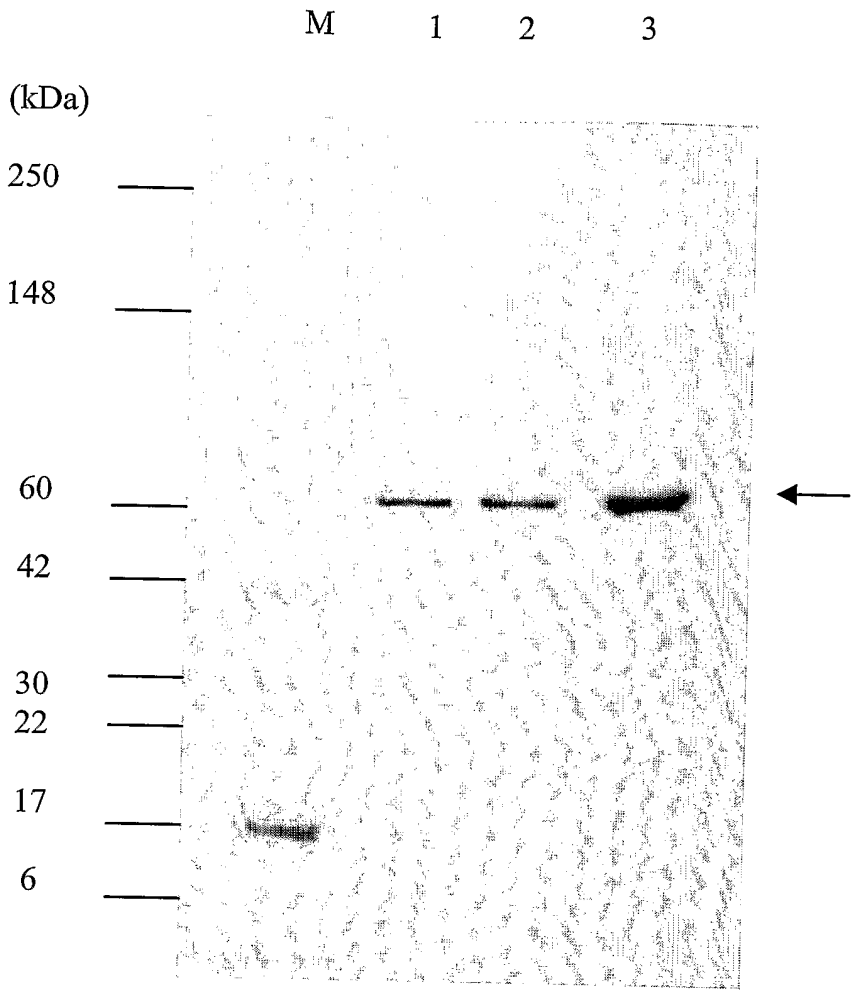
Fig. 3



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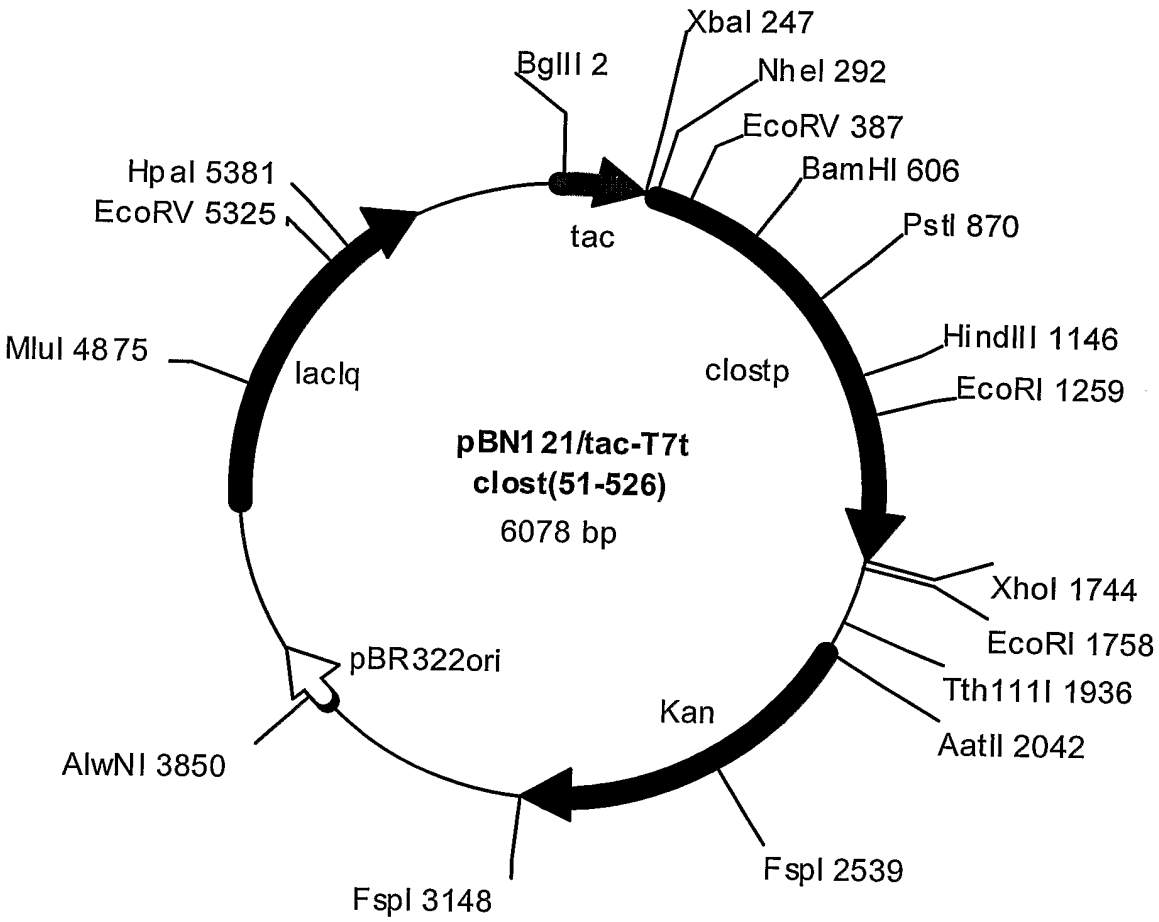
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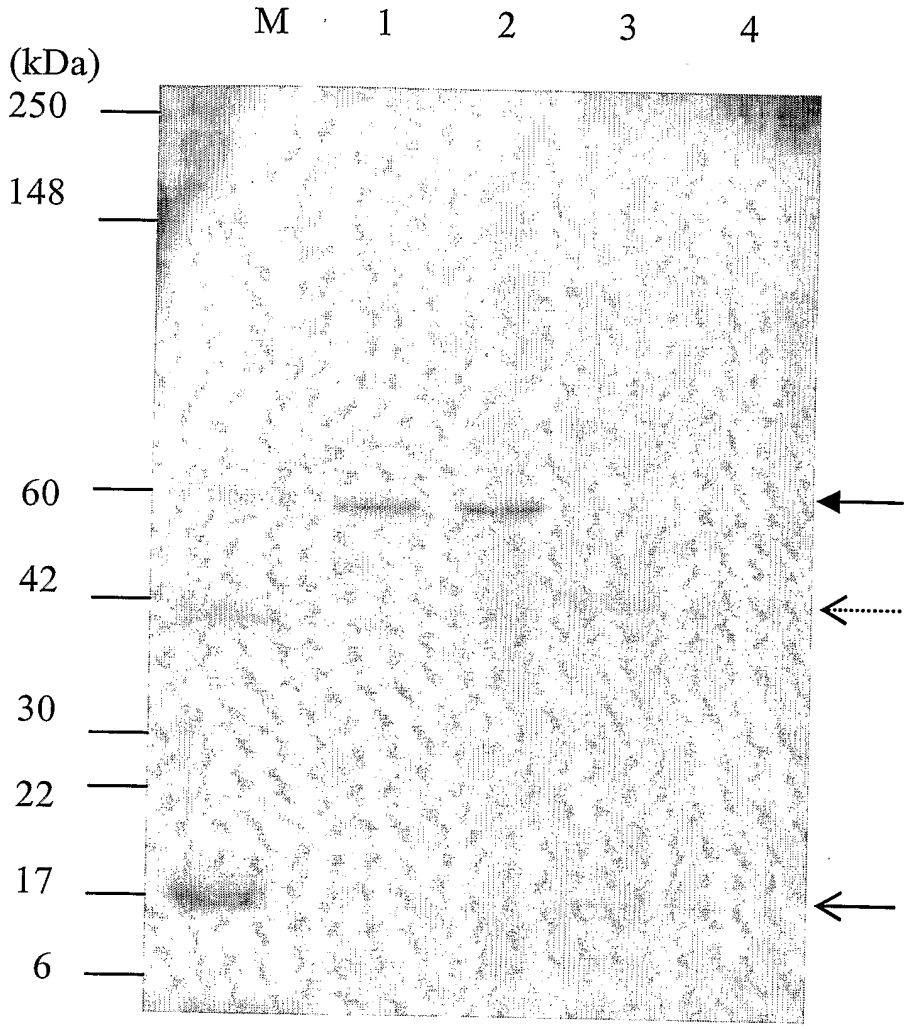
Fig. 5



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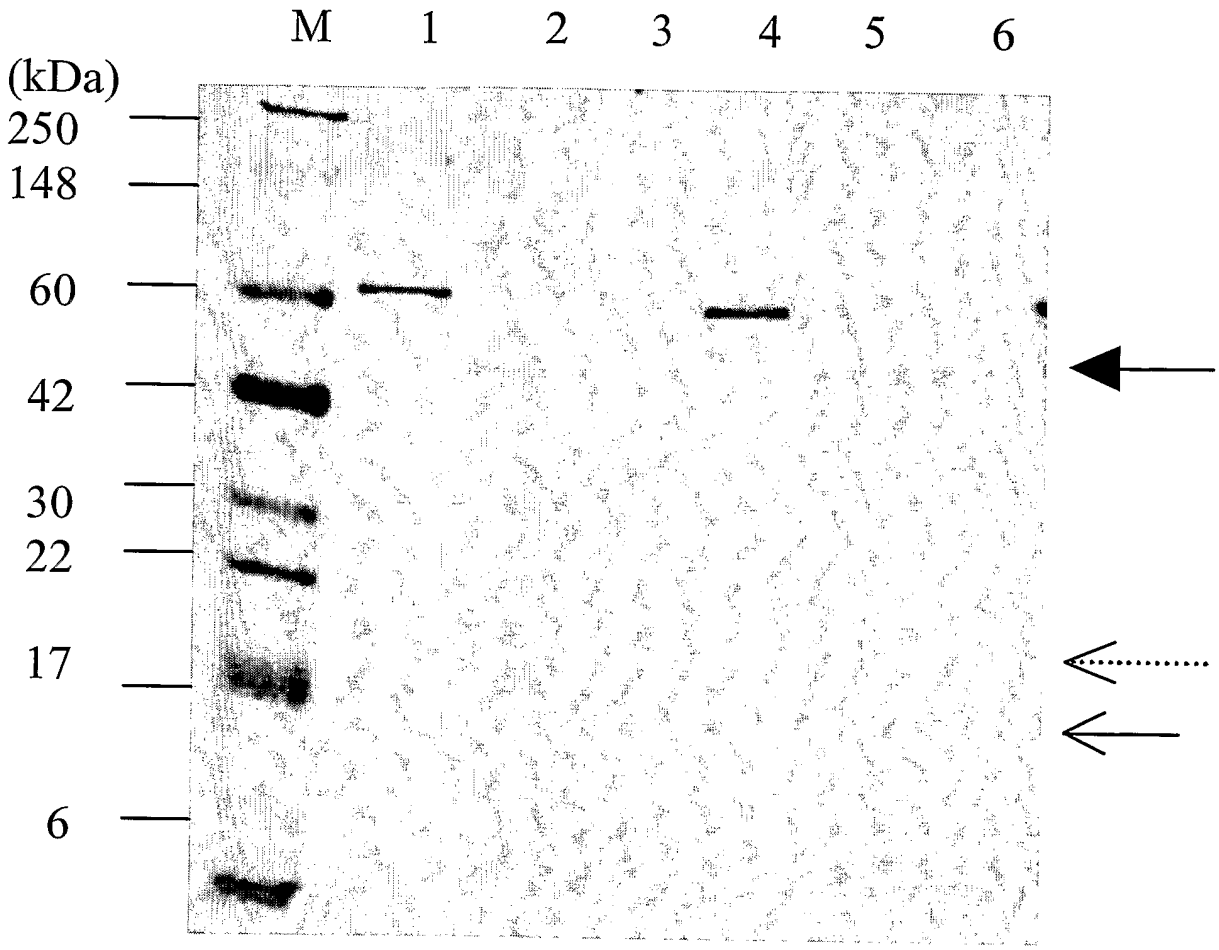

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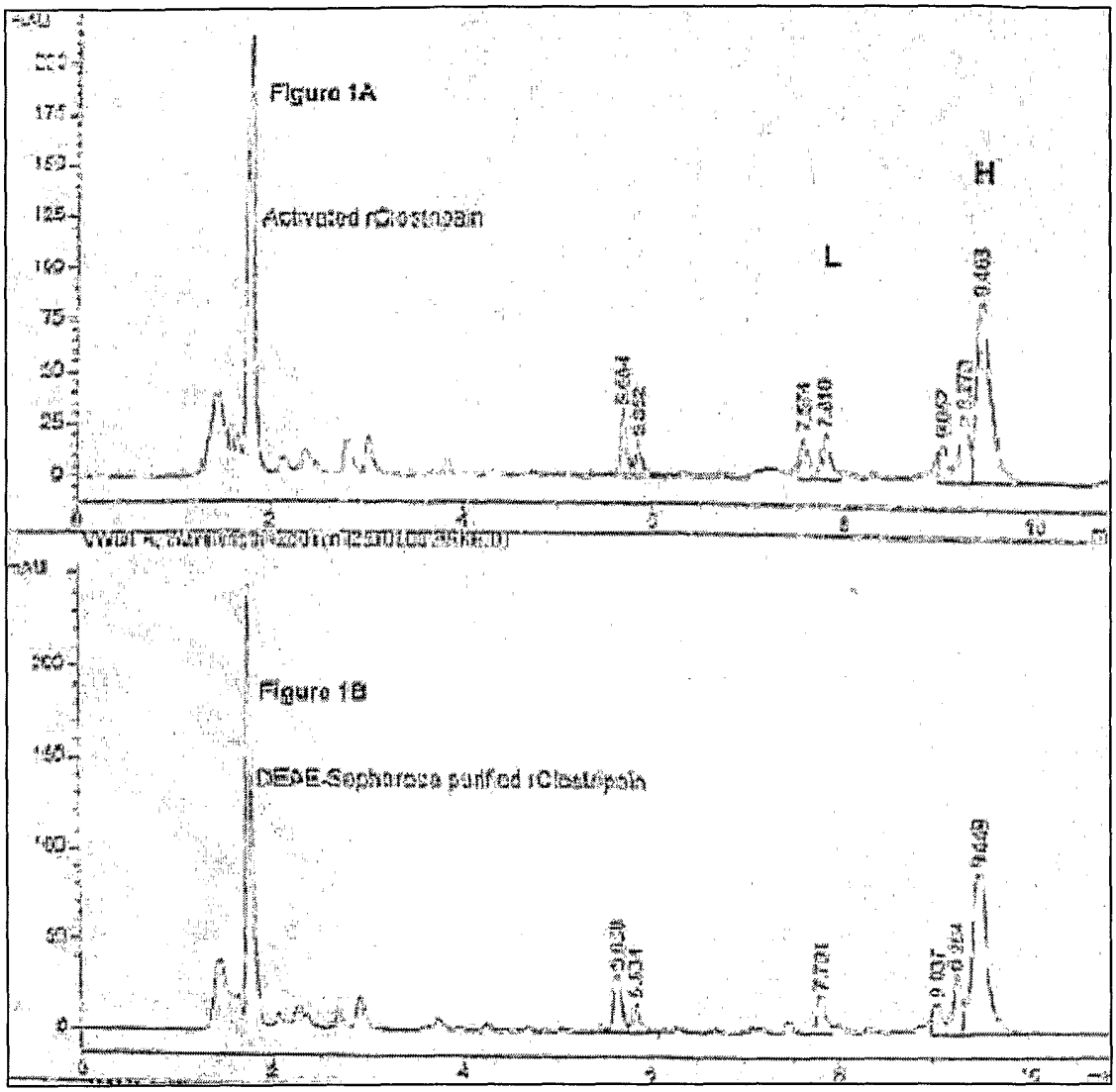
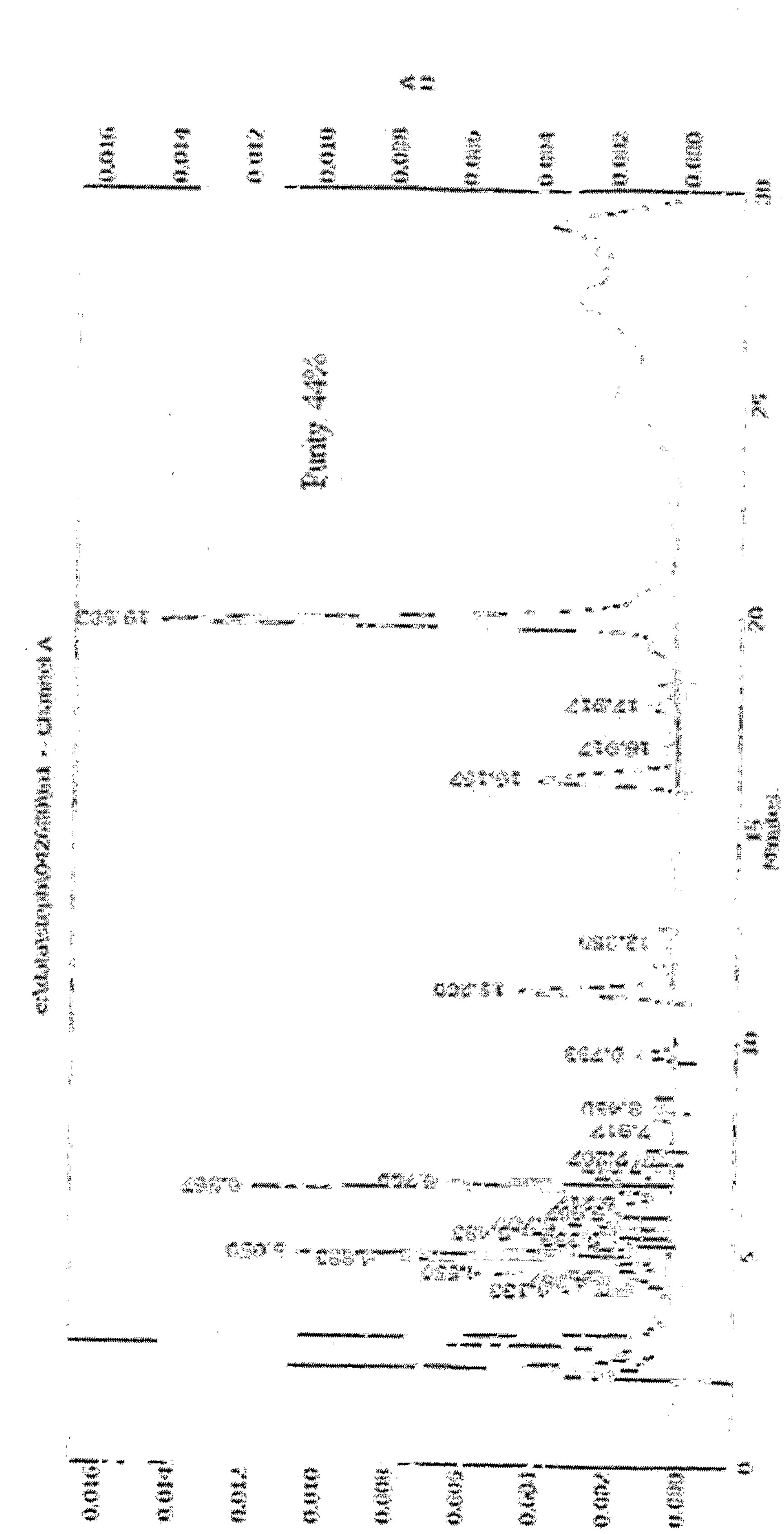


Figure 1A
Activated rClostripain
H
L
Figure 1B
DEAE-Sepharose purified rClostripain

Fig. 8



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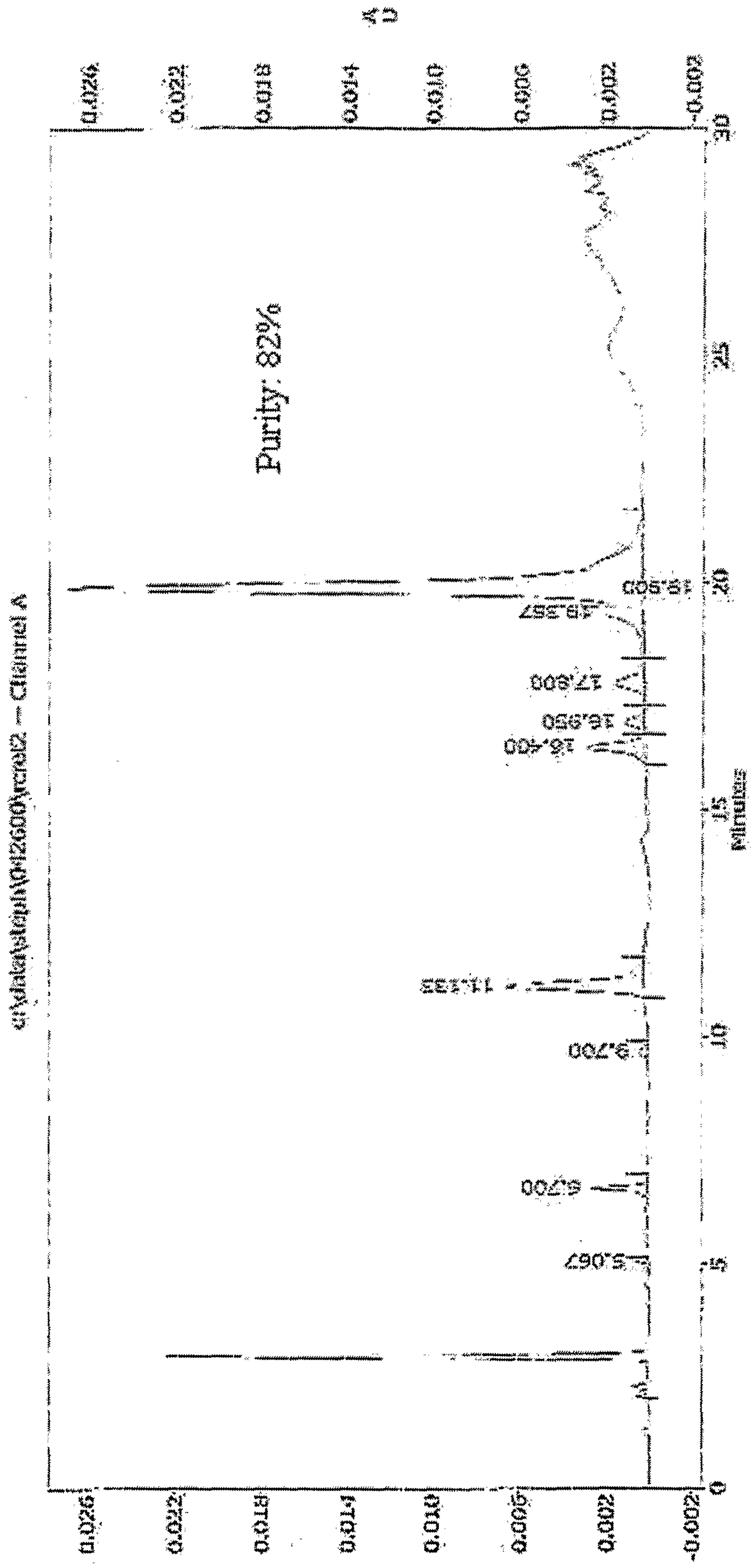
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Fig.10

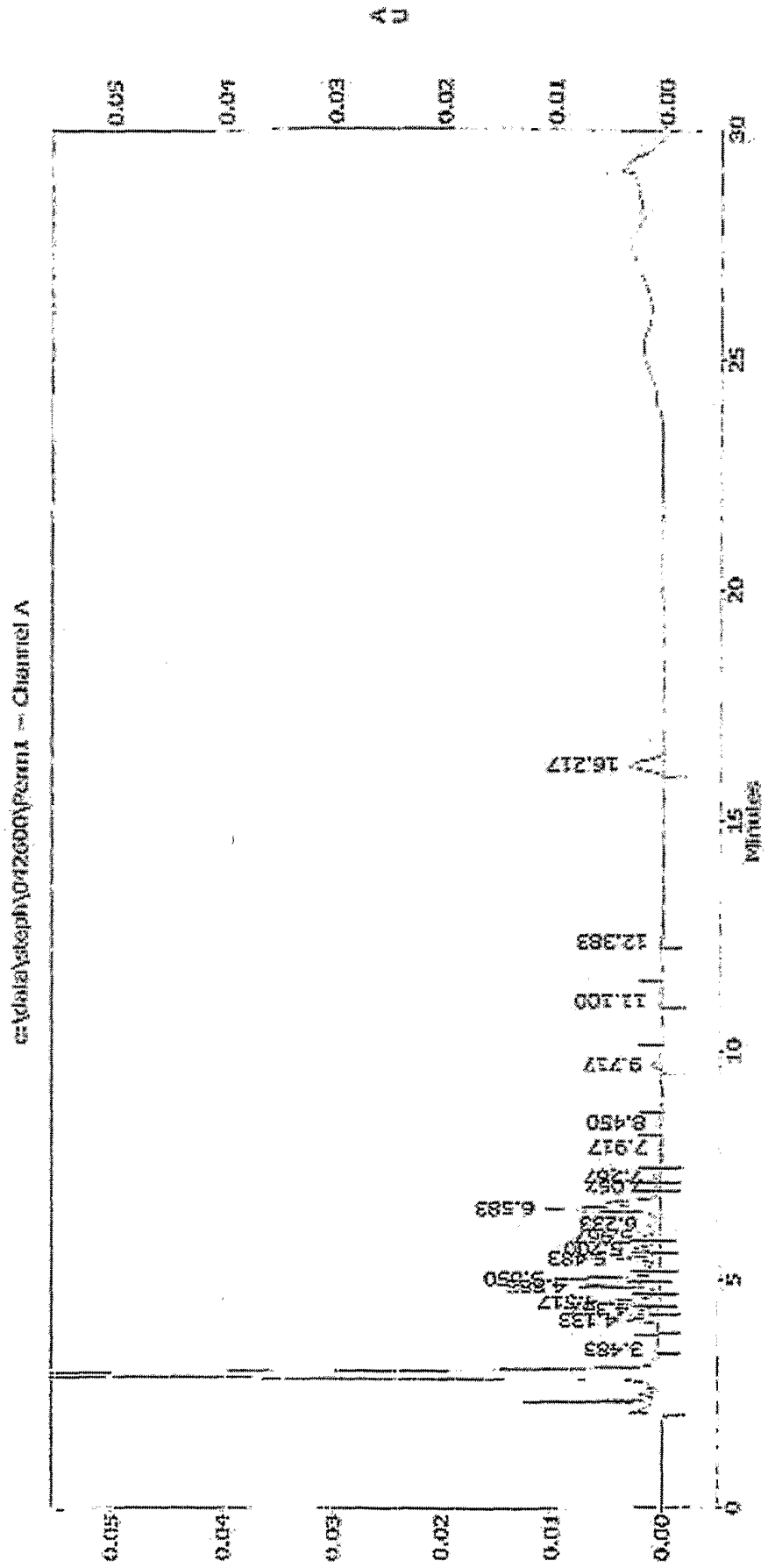
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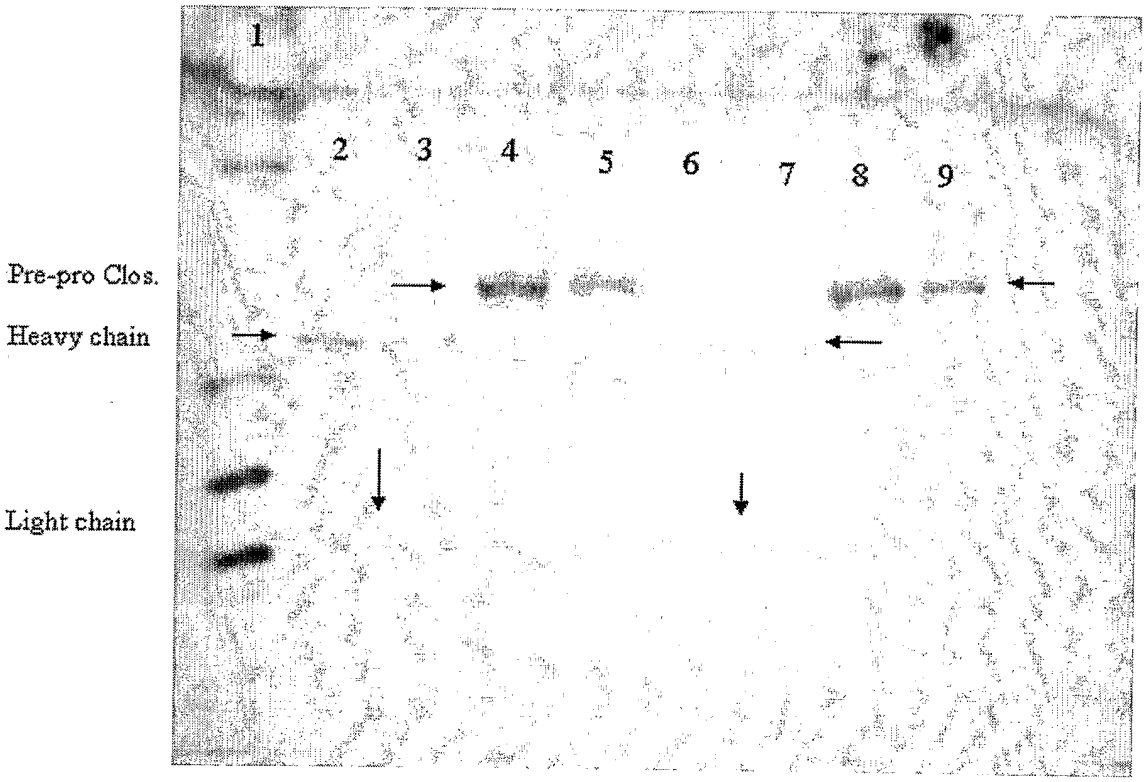
Fig. 11



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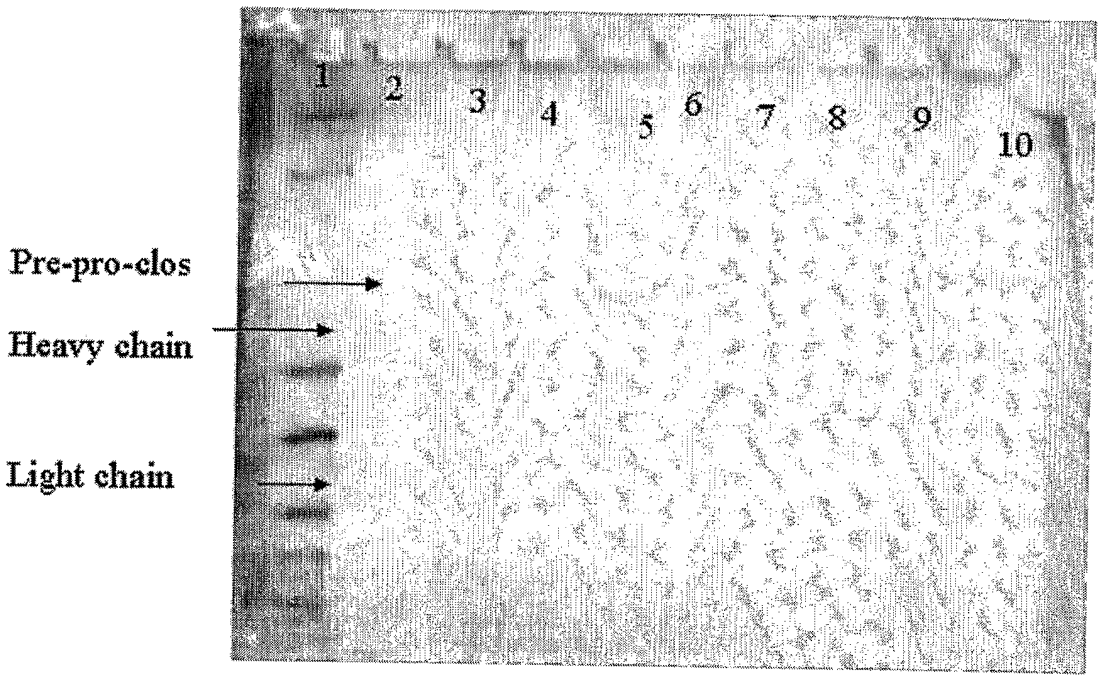

Fig. 13

SEQUENCE LISTING

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Merrifield, Edwin H.
Strydom, Daniel
Xia, Yuannan
Wagner, Fred W.
Holmquist, Barton

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65					70					75					80
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Asp Gly Lys Asn Glu Phe Pro Glu Ile Ser Thr Thr Ser Lys Tyr Glu
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Lys Ser Asn Tyr Glu Ala Asp Lys Tyr Val Leu Ile Met Ala Asn His
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Val Leu Gly Glu Asp Phe Ser Asp Thr Arg Leu Tyr Lys Ile Glu His
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Thr Thr Ser Lys Tyr Glu Ala Asn Met Gly Asp Pro Glu Val Leu Lys
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