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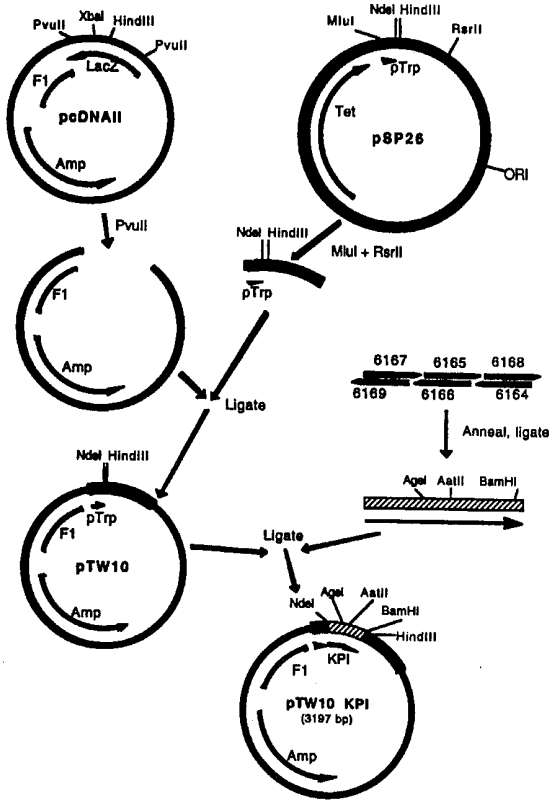
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(57) Abstract

Analogues of the Kunitz Protease Inhibitor (KPI) domain of amyloid precursor protein bind to an inhibit activity of serine proteases, including kallikrein, plasmin and coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa. Pharmaceutical compositions containing the KPI analogs, along with methods for using such compositions, are useful for ameliorating and treating clinical conditions associated with increased serine protease activity, such as blood loss related to cardiopulmonary bypass surgery. Nucleic acid sequences encoding these analogs and systems for expression of the peptides of the invention are provided.



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PROTEASE INHIBITOR PEPTIDES

Background of the Invention

The plasma, or serine, proteases of the blood contact system are known to be activated by interaction with negatively charged surfaces. For example, tissue injury during surgery exposes the vascular basement membrane, causing interaction of the blood with collagen, which is negatively charged at physiological pH. This induces a cascade of proteolytic events, leading to production of plasmin, a fibrinolytic protease, and consequent blood loss.

Perioperative blood loss of this type can be particularly severe during cardiopulmonary bypass (CPB) surgery, in which the patient's blood flow is diverted to an artificial heart-lung machine. CPB is an essential component of a number of life-saving surgical procedures. For example, in the United States, it is estimated that 300,000 patients every year undergo coronary artery bypass grafts involving the use of CPB.

Although necessary and generally safe, CPB is associated with a significant rate of morbidity, some of which may be attributed to a "whole body inflammatory response" caused by activation of plasma protease systems and blood cells through interactions with the artificial surfaces of the heart-lung machine (Butler *et al.*, *Ann. Thorac. Surg.* 55:552 (1993); Edmunds *et al.*, *J. Card. Surg.* 8:404 (1993)). For example, during extracorporeal circulation, exposure of blood to negatively charged surfaces of the artificial bypass circuit, *e.g.*, plastic surfaces in the heart-lung machine, results in direct activation of plasma factor XII.

Factor XII is a single-chain 80 kDa protein that circulates in plasma as an inactive zymogen. Contact with negatively charged nonendothelial surfaces, like those of the bypass circuit, causes surface-bound factor XII to be autoactivated to the active serine protease factor XIIa. *See* Colman, *Agents Actions Suppl.* 42:125 (1993). Surface-activated factor XIIa then processes prekallikrein (PK) to active kallikrein, which in turn cleaves more XIIa from XII in a reciprocal activation reaction that results in a rapid amplification of the contact pathway. Factor XIIa can also activate the first component of complement C1, leading to production of the anaphylatoxin C5a through the classical complement pathway.

The CPB-induced inflammatory response includes changes in capillary permeability and interstitial fluid accumulation. Cleavage of high molecular weight

kininogen (HK) by activated kallikrein generates the potent vasodilator bradykinin, which is thought to be responsible for increasing vascular permeability, resulting in edema, especially in the lung. The lung is particularly susceptible to damage associated with CPB, with some patients exhibiting what has been called "pump lung syndrome" following bypass, a condition indistinguishable from adult respiratory distress. *See* Johnson *et al.*, *J. Thorac. Cardiovasc. Surg.* 107:1193 (1994).

Post-CPB pulmonary injury includes tissue damage thought to be mediated by neutrophil sequestration and activation in the microvasculature of the lung. Butler *et al.*, *supra*; Johnson, *et al.*, *supra*. Activated factor XII can itself stimulate neutrophil aggregation. Factor XIIa-generated kallikrein, and complement protein C5a generated by Factor XIIa activation of the complement cascade, both induce neutrophil chemotaxis, aggregation and degranulation. *See* Edmunds *et al.*, *supra*. Activated neutrophils may damage tissue through release of oxygen-derived free radicals, proteolytic enzymes such as elastase, and metabolites of arachidonic acid. Release of neutrophil products in the lung can cause changes in vascular tone, endothelial injury and loss of vascular integrity.

Intrinsic inhibition of the contact system occurs through inhibition of activated XIIa by C1-inhibitor (C1-INH). *See* Colman, *supra*. During CPB, massive activation of plasma proteases and consumption of inhibitors overwhelm this natural inhibitory mechanism. A potential therapeutic strategy for reducing post-bypass pulmonary injury mediated by neutrophil activation would, therefore, be to block the formation and activity of the neutrophil agonists kallikrein, factor XIIa, and C5a by inhibition of proteolytic activation of the contact system.

Protease inhibitor therapy, which partially attenuates the contact system, is currently employed clinically in CPB. Aprotinin, also known as basic pancreatic protease inhibitor (BPPI), is a small, basic, 58 amino acid polypeptide isolated from bovine lung. It is a broad-spectrum serine protease inhibitor of the Kunitz type, and was first used during bypass in an attempt to reduce the inflammatory response to CPB. *See* Butler *et al.*, *supra*. Aprotinin treatment results in a significant reduction in blood loss following bypass, but does not appear to significantly reduce neutrophil activation. Additionally, since aprotinin is of bovine origin, there is concern that repeated administration to patients could lead to the development of an immune response to aprotinin in the patients, precluding its further use.

The proteases inhibited by aprotinin during CPB appear to include plasma kallikrein and plasmin. *See, e.g.*, Scott, *et al.*, *Blood* 69:1431 (1987). Aprotinin is an inhibitor of plasmin (K_i of 0.23nM), and the observed reduction in blood loss may be due to inhibition of fibrinolysis through the blocking of plasmin action. Although aprotinin inhibits plasma kallikrein (K_i of 20nM), it does not inhibit activated factor XII, and consequently only partially blocks the contact system during CPB.

Another attractive protease target for use of protease inhibitors, such as those of the present invention, is factor XIIa, situated at the very first step of contact activation. By inhibiting the proteolytic activity of factor XIIa, kallikrein production would be prevented, blocking amplification of the contact system, neutrophil activation and bradykinin release. Inhibition of XIIa would also prevent complement activation and production of C5a. More complete inhibition of the contact system during CPB could, therefore, be achieved through the use of a better XIIa inhibitor.

Protein inhibitors of factor XIIa are known. For example, active site mutants of α_1-antitrypsin that inhibit factor XIIa have been shown to inhibit contact activation in human plasma. *See* Patston *et al.*, *J. Biol. Chem.* 265:10786 (1990). The large size and complexity (greater than 400 amino acid residues) of these proteins present a significant challenge for recombinant protein production, since large doses will almost certainly be required during CPB. For example, although it is a potent inhibitor of both kallikrein and plasmin, nearly 1 gram of aprotinin must be infused into a patient to inhibit the massive activation of the kallikrein-kinin and fibrinolytic systems during CPB.

The use of smaller, more potent XIIa inhibitors such as the corn and pumpkin trypsin inhibitors (Wen, *et al.*, *Protein Exp. & Purif.* 4:215 (1993); Pedersen, *et al.*, *J. Mol. Biol.* 236:385 (1994)) could be more cost-effective than the large α_1-antitrypsins, but the infusion of high doses of these non-mammalian inhibitors could result in immunologic reactions in patients undergoing repeat bypass operations. The ideal protein XIIa inhibitor is, therefore, preferably small, potent, and of human sequence origin.

One candidate for an inhibitor of human origin is found in circulating isoforms of the human amyloid β-protein precursor (APPI), also known as protease nexin-2. APPI contains a Kunitz serine protease inhibitor domain known as KPI (Kunitz Protease Inhibitor). *See* Ponte *et al.*, *Nature*, 331:525 (1988); Tanzi *et al.*, *Nature* 331:528 (1988); Johnstone *et al.*, *Biochem. Biophys. Res. Commun.* 163:1248 (1989); Oltersdorf

et al., *Nature* 341:144 (1989). Human KPI shares about 45% amino acid sequence identity with aprotinin. The isolated KPI domain has been prepared by recombinant expression in a variety of systems, and has been shown to be an active serine protease inhibitor. *See*, for example, Sinha, *et al.*, *J. Biol. Chem.* 265:8983 (1990). The measured *in vitro* K_i of KPI against plasma kallikrein is 45nM, compared to 20nM for aprotinin.

Aprotinin, KPI, and other Kunitz-type serine protease inhibitors have been engineered by site-directed mutagenesis to improve inhibitory activity or specificity. Thus, substitution of Lys^{15} of aprotinin with arginine resulted in an inhibitor with a K_i of 0.32nM toward plasma kallikrein, a 100-fold improvement over natural aprotinin. *See* PCT application No. 89/10374. *See also* Norris *et al.*, *Biol. Chem. Hoppe Seyler* 371:3742 (1990). Alternatively, substitution of position 15 of aprotinin with valine or substitution of position 13 of KPI with valine resulted in elastase inhibitors with K_is in the 100 pM range, although neither native aprotinin nor native KPI significantly inhibits elastase. *See* Wenzel *et al.*, in: *Chemistry of Peptides and Proteins, Vol. 3*, (Walter de Gruyter, Berlin, New York, 1986); Sinha *et al.*, *supra*. Methods for substituting residues 13, 15, 37, and 50 of KPI are shown in general terms in European Patent Application No. 0 393 431, but no specific sequences are disclosed, and no protease inhibition data are given.

Phage display methods have been recently used for preparing and screening derivatives of Kunitz-type protease inhibitors. *See* PCT Application No. 92/15605, which describes specific sequences for 34 derivatives of aprotinin, some of which were reportedly active as elastase and cathepsin inhibitors. The amino acid substitutions in the derivatives were distributed throughout almost all positions of the aprotinin molecule.

Phage display methods have also been used to generate KPI variants that inhibit factor VIIa and kallikrein. *See* Dennis *et al.*, *J. Biol. Chem.* 269:22129 and 269:22137 (1994). The residues that could be varied in the phage display selection process were limited to positions 9-11, 13-17, 32, 36 and 37, and several of those residues were also held constant for each selection experiment. One of those variants was said to have a K_i of 1.2nM for kallikrein, and had substitutions at positions 9 (ThrPro), 13 (ArgLys), 15 (MetLeu), and 37 (GlyTyr). None of the inhibitors was tested for the ability to inhibit factor XIIa. PCT application WO 96/39515 used phage display methods to vary the residues at positions 11-19 and 34. Certain of those variants were tested for inhibition of kallikrein; factors Xia, Xa, and VIIa; thrombin; plasmin; and activated protein C. PCT

application WO 96/35788 used phage display methods to vary the residues at positions 9, 11, 13-18, 32, and 37-40. Certain of those variants were tested for inhibition of kallikrein, plasmin, and factors Xa, Xia, and XIIa.

It is apparent, therefore, that new protease inhibitors that can bind to and inhibit the activity of serine proteases are greatly desirable. In particular it is highly desirable to prepare peptides, based on human peptide sequences, that can inhibit selected serine proteases such as kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. It is also highly desirable to prepare novel protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example by reducing pulmonary damage or blood loss during CPB. In addition, it is highly desirable to prepare such novel protease inhibitors with high expression levels, as well as with high yields.

Summary of the Invention

The present invention relates to peptides that can bind to and preferably exhibit inhibition of the activity of serine proteases. Those peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. Particularly, the novel peptides of the present invention preferably exhibit a more potent and specific (*i.e.*, greater) inhibitory effect toward serine proteases of interest in comparison to known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (*e.g.*, kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors.

The novel peptides of the present invention preferably comprise substituting the tyrosine residue at position 48. Such substituted peptides may exhibit an increased level of recombinant expression in comparison to the expression levels of serine proteases that

do not have that substitution. The effect of this substitution may be manifested not only on the substituted KPI peptides of the present invention, but on wild-type KPI as well. Also, the peptides of the present invention that comprise the N-terminal sequence Glu-Val-Val-Arg (residues –4 to –1) may also preferably exhibit increased yields via a substitution of that N-terminal sequence to Asp-Val-Val-Arg.

In achieving the inhibition of serine protease activity, the invention provides protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example, by reducing pulmonary damage or blood loss during CPB.

The present invention relates to protease inhibitors comprising the following sequences: X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^{13}-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile, wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, and Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Pro and Ala; X^4 is selected from Arg, Ala, Leu, Gly, and Met; X^5 is selected from Ile, His, Leu, Lys, Ala, and Phe; X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, and Glu; X^7 is selected from Arg, His, and Ala; X^8 is selected from Phe, Val, Leu, and Gly; X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, and Tyr; X^{10} is selected from Ala, Arg, and Gly; X^{11} is selected from Lys, Ala, and Asn; X^{12} is selected from Ser, Ala, and Arg; X^{13} is selected from His, Gln, Ala, and Asp.

A further aspect of the present invention provides protease inhibitors wherein X^1 is Asp-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Met, X^5 is Ile, X^6 is Ser or Tyr, X^7 is His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Another aspect of the present invention provides protease inhibitors wherein X^1 is Asp-Val-Val-Arg-Glu-, X^2 is Pro, X^4 is Ala, X^5 is Ile, X^6 is Phe, X^7 is Arg, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Yet another aspect of the present invention provides protease inhibitors wherein X^2 is Thr or Val. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr. A further aspect of the present invention provides protease inhibitors wherein X^2 is Val. Another aspect of this invention provides protease inhibitors wherein X^2 is Thr or Val, and X^4 is Ala. A further aspect of the present invention provides protease inhibitors wherein X^2 is Thr or Val, and X^4 is Met. Yet another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is His. A further aspect of the present

invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Ala. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Asp. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Met, X^6 is Ser, and X^{13} is selected from His, Ala, or Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Val, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln. Another aspect of the present invention provides protease inhibitors wherein X^{13} is selected from His or Ala. Another aspect of the present invention provides protease inhibitors wherein X^{13} is selected from His or Ala. Another aspect of the present invention provides protease inhibitors wherein X^{13} is His. A further aspect of the present invention provides protease inhibitors wherein X^{13} is Ala.

A further aspect of the present invention provides an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor of the invention. Another aspect of the present invention provides an isolated DNA molecule operably linked to a regulatory sequence that controls expression of the coding sequence of the protease inhibitor in a host cell. Another aspect of the present invention provides an isolated DNA molecule operably linked to a regulatory sequence that controls expression of the coding sequence of the protease inhibitor in a host cell further comprising a DNA sequence encoding a secretory signal peptide. That secretory signal peptide may preferably comprise the signal sequence of yeast ∝-mating factor. Another aspect of the present invention provides a host cell transformed with a DNA molecule. Another aspect of the present invention provides a host cell transformed with any of the DNA molecules defined above. Such a host cell may preferably comprise *E. coli* or a yeast cell. When said host cell is a yeast cell, the yeast cell may preferably be *Saccharomyces cerevisiae*. When said host cell is a yeast cell, the yeast cell may preferably be *Pichia pastoris*.

Another aspect of this invention provides a method for producing a protease inhibitor, comprising the steps of culturing a host cell as defined above and isolating and purifying said protease inhibitor.

A further aspect of this invention provides a pharmaceutical composition, comprising a protease inhibitor together with a pharmaceutically acceptable sterile vehicle.

An additional aspect of the present invention provides a method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle. That method of treatment may preferably be used to treat the clinical condition of blood loss during surgery.

Yet another aspect of the present invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

Another aspect of the present invention provides protease inhibitors comprising the sequence: X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-Ala-Ile-Tyr-His-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^3-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile, wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr and Val; X^3 is selected from His, Gln, Ala, or Asp.

A further aspect of the present invention relates to protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu. Yet another aspect of the present invention provides for protease inhibitors wherein X^2 is Thr. An additional aspect of the present invention provides protease inhibitors wherein X^2 is Val. Yet another aspect of the present invention provides protease inhibitors wherein X^3 is His. Another aspect of the present invention provides protease inhibitors wherein X^3 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^3 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^3 is Asp. Another aspect of the present invention provides protease inhibitors wherein X^1 is Asp-Val-Val-Arg-Glu. Another

aspect of the present invention provides protease inhibitors wherein X^2 is Thr. Another aspect of the present invention provides protease inhibitors wherein X^2 is Val. Another aspect of the present invention provides protease inhibitors wherein X^3 is His. Another aspect of the present invention provides protease inhibitors wherein X^3 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^3 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^3 is Asp. Another aspect of the present invention provides protease inhibitors wherein X^1 is Glu. A further aspect of the present invention provides protease inhibitors wherein X^2 is Thr. Another aspect of the present invention provides protease inhibitors wherein X^2 is Val. Another aspect of the present invention provides protease inhibitors wherein X^3 is His. Another aspect of the present invention provides protease inhibitors wherein X^3 is Gln.

Another aspect of the present invention provides protease inhibitors wherein X^3 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^3 is Asp. Another aspect of the present invention provides protease inhibitors wherein X^1 is Asp. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr. Another aspect of the present invention provides protease inhibitors wherein X^2 is Val. Another aspect of the present invention provides protease inhibitors wherein X^3 is His. Another aspect of the present invention provides protease inhibitors wherein X^3 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^3 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^3 is Asp.

Another aspect of the present invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Met, X^5 is Ile, X^6 is Ser or Tyr, X^7 is His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr or Val. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr. Another aspect of the present invention provides protease inhibitors wherein X^2 is Val. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr or Val, and X^4 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr or Val, and X^4 is Met. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is His. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Ala.

Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Asp. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Met, X^6 is Ser, and X^{13} is selected from His, Ala, or Gln. Another aspect of the present invention provides a protease inhibitors wherein X^2 is Val, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln. Another aspect of the present invention provides protease inhibitors wherein X^{13} is selected from His or Ala. Another aspect of the present invention provides protease inhibitors wherein X^{13} is selected from His or Ala. Another aspect of the present invention provides protease inhibitors wherein X^{13} is His. Another aspect of the present invention provides protease inhibitors wherein X^{13} is Ala.

Another aspect of the present invention provides an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor. Another aspect of the present invention provides an isolated DNA molecule operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell. Another aspect of the present invention provides an isolated DNA molecule further comprising a DNA sequence encoding a secretory signal peptide. Another aspect of the present invention provides an isolated DNA molecule wherein said secretory signal peptide comprises the signal sequence of yeast ∝-mating factor. Another aspect of the present invention provides a host cell transformed with any of the DNA molecules defined above. Such a host cell may preferably comprise *E. coli* or a yeast cell. When said host cell is a yeast cell, the yeast cell may preferably be *Saccharomyces cerevisiae*. When said host cell is a yeast cell, the yeast cell may preferably be *Pichia pastoris*.

Another aspect of the present invention provides for a method for producing a protease inhibitor, comprising the steps of culturing a host cell as defined above and isolating and purifying said protease inhibitor.

A further aspect of this invention provides a pharmaceutical composition, comprising a protease inhibitor together with a pharmaceutically acceptable sterile vehicle.

An additional aspect of the present invention provides a method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective

amount of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle. That method of treatment may preferably be used to treat the clinical condition of blood loss during surgery.

Another aspect of the present invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

Yet another aspect of the present invention provides a method for increasing the expression levels of recombinant protease inhibitors comprising the step of culturing a host cell transformed with an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor. Such a host cell is *E. coli* or a yeast cell. When such a host cell is a yeast cell, the yeast cell may preferably be *Saccharomyces cerevisiae.* When such a host cell is a yeast cell, the yeast cell may preferably be *Pichia pastoris.*

Another aspect of the present invention provides a method for increasing the yield of recombinant protease inhibitors comprising the step of culturing a host cell transformed with an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1, wherein X^1 is Asp-Val-Val-Arg-Glu-, and isolating and purifying said protease inhibitor. When said host cell is a yeast cell, the yeast cell may preferably be *Saccharomyces cerevisiae.* When said host cell is a yeast cell, the yeast cell may preferably be *Pichia pastoris.*

Another aspect of the present invention provides protease inhibitors comprising the sequence: X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-X^3-Ile-X^4-X^5-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^6-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile, wherein: X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr or Val; X^3 is selected from Arg and Met; X^4 is selected from Ser and Tyr; X^5 is selected from Arg, His, or Ala; and X^6 is selected from His, Gln, Ala or Asp.

A further aspect of the present invention provides protease inhibitors comprising the sequence: X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-Leu-Phe-Lys-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Leu-Gly-Asp-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^2-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile, wherein: X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, and Glu; X^2 is selected from His, Gln, Ala, and Asp.

Yet a further aspect of the present invention provides protease inhibitors wherein X^1 is Asp-Val-Val-Arg-Glu. Another aspect of the present invention provides protease inhibitors wherein X^2 is His. Another aspect of the present invention provides protease inhibitors wherein X^2 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^2 is Asp. Another aspect of the present invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu. Yet another aspect of the present invention provides protease inhibitors wherein X^2 is His. A further aspect of the present invention provides protease inhibitors wherein X^2 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^2 is Asp.

Yet another aspect of the present invention provides protease inhibitors wherein X^1 is Asp. A further aspect of the present invention provides protease inhibitors wherein X^2 is His. Another aspect of the present invention provides protease inhibitors wherein X^2 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^2 is Asp. Yet another aspect of the present invention provides protease inhibitors wherein X^1 is Glu. Another aspect of the present invention provides protease inhibitors wherein X^2 is His. A further aspect of the present invention provides protease inhibitors wherein X^2 is Gln. Another aspect of the present invention provides protease inhibitors wherein X^2 is Ala. Another aspect of the present invention provides protease inhibitors wherein X^2 is Asp. Another aspect of the present invention provides protease inhibitors comprising the sequence: Asp-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-Leu-Phe-Lys-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Leu-Gly-Asp-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 shows the strategy for the construction of plasmid pTW10:KPI.

Figure 2 shows the sequence of the synthetic gene for KPI (1→57) fused to the bacterial *pho*A secretory signal sequence.

Figure 3 shows the strategy for construction of plasmid pKPI-61.

Figure 4 shows the 192 bp *Xba*I-*Hind*III synthetic gene fragment encoding KPI (1→57) and four amino acids from yeast ∝-mating factor.

Figure 5 shows the synthetic 201 bp *Xba*I-*Hind*III fragment encoding KPI (-4→57) in PKPI-61.

Figure 6 shows the strategy for the construction of plasmid pTW113.

Figure 7 shows plasmid pTW113, encoding the 445 bp synthetic gene for yeast ∝-factor-KPI(-4→57) fusion.

Figure 8 shows the amino acid sequence for KPI (-4→57).

Figure 9 shows the strategy for constructing plasmid pTW6165.

Figure 10 shows plasmid, pTW6165, encoding the 445 bp synthetic gene for yeast ∝-factor-KPI(-4→57; M15A, S17W) fusion.

Figure 11 shows the sequences of the annealed oligonucleotide pairs used to construct plasmids pTW6165, pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, and pTW6174.

Figure 12 shows the sequence of plasmid pTW6166 encoding the fusion of yeast ∝-factor and KPI(-4→57; M15A, S17Y).

Figure 13 shows the sequence of plasmid pTW6175 encoding the fusion of yeast ∝-factor and KPI(-4→57; M15L, S17F).

Figure 14 shows the sequence of plasmid pBG028 encoding the fusion of yeast ∝-factor and KPI(-4→57; M15L, S17Y).

Figure 15 shows the sequence of plasmid pTW6183 encoding the fusion of yeast ∝-factor and KPI(-4→57; I16H, S17F).

Figure 16 shows the sequence of plasmid pTW6184 encoding the fusion of yeast ∝-factor and KPI(-4→57; I16H, S17Y).

Figure 17 shows the sequence of plasmid pTW6185 encoding the fusion of yeast ∝-factor and KPI(-4→57; I16H, S17W).

Figure 18 shows the sequence of plasmid pTW6173 encoding the fusion of yeast ∝-factor and KPI(-4→57; M15A, I16H).

Figure 19 shows the sequence of plasmid pTW6174 encoding the fusion of yeast ∝-factor and KPI(-4→57; M15L, I16H).

Figure 20 shows the sequence of plasmid pBG022 encoding the fusion of yeast ∝-factor and KPI (-4→57; M15A, S17Y, R18H, Y48H).

Figure 21shows the sequence of plasmid pBG033 encoding the fusion of yeast ∝-factor and KPI (-4→57; T9V, M15A, R18H, Y48H).

Figure 22 shows the sequence of plasmid pBG048 encoding the fusion of yeast ∝-factor and KPI (-4→57; Y48H).

Figure 23 shows the sequence of plasmid pBG049 encoding the fusion of yeast ∝-factor and KPI (-4→57; M15A, S17Y, R18H).

Figure 24 shows the sequence of plasmid pBG050 encoding the fusion of yeast ∝-factor and KPI (-4→57; T9V, M15A, S17Y, R18H).

Figure 25 shows the sequence of the coding region for phoA signal: KPI-BG022: gIII protein contained within the phage display vector pDW1-L6-16.

Figure 26 shows the sequence of the coding region for yeast ∝-factor and KPI-P48 library contained within the P48 library.

Figure 27 shows the amino acid sequence of KPI (-4→57; M15A, S17W).

Figure 28 shows the amino acid sequence of KPI (-4→57; M15A, S17Y).

Figure 29 shows the amino acid sequence of KPI (-4→57; M15L, S17F).

Figure 30 shows the amino acid sequence of KPI (-4→57; M15L, S17Y).

Figure 31 shows the amino acid sequence of KPI (-4→57; I16H, S17F).

Figure 32 shows the amino acid sequence of KPI (-4→57; I16H, S17Y).

Figure 33 shows the amino acid sequence of KPI (-4→57; I16H, S17W).

Figure 34 shows the amino acid sequence of KPI (-4→57; M15A, S17F).

Figure 35 shows the amino acid sequence of KPI (-4→57; M15A, I16H).

Figure 36 shows the amino acid sequence of KPI (-4→57; M15L, I16H).

Figure 37 shows the amino acid sequence of KPI (-4→57; M15A, S17Y, R18H, Y48H).

Figure 38 shows the amino acid sequence of KPI (-4→57; T9V, M15A, R18H, Y48H).

Figure 39 shows the amino acid sequence of KPI (-4→57; Y48H).

Figure 40 shows the amino acid sequence of KPI (-4→57; M15A, S17Y, R18H).

Figure 41 shows the amino acid sequence of KPI (-4→57; T9V, M15A, S17Y, R18H).

Figure 42 shows the amino acid sequence of KPI-P48 library (-4→57; M15A, S17Y, R18H, Y28X) encoded by the P48 library.

Figure 43 shows the construction of plasmid pSP26:*Amp*:F1.

Figure 44 shows the construction of plasmid pgIII.

Figure 45 shows the construction of plasmid p*Pho*A:KPI:gIII.

Figure 46 shows the construction of plasmid pLG1.

Figure 47 shows the construction of plasmid pAL51.

Figure 48 shows the construction of plasmid pAL53.

Figure 49 shows the construction of plasmid pSP26:*Amp*:F1:*Pho*A:KPI:gIII.

Figure 50 shows the construction of plasmid pDW1 #14.

Figure 51 shows the construction of plasmid pBG022.

Figure 52 shows the construction of plasmid pBG048.

Figure 53 shows the construction of plasmid pBG049.

Figure 54 shows the construction of plasmid pBG050.

Figure 55 shows the construction of the P48 library.

Figure 56 shows the coding region for the fusion of *pho*A-KPI (155)-geneIII.

Figure 57 shows the construction of plasmid pDW1 14-2.

Figure 58 shows the construction of KPI Library 16-19.

Figure 59 shows the expression unit encoded by the members of KPI Library 16-19.

Figure 60 shows the *pho*A-KPI(155)-geneIII region encoded by the most frequently occurring randomized KPI region.

Figure 61 shows the construction of pDD185 KPI (-4→57; M15A, S17F).

Figure 62 shows the sequence of yeast ∝-factor fused to KPI (-4→57; M15A, S17F).

Figure 63 shows the inhibition constants (K_is) determined for purified KPI variants against the selected serine proteases kallikrein, factor Xa, and factor XIIa.

Figure 64 shows the inhibition constants (K_is) determined for KPI variants against kallikrein, plasmin, and factors Xa, XIa, and XIIa.

Figure 65 shows the post-surgical blood loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 66 shows the post-surgical hemoglobin loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 67 shows the oxygen tension in the presence and absence of KPI, before CPB, immediately after CPB, and at 60 and 180 minutes after the end of CPB.

Figure 68 summarizes the results shown in Figures 65-67.

Figure 69 shows the inhibitor constants (Kis) determined for KPI variants against kallikrein in nM and expression levels (mg/ml) of those variants.

Figure 70 shows a comparison of the survival time of rat xenografts in the presence and absence of KPI-BG022.

Figure 71 shows a comparison of damage in a rat model of TNBS (trinitrobenzene sulfonic acid) induced colitis in the presence and absence of KPI-BG022.

Figure 72 shows a comparison of the HPLC traces, after lyophilization, of KPI having the N-terminus sequence Glu-Val-Val-Arg (E-KPI) and KPI having the N-terminus sequence Asp-Val-Val-Arg (D-KPI).

Detailed Description

The present invention provides peptides that can bind to and preferably inhibit the activity of serine proteases. These inhibitory peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. The novel peptides of the present invention preferably exhibit a more potent and specific (*i.e.*, greater) inhibitory effect toward serine proteases of interest than known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as

factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (*e.g.*, kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors. In addition, the peptides of the present invention preferably comprise a substitution at position 48. Such position 48 substituted peptides may exhibit an increased level of expression in comparison to the expression levels of serine proteases that do not have that substitution. The effect of this substitution may be manifested not only on the substituted KPI peptides of the present invention, but on wild-type KPI as well. Also, the peptides of the present invention that comprise the N-terminal sequence Glu-Val-Val-Arg (residues –4 to –1) may also preferably exhibit increased yields via a substitution to Asp-Val-Val-Arg.

Peptides of the present invention may be used to reduce the tissue damage caused by activation of the proteases of the contact pathway of the blood during surgical procedures such as cardiopulmonary bypass (CPB). Inhibition of contact pathway proteases reduces the "whole body inflammatory response" that can accompany contact pathway activation, and that can lead to tissue damage, and possibly death. The peptides of the present invention may also be used in conjunction with surgical procedures to reduce activated serine protease-associated perioperative and postoperative blood loss. For instance, perioperative blood loss of this type may be particularly severe during CPB surgery. Pharmaceutical compositions comprising the peptides of the present invention may be used in conjunction with surgery such as CPB; administration of such compositions may occur preoperatively, perioperatively or postoperatively. Examples of other clinical conditions associated with increased serine protease activity for which the peptides of the present invention may be used include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of preferable uses of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The invention is based upon the novel substitution of amino acid residues in the peptide corresponding to the naturally occurring KPI protease inhibitor domain of

human amyloid β-amyloid precursor protein (APPI). These substitutions produce peptides that can bind to serine proteases and preferably exhibit an inhibition of the activity of serine proteases. The peptides also preferably exhibit a more potent and specific serine protease inhibition than known serine protease inhibitors. In accordance with the invention, peptides are provided that may exhibit a more potent and specific inhibition of one or more serine proteases of interest, *e.g.*, kallikrein, plasmin and factors Xa, XIa, XIIa, and XIIa.

The present invention also includes pharmaceutical compositions comprising an effective amount of at least one of the peptides of the invention, in combination with a pharmaceutically acceptable sterile vehicle, as described in REMINGTON'S PHARMACEUTICAL SCIENCES: DRUG RECEPTORS AND RECEPTOR THEORY, (18th ed.), Mack Publishing Co., Easton, PA (1990).

A. **Selection of sequences of KPI variants**

The sequence of KPI is shown in Table 1. Table 2 shows a comparison of this sequence with that of aprotinin, with which it shares about 45% sequence identity. The numbering convention for KPI shown in Table 1 and used hereinafter designates the first glutamic acid residue of KPI as residue 1. This corresponds to residue number 3 using the standard numbering convention for aprotinin.

The crystal structure for KPI complexed with trypsin has been determined. *See* Perona *et al.*, *J. Mol. Biol.* 230:919 (1993). The three-dimensional structure reveals two binding loops within KPI that contact the protease. The first loop extends from residue Thr^{9} to Ile^{16}, and the second loop extends from residue Phe^{32} to Gly^{37}. The two protease binding loops are joined through the disulfide bridge extending from Cys^{12} to Cys^{36}. KPI contains two other disulfide bridges, between Cys^{3} and Cys^{53}, and between Cys^{28} to Cys^{49}.

This structure was used as a guide to inform our strategy for making the amino acid residue substitutions that will be most likely to affect the protease inhibitory properties of KPI. Our examination of the structure indicated that certain amino acid residues, including residues 9, 11, 13-18, 32, and 37-40 appear to be of particular significance in determining the protease binding properties of the KPI peptides of the present invention. It was also found that certain position 48 substitutions positively affected the expression levels of the peptide by the transformed host. In a preferred

embodiment of the invention one or more of those KPI peptide residues are substituted, such substitutions preferably occurring among residues 9, 11, 13-18, 32, 37-40, and 48. In particular, those substituted peptides, including peptides comprising substitutions at position 9, substitutions of at least two of the four residues at positions 15-18 and substitutions at position 48 may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain.

Specifically, replacement of arginine at position 18 of the native KPI peptide with histidine (R18H) in combination with one or more additional substitutions at residues 9, 15 and 17 were found to exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than the native KPI peptide. In particular, the specific substitutions T9V, M15A, S17Y and M15A ,S17Y in the context of the R18H substitution exhibited such potent serine protease inhibition. *See* Figures 63, 64 and 69D.

In addition, the peptides of the present invention preferably comprise a substitution at position 48. Such position 48 substituted peptides may exhibit an increased level of expression in comparison to the expression levels of serine proteases that do not have that substitution. The effect of this substitution may be manifested not only on the substituted KPI peptides of the present invention, but on wild-type KPI as well. Also, the peptides of the present invention that comprise the N-terminal sequence Glu-Val-Val-Arg (residues –4 to –1) may also preferably exhibit increased yields via a substitution to Asp-Val-Val-Arg.

Specifically, substitutions at position 48 may exhibit an increased level of expression of KPI peptides in comparison to the expression levels of such peptides not having such a substitution. These substituted peptides exhibiting an increased level of expression also may preferably comprise one or more additional substitutions at residues 9, 11, 13-18, 32 and 37-40; in particular, such peptides may preferably comprise a substitution at positions 9 or 37 and/or substitution of at least two of the four residues at positions 15-18. Those additionally substituted peptides may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain as well as increased expression levels.

One specific embodiment of the invention is based upon a finding that an expression vector prepared to express the KPI variant M15A, S17Y, R18H underwent a spontaneous mutation at position 48 which changed the native tyrosine to histidine

(Y48H) and that this mutation conferred beneficial properties. To assess the effect of this mutation, the KPI variant M15A, S17Y, R18H (pBG049) was constructed using methods known to those skilled in the art and its expression levels compared with the KPI variant M15A, S17Y, R18H, Y48H (pBG022). As detailed *infra*, the expression level of KPI variant M15A, S17Y, R18H was increased over five-fold by replacing the native tyrosine at position 48 with histidine. *See* Figures 69A and B. Moreover, it has been determined that this Y48H substitution confers improvements in expression levels upon KPI variants as well as upon native sequence KPI.

As an additional example of the position 48 substitution effect on expression of the recombinant peptides of the present invention, and as delineated in detail *infra*, the expression level of wild-type KPI (pTW113) was increased on the average approximately five to six-fold by replacing the native tyrosine at position 48 with histidine (pBG048;Y48H), glutamine (pBG072; Y48Q) or alanine (pBG073; Y48A). *See* Figures 69B and F.

In an additional preferred embodiment of the invention, it was found that replacement of arginine at position 18 of the native KPI peptide with histidine (R18H), in combination with one or more additional substitutions at residues 9, 15 and 17, exhibited more potent and specific serine protease inhibition toward selected serine proteases of interest than the native KPI peptide. In particular, the specific substitutions T9V, M15A, S17Y and M15A; S17Y exhibited particularly potent serine protease inhibition in the context of the R18H substitution. *See* Figures 63 and 64. Additionally, the R18H substitution conferred an increased level of expression in comparison to the expression levels of the corresponding peptides lacking the position 48 substitution. To assess the effect of the position 48 substitution on these R18H substituted peptides, Library P48 was constructed for expression of KPI (M15A, S17Y, R18H) in which the amino acids exhibiting at position 48 are randomized. *See* Figure 55. The amino acid sequences of the KPI-P48 Library contained within the P48 Library are shown in Figure 26. Those substituted peptides included substituting the native tyrosine at position 48 with histidine (pBG022; 50D4, 50B6,Y48H), glutamine (50B6, 50L1, 50M1; Y48Q), alanine (50P5, 50C4; Y48A) and aspartic acid (50N1; Y48D). *See* Figures 69B, E and F.

In yet another preferred embodiment, the KPI peptides of the present invention may also comprise a substitution at its N-terminus. Specifically, such a substitution was found to alleviate the problems associated with the purification and subsequent isolation

of the expressed peptides of the present invention having a glutamic acid residue at its N-terminus. This specific substitution changes the additional N-terminal amino acids from the KPI protein sequence (Glu-Val-Val-Arg, designated residues –4 to –1) immediately proceeding the KPI domain in APPI to Asp-Val-Val-Arg. Specifically, this substitution is thought to prevent cyclization of the N-terminus glutamic acid during purification of the expressed peptides of the present invention. In a preferred embodiment of the invention, and as described *supra*, one or more additional KPI peptide residues are substituted, such substitutions preferably occurring among residues 9, 11, 13-18, 32, 37-40, and 48. In particular, those substituted peptides, including peptides comprising substitutions at position 9, substitutions of at least two of the four residues at positions 15-18 and substitutions at position 48 preferably exhibit the desired potency and specificity as well as an increased level of expression in comparison to the expression levels of other serine proteases without those specific substitutions.

In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (*e.g.*, kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors as well as an increased level of expression in comparison to the expression levels of other serine proteases without those specific substitutions. That greater potency and specificity may be manifested by the peptides of the present invention by exhibiting binding constants for serine proteases of interest that are less than the binding constants exhibited by native KPI, or other known serine protease inhibitors, for such proteases.

By way of example, and as set forth in greater detail below, the serine protease inhibitory properties of peptides of the present invention were measured for the serine proteases of interest kallikrein, plasmin and factors Xa, XIa, and XIIa. Methodologies for measuring the inhibitory properties of the KPI variants of the present invention are known to those skilled in the art, *e.g.*, by determining the inhibition constants of the variants toward serine proteases of interest, as described in Example 4, *infra*. Such studies measure the ability of the novel peptides of the present invention to bind to one or more serine proteases of interest and to preferably exhibit a greater potency and specificity for inhibiting one or more serine protease of interest than known serine protease inhibitors such as native KPI.

The ability of the peptides of the present invention to bind one or more serine proteases of interest, particularly the ability of the peptides to exhibit such greater

potency and specificity toward serine proteases of interest, manifest the clinical and therapeutic applications of such peptides. The clinical and therapeutic efficacy of the peptides of the present invention can be assayed by *in vitro* and *in vivo* methodologies known to those skilled in the art, *e.g.*, as described in Examples 5-8, *infra*.

Table 1: SEQUENCE OF KPI:

```
 1                  10                  20                  30
VREVCSEQAETGPCRAMISRWYFDVTEGKCAP

                   40                  50
FFYGGCGGNRNNFDTEEYCMAVCGSAI
```

Table 2: COMPARISON OF KPI AND APROTININ SEQUENCES:

```
          1         10        20        30        40        50
KPI:  VREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI
          | |   ||||  | | |       | |  | || ||   ||||   | ||   ||| .
BPTI: RPDFCLEPPYTGPCKARIIRYFYNAKA GL CQTFVYGGCRAKRNNFKSAEDCMRTCGGA
          1         10        20        30        40        50
```

B. Methods of producing KPI variants

The peptides of the present invention can be created by synthetic techniques or recombinant techniques which employ genomic or cDNA cloning methods.

1. Production by chemical synthesis

Peptides of the present invention can be routinely synthesized using solid phase or solution phase peptide synthesis. Methods of preparing relatively short peptides such as KPI by chemical synthesis are well known in the art. KPI variants could, for example be produced by solid-phase peptide synthesis techniques using commercially available equipment and reagents such as those available from Milligen (Bedford, MA) or Applied Biosystems-Perkin Elmer (Foster City, CA). Alternatively, segments of KPI variants could be prepared by solid-phase synthesis and linked together using segment condensation methods such as those described by Dawson *et al.*, *Science* 266:776

(1994). During chemical synthesis of the KPI variants, substitution of any amino acid can be achieved simply by replacement of the residue that is to be substituted with a different amino acid monomer.

2. Production by recombinant DNA technology

(a) Preparation of genes encoding KPI variants

In a preferred embodiment of the invention, KPI variants are produced by recombinant DNA technology. *See* PCT application WO 96/35788, hereby incorporated in its entirety. This requires the preparation of genes encoding each KPI variant that is to be made. Suitable genes can be constructed by oligonucleotide synthesis using commercially available equipment, such as that provided by Milligen and Applied Biosystems, *supra*. The genes can be prepared by synthesizing the entire coding and non-coding strands, followed by annealing the two strands. Alternatively, the genes can be prepared by ligation of smaller synthetic oligonucleotides by methods well known in the art. Genes encoding KPI variants are produced by varying the nucleotides introduced at any step of the synthesis to change the amino acid sequence encoded by the gene.

Preferably, however, KPI variants are made by site-directed mutagenesis of a gene encoding KPI. Methods of site-directed mutagenesis are well known in the art. *See*, for example, Ausubel *et al.*, (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience, 1987); PROTEIN ENGINEERING (Oxender & Fox eds., A. Liss, Inc. 1987). These methods require the availability of a gene encoding KPI or a variant thereof, which can then be mutagenized by known methods to produce the desired KPI variants. In addition, linker-scanning and polymerase chain reaction ("PCR") mediated techniques can be used for purposes of mutagenesis. *See* PCR TECHNOLOGY (Erlich ed., Stockton Press 1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, *loc. cit.*

A gene encoding KPI can be obtained by cloning the naturally occurring gene, as described for example in U.S. Patents Nos. 5,223,482 and 5,187,153, which are hereby incorporated by reference in their entireties. In particular, see columns 6-9 of U.S. Patent No. 5,187,153. *See also* PCT Application No. 93/09233. In a preferred embodiment of the invention a synthetic gene encoding KPI is produced by chemical synthesis, as described above. The gene may encode the 57-amino acid KPI domain shown in Table 1, or it may also encode additional N-terminal amino acids from the

APPI protein sequence, such as the four amino acid sequence (Glu-Val-Val-Arg or Asp-Val-Val-Arg, designated residues -4 to -1) immediately preceding the KPI domain in APPI.

Production of the gene by synthesis allows the codon usage of the KPI gene to be altered to introduce convenient restriction endonuclease recognition sites, without altering the sequence of the encoded peptide. In a preferred embodiment of the invention, the synthetic KPI gene contains restriction endonuclease recognition sites that facilitate excision of DNA cassettes from the KPI gene. These cassettes can be replaced with small synthetic oligonucleotides encoding the desired changes in the KPI peptide sequence. *See* Ausubel, *supra*.

This method also allows the production of genes encoding KPI as a fusion peptide with one or more additional peptide or protein sequences. The DNA encoding these additional sequences is arranged in-frame with the sequence encoding KPI such that, upon translation of the gene, a fusion protein of KPI and the additional peptide or protein sequence is produced. Methods of making such fusion proteins are well known in the art. Examples of additional peptide sequences that can be encoded in the genes are secretory signal peptide sequences, such as bacterial leader sequences, for example *omp*A and *pho*A, that direct secretion of proteins to the bacterial periplasmic space. In a preferred embodiment of the invention, the additional peptide sequence is a yeast secretory signal sequence, such as α-mating factor, that directs secretion of the peptide when produced in yeast.

Additional genetic regulatory sequences can also be introduced into the synthetic gene that are operably linked to the coding sequence of the gene, thereby allowing synthesis of the protein encoded by the gene when the gene is introduced into a host cell. Examples of regulatory genetic sequences that can be introduced are: promoter and enhancer sequences and transcriptional and translational control sequences. Other regulatory sequences are well known in the art. *See* Ausubel *et al., supra*, and Sambrook *et al., supra*.

Sequences encoding other fusion proteins and genetic elements are well known to those of skill in the art. In a preferred embodiment of the invention, the KPI sequence is prepared by ligating together synthetic oligonucleotides to produce a gene encoding an in-frame fusion protein of yeast α-mating factor with either KPI (1→57) or KPI (-4→57).

The gene constructs prepared as described above are conveniently manipulated in host cells using methods of manipulating recombinant DNA techniques that are well known in the art. *See*, for example Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989), and Ausubel, *supra*. In a preferred embodiment of the invention the host cell used for manipulating the KPI constructs is *E. coli*. For example, the construct can be ligated into a cloning vector and propagated in *E. coli* by methods that are well known in the art. Suitable cloning vectors are described in Sambrook, *supra*, or are commercially available from suppliers such as Promega (Madison, WI), Stratagene (San Diego, CA) and Life Technologies (Gaithersburg, MD).

Once a gene construct encoding KPI has been obtained, genes encoding KPI variants are obtained by manipulating the coding sequence of the construct by standard methods of site-directed mutagenesis, such as excision and replacement of small DNA cassettes, as described *supra*. *See* Ausubel, *supra*, and Sinha *et al.*, *supra*. *See also* U.S. Patent 5,373,090, which is herein incorporated by reference in its entirety. *See* particularly, columns 4-12 of U.S. Patent 5,272,090. These genes are then used to produce the KPI variant peptides as described below.

Alternatively, KPI variants can be produced using phage display methods. *See*, for example, Dennis *et al.*, *supra*, which is hereby incorporated by reference in its entirety. *See also* U.S. Patent Nos. 5,223,409 and 5,403,484, which are hereby also incorporated by reference in their entireties. In these methods, libraries of genes encoding variants of KPI are fused in-frame to genes encoding surface proteins of filamentous phage, and the resulting peptides are expressed (displayed) on the surface of the phage. The phage are then screened for the ability to bind, under appropriate conditions, to serine proteases of interest immobilized on a solid support. Large libraries of phage can be used, allowing simultaneous screening of the binding properties of a large number of KPI variants. Phage that have desirable binding properties are isolated and the sequences of the genes encoding the corresponding KPI variants is determined. These genes are then used to produce the KPI variant peptides as described below.

(b) Expression of KPI variant peptides

Once genes encoding KPI variants have been prepared, they are inserted into an expression vector and used to produce the recombinant peptide. Suitable expression vectors and corresponding methods of expressing recombinant proteins and peptides are

well known in the art. Methods of expressing KPI peptides are described in U.S. Patent 5,187,153, columns 9-11, U.S. Patent 5,223,482, columns 9-11, PCT application 93/09233, pp. 49-67, and PCT application 96/35788, pp. 31-33. *See also* Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*. The gene can be expressed in any number of different recombinant DNA expression systems to generate large amounts of the KPI variant, which can then be purified and tested for its ability to bind to and inhibit serine proteases of interest. Within the context of the present invention, substitutions at position 48 may exhibit an increased level of expression of KPI peptides, both wild-type and substituted, in comparison to the expression levels of such peptides not having such a substitution. Such peptides having a substitution at position 48 also may preferably comprise one or more additional substitutions at residues 9, 11, 13-18, 32 and 37-40; in particular, such peptides may preferably comprise a substitution at positions 9 or 37 and/or substitution of at least two of the four residues at positions 15-18. Those additionally substituted peptides may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain as well as increased expression levels.

In particular, replacement of arginine at position 18 of the native KPI peptide with histidine (R18H) in combination with one or more additional substitutions at residues 9, 15 and 17 was found to exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than the native KPI peptide. In particular, the specific substitutions T9V, M15A, S17Y and M15A, S17Y in the context of the R18H substitution exhibited such potent serine protease inhibition. Placing this position 48 substitution in such substituted peptides resulted in increased expression levels of these peptides in comparison to the expression levels of the peptides without the position 48 substitution.

Examples of expression systems known to the skilled practitioner in the art include bacteria such as *E. coli*, yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in Cos or CHO cells. In a preferred embodiment, KPI variants are expressed in *S. cerevisiae*. In another preferred embodiment the KPI variants are cloned into expression vectors to produce a chimeric gene encoding a fusion protein of the KPI variant with yeast α-mating factor. The mating factor acts as a signal sequence to direct secretion of the fusion protein from the yeast cell, and is then cleaved from the fusion protein by a membrane-bound protease

during the secretion process. The expression vector is transformed into *S. cerevisiae*, the transformed yeast cells are cultured by standard methods, and the KPI variant is purified from the yeast growth medium.

Recombinant bacterial cells expressing the peptides of the present invention, for example, *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant antigen induced by adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed. If the recombinant peptide is expressed in inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to assist in the removal of any contaminating host proteins, then solubilized in solutions containing high concentrations of urea (*e.g.*, 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as β-mercaptoethanol or DTT (dithiothreitol).

At this stage it may be advantageous to incubate the peptides of the present invention for several hours under conditions suitable for the peptides to undergo a refolding process into a conformation which more closely resembles that of native KPI. Such conditions generally include low protein concentrations less than 500 μg/ml, low levels of reducing agent, concentrations of urea less than 2M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule. The refolding process can be monitored, for example, by SDS-PAGE or with antibodies, which are specific for the native molecule (which can be obtained from animals vaccinated with the native molecule isolated from parasites). Following refolding, the peptide can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

Purification of KPI variants can be achieved by standard methods of protein purification, *e.g.*, using various chromatographic methods including high performance

liquid chromatography and adsorption chromatography. The purity and the quality of the peptides can be confirmed by amino acid analyses, molecular weight determination, sequence determination and mass spectrometry. *See*, for example, PROTEIN PURIFICATION METHODS: A PRACTICAL APPROACH, Harris *et al.*, eds. (IRL Press, Oxford, 1989). In a preferred embodiment, the yeast cells are removed from the growth medium by filtration or centrifugation, and the KPI variant is purified by affinity chromatography on a column of trypsin-agarose, followed by reversed-phase HPLC.

In yet another embodiment, the KPI peptides of the present invention may also comprise a substitution at its N-terminus. Placing the amino acid sequence Asp-Val-Val-Arg (designated residues –4 to –1) immediately before the KPI domain was found to alleviate the problems associated with the purification and subsequent isolation of the expressed peptides of the present invention having a glutamic acid residue at its N-terminus. In a preferred embodiment, this substitution changes the additional N-terminal amino acids from the KPI protein sequence (Glu-Val-Val-Arg, designated residues –4 to –1) immediately proceeding the KPI domain to Asp-Val-Val-Arg. Specifically, this substitution is thought to prevent cyclization of the N-terminus glutamic acid in the unsubstituted variant during purification of the expressed peptides of the present invention and is thought to be applicable to the substituted KPI variants of the present invention, as well as wild-type KPI. By way of example, Figure 72 provides a comparison of the HPLC traces, after lyophilization, of KPI having the N-terminal sequence Glu-Val-Val-Arg (E-KPI) and KPI having the N-terminus sequence Asp-Val-Val-Arg (D-KPI). Those KPI samples were injected onto a YMC-Phenyl HPLC column (Cat. No.:PH12SO30504WTA, 4x50 mm, 3μ particle size, 50 angstrom pore size). Mobile Phase A was 40 nM ammonium phosphate (pH 6.5), 10% acetonitrile, and 90% water. Mobile Phase B was 40 nM ammonium phosphate (pH 6.5), 60% acetonitrile, and 40% water. The KPI-185 elution point was at approximately 21% acetonitrile. The HPLC of E-KPI exhibits an additional peak after 10 minutes, which is the product of the cyclization of the N-terminus glutamic acid of E-KPI. The HPLC of D-KPI exhibits no such peak and thus no such cyclization product.

C. Measurement of protease inhibitory properties of KPI variants

Once KPI variants have been purified, they are tested for their ability to bind to and inhibit serine proteases of interest *in vitro*. The peptides of the present invention

preferably exhibit a more potent and specific inhibition of serine proteases of interest than known serine protease inhibitors, such as the natural KPI peptide domain. Such binding and inhibition can be assayed for by determining the inhibition constants for the peptides of the present invention toward serine proteases of interest and comparing those constants with constants determined for known serine protease inhibitors, *e.g.*, the native KPI domain, toward those proteases. Methods for determining inhibition constants of protease inhibitors are well known in the art. *See* Fersht, ENZYME STRUCTURE AND MECHANISM, 2nd ed., W.H. Freeman and Co., New York, (1985).

In a preferred embodiment the inhibition experiments are carried out using a chromogenic synthetic protease substrate, as described, for example, in Bender *et al.*, *J. Amer. Chem. Soc.* 88:5890 (1966). Measurements taken by this method can be used to calculate inhibition constants (K_i values) of the peptides of the present invention toward serine proteases of interest. *See* Bieth in BAYER-SYMPOSIUM V "PROTEINASE INHIBITORS", Fritz *et al.*, eds., pp. 463-69, Springer-Verlag, Berlin, Heidelberg, New York, (1974). KPI variants that exhibit potent and specific inhibition of one or more serine proteases of interest may subsequently be tested *in vivo*. *In vitro* testing, however, is not a prerequisite for *in vivo* studies of the peptides of the present invention.

D. Testing of KPI variants *in vivo*

The peptides of the present invention may be tested, alone or in combination, for their therapeutic efficacy by various *in vivo* methodologies known to those skilled in the art, *e.g.*, the ability of KPI variants to reduce postoperative bleeding can be tested in standard animal models. For example, cardiopulmonary bypass surgery can be carried out on animals such as pigs in the presence of KPI variants, or in control animals where the KPI variant is not used. The use of pigs as a model for studying the clinical effects associated with CPB has previously been described. *See* Redmond *et al.*, *Ann. Thorac. Surg.* 56:474 (1993).

The KPI variant is supplied to the animals in a pharmaceutical sterile vehicle by methods known in the art, for example by continuous intravenous infusion. Chest tubes can be used to collect shed blood for a defined period of time. The shed blood, together with the residual intrathoracic blood found after sacrifice of the animal can be used to calculate hemoglobin (Hgb) loss. The postoperative blood and Hgb loss is then compared between the test and control animals to determine the effect of the KPI variants.

E. Therapeutic use of KPI variants

KPI variants of the present invention found to exhibit therapeutic efficacy (*e.g.*, reduction of blood loss following surgery in animal models) may preferably be used and administered, alone or in combination or as a fusion protein, in a manner analogous to that currently used for aprotinin or other known serine protease inhibitors. *See* Butler *et al., supra.* Peptides of the present invention generally may be administered in the manner that natural peptides are administered. A therapeutically effective dose of the peptides of the present invention preferably affects the activity of the serine proteases of interest such that the clinical condition may be treated, ameliorated or prevented. Therapeutically effective dosages of the peptides of the present invention can be determined by those skilled in the art, *e.g.*, through *in vivo* or *in vitro* models. Generally, the peptides of the present invention may be administered in total amounts of approximately 0.01 to approximately 500, specifically 0.1 to 100 mg/kg body weight, if desired in the form of one or more administrations, to achieve therapeutic effect. It may, however, be necessary to deviate from such administration amounts, in particular depending on the nature and body weight of the individual to be treated, the nature of the medical condition to be treated, the type of preparation and the administration of the peptide, and the time interval over which such administration occurs. Thus, it may in some cases be sufficient to use less than the above amount of the peptides of the present invention, while in other cases the above amount is preferably exceeded. The optimal dose required in each case and the type of administration of the peptides of the present invention can be determined by one skilled in the art in view of the circumstances surrounding such administration. Such peptides can be administered by intravenous injections, *in situ* injections, local applications, inhalation, oral administration using coated polymers, dermal patches or other appropriate means. Compositions comprising peptides of the present invention are advantageously administered in the form of injectable compositions. Such peptides may be preferably administered to patients via continuous intravenous infusion, but can also be administered by single or multiple injections. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described in REMINGTON'S PHARMACEUTICAL SCIENCES, pp. 1405-12 and 1461-87 (1975) and THE NATIONAL FORMULARY XIV., 14th Ed. Washington: American

Pharmaceutical Association (1975). Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobials, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the composition are adjusted according to routine skills in the art. *See* GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS FOR THERAPEUTICS (7th ed.). The peptides of the present invention may be present in such pharmaceutical preparations in a concentration of approximately 0.1 to 99.5% by weight, specifically 0.5 to 95% by weight, relative to the total mixture. Such pharmaceutical preparations may also comprise other pharmaceutically active substances in addition to the peptides of the present invention. Other methods of delivering the peptides to patients will be readily apparent to the skilled artisan.

Examples of mammalian serine proteases that may exhibit inhibition by the peptides of the present invention include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as thrombin and factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. Examples of conditions associated with increased serine protease activity include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of the use of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The inhibitors of the present invention may also be used for inhibition of serine protease activity *in vitro*, for example during the preparation of cellular extracts to prevent degradation of cellular proteins. For this purpose the inhibitors of the present invention may preferably be used in a manner analogous to the way that aprotinin, or other known serine protease inhibitors, are used. The use of aprotinin as a protease inhibitor for preparation of cellular extracts is well known in the art, and aprotinin is sold commercially for this purpose.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

Example 1. Expression of wild-type KPI (-4→57)

A. Construction of pTW10:KPI

Plasmid pTW10:KPI is a bacterial expression vector encoding the 57 amino acid form of KPI fused to the bacterial *pho*A signal sequence. The strategy for the construction of pTW10:KPI is shown in Figure 1.

Plasmid pcDNAII (Invitrogen, San Diego, CA) was digested with *Pvu*II and the larger of the two resulting *Pvu*II fragments (3013 bp) was isolated. Bacterial expression plasmid pSP26 was digested with *Mlu*I and *Rsr*II, and the 409 bp *Mlu*I-*Rsr*II fragment containing the pTrp promoter element and transcription termination signals was isolated by electrophoresis in a 3% NuSieve Agarose gel (FMC Corp., Rockland, ME). Plasmid pSP26, containing a heparin-binding EGF-like growth factor (HB-EGF) insert between the *Nde*I and *Hind*III sites, is described as pNA28 in Thompson *et al.*, *J. Biol. Chem.* 269:2541 (1994). Plasmid pSP26 was deposited in host *E. coli* W3110, pSP26 with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209, USA under the conditions specified by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (Budapest Treaty). Host *E. coli* W3110, pSP26 was deposited on 3 May 1995 and given Accession No. 69800. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The ends of the *Mlu*I-*Rsr*II fragment were blunted using DNA polymerase Klenow fragment by standard techniques. The blunted fragment of pSP26 was then ligated into the large *Pvu*II fragment of plasmid pCDNAII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to isolate plasmid pTW10 by standard techniques.

A synthetic gene was constructed encoding the bacterial *pho*A secretory signal sequence fused to the amino terminus of KPI(1→57). The synthetic gene contains cohesive ends for *Nde*I and *Hind*III, and also incorporates restriction endonuclease recognition sites for *Age*I, *Rsr*II, *Aat*II and *Bam*HI, as shown in Figure 2. The synthetic

*pho*A-KPI gene was constructed from 6 oligonucleotides of the following sequences (shown 5'-3'):

6167:

TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTAC
CCCTGTGACAAAAGCCGAGGTGTGCTCTGAA

6169:

CTCGGCTTTTGTCACAGGGGTAAACAGTAACGGTAAGAGTGCCAGTG
CAATAGTGCTTTGTTTCATA

6165:

CAAGCTGAGACCGGTCCGTGCCGTGCAATGATCTCCCGCTGGTACTTT
GACGTCACTGAAGGTAAGTGCGCTCCATTCTTT

6166:

GCACTTACCTTCAGTGACGTCAAAGTACCAGCGGGAGATCATTGCAC
GGCACGGACCGGTCTCAGCTTGTTCAGAGCACAC

6168:

TACGGCGGTTGCGGCGGCAACCGTAACAACTTTGACACTGAAGAGTA
CTGCATGGCAGTGTGCGGATCCGCTATTTAAGCT

6164:

AGCTTAAATAGCGGATCCGCACACTGCCATGCAGTACTCTTCAGTGTC
AAAGTTGTTACGGTTGCCGCCGCAACCGCCGTAAAAGAATGGAGC

The oligonucleotides were phosphorylated and annealed in pairs: 6167 + 6169, 6165 + 6166, 6168 + 6164. In 20 µl T4 DNA Ligase Buffer (New England Biolabs, Beverly, MA), 1 g of each oligonucleotide pair was incubated with 10 U T4 Polynucleotide Kinase (New England Biolabs) for 1 h at 37°C, then heated to 95°C for 1 minute, and slow-cooled to room temperature to allow annealing. All three annealed

oligo pairs were then mixed for ligation to one another in a total volume of 100 l T4 DNA Ligase Buffer, and incubated with 400 U T4 DNA Ligase (New England Biolabs) overnight at 15°C. The ligation mixture was extracted with an equal volume of phenol:$CHCl_3$ (1:1), ethanol-precipitated, resuspended in 50 Restriction Endonuclease Buffer #4 (New England Biolabs) and digested with *Nde*I and *Hind*III. The annealed, ligated and digested oligos were then subjected to electrophoresis in a 3% NuSieve Agarose gel, and the 240 bp *Nde*I-*Hind*III fragment was excised. This gel-purified synthetic gene was ligated into plasmid pTW10, which had previously been digested with *Nde*I and *Hind*III, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to prepare plasmid pTW10:KPI. This plasmid contains the *pho*A-KPI(1→57)-fusion protein inserted between the pTrp promoter element and the transcription termination signals.

B. Construction of pKPI-61

The strategy for constructing pKPI-61 is shown in Figure 3. Plasmid pTW10:KPI was digested with *Age*I and *Hind*III; the resulting 152 bp *Age*I-*Hind*III fragment containing a portion of the KPI synthetic gene was isolated by preparative gel electrophoresis. An oligonucleotide pair (129 + 130) encoding the 9 amino-terminal residues of KPI(1→57) and 4 amino acids of yeast α-mating factor was phosphorylated and annealed as described above.

129: CTAGATAAAAGAGAGGTGTGCTCTGAACAAGCTGAGA

130: CCGGTCTCAGCTTGTTCAGAGCACACCTCTCTTTTAT

The annealed oligonucleotides were then ligated to the *Age*I-*Hind*III fragment of the KPI (1→57) synthetic gene. The resulting 192 bp *Xba*I-*Hind*III synthetic gene (shown in Figure 4) was purified by preparative gel electrophoresis, and ligated into plasmid pUC19 which had previously been digested with *Xba*I and *Hind*III. The ligation products were used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were picked and used to prepare plasmid pKPI-57 by standard methods. To create a synthetic gene encoding KPI(-4→57), pKPI-57 was digested with *Xba*I and *Age*I and the smaller fragment replaced with annealed oligos 234 + 235, which encode 4 amino acid

residues of yeast α-mating factor fused a 4 amino acid residue amino-terminal extension of KPI(1→57).

234:

CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCTGAGA

235: CCGGTCTCAGCTTGTTCAGAGCACACCTCTCTAACAACCTCTCTTTTAT

The 4 extra amino acids are encoded in the amyloid β-protein precursor/protease nexin-2 (APPI) which contains the KPI domain. The synthetic 201 bp *Xba*I-*Hind*III fragment encoding KPI(-4→57) in pKPI-61 is shown in Figure 5.

C. Assembly of pTW113

The strategy for the construction of pTW113 is shown in Figure 6. Plasmid pSP35 was constructed from yeast expression plasmid pYES2 (Invitrogen, San Diego, CA) as follows. A 267 bp *Pvu*II-*Xba*I fragment was generated by PCR from yeast α-mating factor DNA using oligos 6274 and 6273:

6274: GGGGGCAGCTGTATAAACGATTAAAA

6273: GGGGGTCTAGAGATACCCCTTCTTCTTTAG

This PCR fragment, encoding an 82 amino acid portion of yeast α-mating factor, including the secretory signal peptide and pro-region, was inserted into pYES2 that had been previously digested with *Pvu*II and *Xba*I. The resulting plasmid is denoted pSP34.

Two oligonucleotide pairs, 6294 + 6292 were then ligated to 6290 + 6291, and the resulting 135 bp fragment was purified by gel electrophoresis.

6294: CTAGATAAAAGAGAGGCTGAGGCTCACGCTGAAGGTACTTTCACTTC

6290:

TGACGTCTCTTCTTACTTGGAAGGTCAAGCTGCTAAGGAATTCATCG

CTTGGTTGGTCAAAGGTAGAGGTTAAGCTTA

6291:

CTAGTAAGCTTAACCTCTACCTTTGACCAACCAAGCGATGAATTCCT
TAGCA

6292:

GCTTGACCTTCCAAGTAAGAAGAGACGTCAGAAGTGAAAGTACCTT
CAGCGTGAGCCTCAGCCTCTCTTTTAT

The resulting synthetic fragment was ligated into the *Xba*I site of pSP34, resulting in plasmid pSP35. pSP35 was digested with *Xba*I and *Hind*III to remove the insert, and ligated with the 201 bp *Xba*I-*Hind*III fragment of pKPI-61, encoding KPI (-4→57). The resulting plasmid pTW113, encodes the 445 bp synthetic gene for the α-factor-KPI(-4→57) fusion. *See* Figure 7.

D. Transformation of yeast with pTW113

Saccharomyces cerevisiae strain ABL115 was transformed with plasmid pTW113 by electroporation by the method of Becker *et al., Methods Enzymol.* 194:182 (1991). An overnight culture of yeast strain ABL115 was used to inoculate 200 ml YPD medium. The inoculated culture was grown with vigorous shaking at 30°C to an OD_{600} of 1.3-1.5, at which time the cells were harvested by centrifugation at 5000 rpm for 5 minutes. The cell pellet was resuspended in 200 ml ice-cold water, respun, and resuspended in 100 ml ice-cold water, then pelleted again. The washed cell pellet was resuspended in 10 ml ice-cold 1M sorbitol, recentrifuged, then resuspended in a final volume of 0.2 ml ice-cold 1M sorbitol. A 40 ml aliquot of cells was placed into the chamber of a cold 0.2 cm electroporation cuvette (Invitrogen), along with 100 ng plasmid DNA for pTW113. The cuvette was placed into an Invitrogen Electroporator II and pulsed at 1500 V, 25F, 100 Ω. Electroporated cells were diluted with 0.5 ml 1M sorbitol, and 0.25 ml was spread on an SD agar plate containing 1M sorbitol. After 3 days' growth at 30°C, individual colonies were streaked on SD + CAA agar plates.

E. Induction of pTW113/ABL115, purification of KPI(-4→57)

Yeast cultures were grown in a rich broth and the galactose promoter of the KPI expression vector induced with the addition of galactose as described by Sherman, *Methods Enzymol.* 194:3 (1991). A single well-isolated colony of pTW113/ABL115

was used to inoculate a 10 ml overnight culture in Yeast Batch Medium. The next day, 1L Yeast Batch Medium which had been made 0.2% glucose was inoculated to an OD_{600} of 0.1 with the overnight culture. Following 24 hours at 30°C with vigorous shaking, the 1L culture was induced by the addition of 20 ml Yeast Galactose Feed Medium. Following induction, the culture was fed every 12 hours with the addition of 20 ml Yeast Galactose Feed Medium. At 48 hours after induction, the yeast broth was harvested by centrifugation, then adjusted to pH 7.0 with 2M Tris, pH 10. The broth was subjected to trypsin-Sepharose affinity chromatography, and bound KPI(-4→57) was eluted with 20mM Tris pH 2.5. *See* Schilling *et al., Gene* 98:225 (1991). Final purification of KPI(-4→57) was accomplished by HPLC chromatography on a semi-prep Vydac C4 column in a gradient of 20% to 35% acetonitrile. The sample was dried and resuspended in PBS at 1-2 mg/ml. The amino acid sequence of KPI(-4→57) is shown in Figure 8.

Example 2. Recombinant Expression of site-directed KPI(-4→57) variants

Expression vectors for the production of specific variants of KPI(-4→57) were all constructed using the pTW113 backbone as a starting point. For each KPI variant, an expression construct was created by replacing the 40 bp *Rsr*II-*Aat*II fragment of the synthetic KPI gene contained in pTW113 with a pair of annealed oligonucleotides which encode specific codons mutated from the wild-type KPI(-4→57) sequence. In the following examples, the convention used for designating the amino substituents in the KPI variants indicates first the single letter code for the amino acid found in wild-type KPI, followed by the position of the residue, followed by the code for the replacement amino acid. Thus, for example, M15R indicates that the methionine residue at position 15 is replaced by an arginine.

A. Construction of pTW6165

The strategy for constructing pTW6165 is shown in Figure 9. Plasmid pTW113 was digested with *Rsr*II and *Aat*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (812 + 813) was phosphorylated, annealed and gel-purified as described above.

812: GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT

813: CAAAGTACCAGCGCCAGATAGCTGCACGGCACG

The annealed oligonucleotides were ligated into the *Rsr*II and *Aat*II-digested pTW113, and the ligation product was used to transform *E. coli* strain MC1061. Transformed colonies were selected by ampicillin resistance. The resulting plasmid, pTW6165, encodes the 445 bp synthetic gene for the α-factor-KPI(-4→57; M15A, S17W) fusion. *See* Figure 10.

B. Construction of pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, pTW6174.

Construction of the following KPI(-4→57) variants was accomplished exactly as outlined for pTW6165. The oligonucleotides utilized for each construct are denoted below, and the sequences of annealed oligonucleotide pairs are shown in Figure 11. Figures 12-19 show the synthetic genes for the α-factor fusions with each KPI(-4→57) variant.

pTW6166: KPI(-4→57; M15A, S17Y) See Figure 12.

814: GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT

815: CAAAGTACCAGCGGTAGATAGCTGCACGGCACG

pTW6175: KPI(-4→57; M15L, S17F) See Figure 13.

867: GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT

868: CAAAGTACCAGCGGAAGATCAATGCACGGCACG

pBG028: KPI(-4→57; M15L, S17Y) See Figure 14.

1493: GTCCGTGCCGTGCTTTGATCTACCGCTGGTACTTTGACGT

1494: CAAAGTACCAGCGGTAGATCAAAGCACGGCACG

pTW6183: KPI(-4→57; I16H, S17F) See Figure 15.

925: GTCCGTGCCGTGCAATGCACTTCCGCTGGTACTTTGACGT

926: CAAAGTACCAGCGGAAGTGCATTGCACGGCACG

pTW6184: KPI(-4→57; I16H, S17Y) See Figure 16.

927: GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT

928: CAAAGTACCAGCGGTAGTGCATTGCACGGCACG

pTW6185: KPI(-4→57; I16H, S17W) See Figure 17.

929: GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT

930: CAAAGTACCAGCGCCAGTGCATTGCACGGCACG

pTW6173: KPI(-4→57; M15A, I16H) See Figure 18.

863: GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT

864: CAAAGTACCAGCGGGAGTGAGCTGCACGGCACG

pTW6174: KPI(-4→57; M15L, I16H) See Figure 19.

865: GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT

866: CAAAGTACCAGCGGGAGTGCAATGCACGGCACG

C. Transformation of yeast with expression vectors

Yeast strain ABL115 was transformed by electroporation exactly according to the protocol described for transformation by pTW113.

D. Induction of transformed yeast strains, purification of KPI(-4→57) variants.

Cultures of yeast strains were grown and induced, and recombinant secreted KPI (-4→57) variants were purified according to the procedure described for KPI (-4→57). The amino acid sequences of KPI(-4→57) variants are shown in Figures 27-36.

Example 3. Identification of KPI (-4→57; M15A, S17F) DD185 by phage display.

A. Construction of vector pSP26:Amp:F1

The construction of pSP26:*Amp*:F1 is outlined in Figure 43. Vector pSP26:*Amp*:F1 contributes the basic plasmid backbone for the construction of the phage display vector for the *pho*A:KPI fusion, pDW1 #14. pSP26:*Amp*:F1 contains a low-copy number origin of replication, the ampicillin-resistance gene (*Amp*) and the F1 origin for production of single-stranded phagemid DNA.

The ampicillin-resistance gene (*Amp*) was generated through polymerase chain reaction (PCR) amplification from the plasmid genome of PUC19 using oligonucleotides 176 and 177.

176: GCCATCGATGGTTTCTTAAGCGTCAGGTGGCACTTTTC

177: GCGCCAATTCTTGGTCTACGGGGTCTGACGCTCAGTGGAACGAA

The PCR amplification of *Amp* was done according to standard techniques, using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification from plasmid pUC19 with these oligonucleotides yielded a fragment of 1159 bp, containing *Pfl*MI and *Cla*I restriction sites. The PCR product was digested with *Pfl*MI and *Cla*I and purified by agarose gel electrophoresis in 3% NuSieve Agarose (FMC Corp.). Bacterial expression vector pSP26 (*supra)* was digested with *Pfl*MI and *Cla*I and the larger vector

fragment was purified. The *Pfl*MI-*Cla*I PCR fragment was ligated into the previously digested pSP26 containing the *Amp* gene. The ligation product was used to transform *E. coli* strain MC1061 and colonies were selected by ampicillin resistance. The resulting plasmid is denoted pSP26:*Amp*.

The F1 origin of replication from the mammalian expression vector pcDNAII (Invitrogen) was isolated in a 692 bp *Ear*I fragment. Plasmid pcDNAII was digested with *Ear*I and the resulting 692 bp fragment purified by agarose gel electrophoresis. *Ear*I-*Not*I adapters were added to the 692 bp *Ear*I fragment by ligation of two annealed oligonucleotide pairs, 179 + 180 and 181 + 182. The oligo pairs were annealed as described above.

179: GGCCGCTCTTCC
180: AAAGGAAGAGC
181: CTAGAATTGC
182: GGCCGCAATTC

The oligonucleotide-ligated fragment was then ligated into the single *Not*I site of PSP26:*Amp* to yield the vector pSP26:*Amp*:F1.

B. Construction of vector pgIII

The construction of pgIII is outlined in Figure 44. The portion of the phage geneIII protein gene contained by the PDW1 #14 phagemid vector was originally obtained as a PCR amplification product from vector m13mp8. A portion of m13mp8 geneIII encoding the carboxyl-terminal 158 amino acid residues of the geneIII product was isolated by PCR amplification of m13mp8 nucleotide residues 2307-2781 using PCR oligos 6162 and 6160.

6162: GCCGGATCCGCTATTTCCGGTGGTGGCTCTGGTTCC
6160: GCCAAGCTTATTAAGACTCCTTATTACGCAG

The PCR oligos contain *Bam*HI and *Hind*III restriction recognition sites such that PCR from m13mp8 plasmid DNA with the oligo pair yielded a 490 bp *Bam*HI-*Hind*III fragment encoding the appropriate portion of geneIII. The PCR product was

ligated between the *Bam*HI and *Hind*III sites within the polylinker of PUC19 to yield plasmid pgIII.

C. Construction of pPhoA:KPI:gIII

Construction of p*Pho*A:KPI:gIII is outlined in Figure 45. A portion of the *pho*A signal sequence and KPI fusion encoded by the phage display vector PDW1 #14 originates with p*Pho*A:KPI:gIII. The 237 bp *Nde*I-*Hind*III fragment of pTW10:KPI encoding the entire *pho*A:KPI (1→57) fusion was isolated by preparative agarose gel electrophoresis, and inserted between the *Nde*I and *Hind*III sites of pUC19 to yield plasmid p*Pho*A:KPI. The 490 bp *Bam*HI-*Hind*III fragment of pgIII encoding the C-terminal portion of the geneIII product was then isolated and ligated between the *Bam*HI and *Hind*III sites of p*Pho*A:KPI to yield vector p*Pho*a:KPI:gIII. The p*Pho*A:KPI:gIII vector encodes a 236 amino acid residue fusion of the *pho*A signal peptide, KPI (1→57) and the carboxyl-terminal portion of the geneIII product.

D. Construction of pLG1

Construction of pLG1 is illustrated in Figure 46. The exact geneIII sequences contained in vector PDW1 #14 originate with phage display vector pLG1. A modified geneIII segment was generated by PCR amplification of the geneIII region from pgIII using PCR oligonucleotides 6308 and 6305.

6308: AGCTCCGATCTAGGATCCGGTGGTGGCTCTGGTTCCGGT

6305: GCAGCGGCCGTTAAGCTTATTAAGACTCCT

PCR amplification from pgIII with these oligonucleotides yielded a 481 bp *Bam*HI-*Hind*III fragment encoding a geneIII product shortened by 3 amino acid residues at the amino-terminal portion of the segment of the geneIII fragment encoded by pgIII. A 161 bp *Nde*I-*Bam*HI fragment was generated by PCR amplification from bacterial expression plasmid pTHW05 using oligonucleotides 6306 and 6307.

6306: GATCCTTGTGTCCATATGAAACAAAGC

6307: CACGTCGGTCGAGGATCCCTAACCACGGCCTTTAACCAG

The 161 bp *Nde*I-*Bam*HI fragment and the 481 bp *Bam*HI-*Hind*III fragment were gel-purified, and then ligated in a three-way ligation into pTW10 which had previously been digested with *Nde*I and *Hind*III. The resulting plasmid pLG1 encodes a *pho*A signal peptide-insert-geneIII fusion for phage display purposes.

E. Construction of pAL51

Construction of pAL51 is illustrated in Figure 47.

Vector pAL51 contains the geneIII sequences of pLG1 which are to be incorporated in vector pDW1 #14.

A 1693 bp fragment of plasmid pBR322 was isolated, extending from the *Bam*HI site at nucleotide 375 to the *Pvu*II site at position 2064. Plasmid pLG1 was digested with *Asp*718I and *Bam*HI, removing an 87 bp fragment. The overhanging *Asp*718I end was blunted by treatment with Klenow fragment, and the *Pvu*II-*Bam*HI fragment isolated from pBR322 was ligated into this vector, resulting in the insertion of a 1693 bp "stuffer" region between the *Asp*718I and *Bam*HI sites. The 78 bp *Nde*I-*Asp*718I region of the resulting plasmid was removed and replaced with the annealed oligo pair 6512 + 6513.

6512:

TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTA
CCCCGGTGACCAAAGCCCACGCTGAAG

6513:

GTACCTTCAGCGTGGGCTTTGGTCACCGGGGTAAACAGTAACGGTAA
GAGTGCCAGTGCAATAGTGCTTTGTTTCA

The newly created 74 bp *Nde*I-*Asp*718I fragment encodes the *pho*A signal peptide, and contains a *Bst*EII cloning site. The resulting plasmid is denoted pAL51.

F. Construction of pAL53

Construction of pAL53 is outlined in Figure 48. Plasmid pAL53 contributes most of the vector sequence of pDW1 #14, including the basic vector backbone with *Amp* gene, F1 origin, low copy number origin of replication, geneIII segment, *pho*A promoter and *pho*A signal sequence.

Plasmid pAL51 was digested with *Nde*I and *Hind*III and the resulting 2248 bp *Nde*I-*Hind*III fragment encoding the *pho*A signal peptide, stuffer region and geneIII region was isolated by preparative agarose gel electrophoresis. The *Nde*I-*Hind*III fragment was ligated into plasmid pSP26:*Amp*:F1 between the *Nde*I and *Hind*III sites, resulting in plasmid pAL52.

The *pho*A promoter region and signal peptide was generated by amplification of a portion of the *E. coli* genome by PCR, using oligonucleotide primers 405 and 406.

405: CCGGACGCGTGGAGATTATCGTCACTG

406: GCTTTGGTCACCGGGGTAAACAGTAACGG

The resulting PCR product is a 332 bp *Mlu*I-*Bst*EII fragment, which contains the *pho*A promoter region and signal peptide sequence. This fragment was used to replace the 148 bp *Mlu*I-*Bst*EII segment of pAL52, resulting in vector pAL53.

G. Construction of pSP26:Amp:F1:PhoA:KPI:gIII

Construction of pSP26:*Amp*:F1:*Pho*A:KPI:gIII is illustrated in Figure 49. This particular vector is the source of the KPI coding sequence found in vector pDW1 #14. Plasmid p*Pho*a:KPI:gIII was digested with *Nde*I and *Hind*III, and the resulting 714 bp *Nde*I-*Hind*III fragment was purified, and then inserted into vector pSP26:*Amp*:F1 between the *Nde*I and *Hind*III sites. The resulting plasmid is denoted pSP26:*Amp*:F1:*Pho*A:KPI:gIII.

H. Construction of pDW1 #14

Construction of pDW1 #14 is illustrated in Figure 50. The sequences encoding KPI were amplified from plasmid pSP26:*Amp*:F1:*Pho*A:KPI:gIII by PCR, using oligonucleotide primers 424 and 425.

424: CTGTTTACCCCGGTGACCAAAGCCGAGGTGTGCTCTGAACAA

425: AATAGCGGATCCGCACACTGCCATGCAGTACTCTTC

The resulting 172 bp *Bst*EII-*Bam*HI fragment encodes most of KPI (155). This fragment was used to replace the stuffer region in pAL53 between the *Bst*EII and *Bam*HI sites. The resulting plasmid, pDW1 #14, is the parent KPI phage display vector for

preparation of randomized KPI phage libraries. The coding region for the *pho*A-KPI (155)-geneIII fusion is shown in Figure 56.

I. Construction of pDW1 14-2

Construction of pDW1 14-2 is illustrated in Figure 57. The first step in the construction of the KPI phage libraries in pDW1 #14 was the replacement of the *Age*I-*Bam*HI fragment within the KPI coding sequence with a stuffer fragment. This greatly aids in preparation of randomized KPI libraries, which are substantially free of contamination of phagemid genomes encoding wild-type KPI sequence.

Plasmid pDW1 #14 was digested with *Age*I and *Bam*HI, and the 135 bp *Age*I-*Bam*HI fragment encoding KPI was discarded. A stuffer fragment was created by PCR amplification of a portion of the pBR322 *Tet* gene, extending from the *Bam*HI site at nucleotide 375 to nucleotide 1284, using oligo primers 266 and 252.

266: GCTTTAAACCGGTAGGTGGCCCGGCTCCATGCACC

252: CGAATTCACCGGTGTCATCCTCGGCACCGTCACCCT

The resulting 894 bp *Age*I-*Bam*HI stuffer fragment was then inserted into the *AgeI/Bam*HI-digested pDW1 #14 to yield the phagemid vector pDW1 14-2. This vector was the starting point for construction of the randomized KPI libraries.

J. Construction of KPI Library 16-19

Construction of KPI Library 16-19 is outlined in Figure 58. Library 16-19 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Met^{15}, Ile^{16} and Ser^{17} are randomized. For preparation of the library, plasmid pDW1 14-2 was digested with *Age*I and *Bam*HI to remove the stuffer region, and the resulting vector was purified by preparative agarose gel electrophoresis. Plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 544 and 551.

544: GGGCTGAGACCGGTCCGTGCCGT$(NNS)_4$CGCTGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 544 contains four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop codon, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was purified by preparative agarose gel electrophoresis and ligated into the *AgeI/Bam*HI digested pDW1 14-2 vector. The ligation mixture was used to transform *E. coli* Top10F' cells (Invitrogen) by electroporation according to the manufacturer's directions. The resulting Library 16-19 contained approximately 400,000 independent clones. The potential size of the library, based upon the degeneracy of the priming PCR oligo #544 was 1,048,576 members. The expression unit encoded by the members of Library 16-19 is shown in Figure 59.

K. Selection of Library 16-19 with human plasma kallikrein

KPI phage were prepared and amplified by infecting transformed cells with M13KO7 helper phage as described by Matthews *et al.*, *Science* 260:1113 (1993). Human plasma kallikrein (Enzyme Research Laboratories, South Bend, IN), was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized kallikrein resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, $CaCl_2$, $MgCl_2$, 0.1% gelatin, and 0.05% Triton X-100). Approximately $5x10^9$ phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 µl kallikrein resin containing 15 pmoles of active human plasma kallikrein in a total volume of 250 µl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the kallikrein resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on kallikrein-Sepharose, phagemid DNA was isolated from 22 individual colonies and subjected to DNA sequence analysis.

The most frequently occurring randomized KPI region encoded: Ala^{14}-Ala^{15}-Ile^{16}-Phe^{17}. The *pho*A-KPI-geneIII region encoded by this class of selected KPI phage is

shown in Figure 60. The KPI variant encoded by these phagemids is denoted KPI (155; M15A, S17F).

L. Construction of pDD185 KPI (-4→57; M15A, S17F)

Figure 61 outlines the construction of pDD185 KPI (-4→57; M15A, S17F). The sequences encoding KPI (155; M15A, S17F) were moved from one phagemid vector, pDW1 (16-19) 185, to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of pDW1 (16-19) 185 was isolated and ligated into the yeast vector to yield plasmid pDD185, encoding α-factor fused to KPI (-4→57; M15A, S17F). *See* Figure 62.

M. Purification of KPI (-4→57; M15A, S17F) pDD185

Transformation of yeast strain ABL115 with pDD185, induction of yeast cultures, and purification of KPI (-4→57; M15A, S17F) pDD185 was accomplished as described for the other KPI variants.

N. Construction of KPI Library 6 M15A, with residues 14, 16-18 random.

Library 6 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Ile^{16}, Ser^{17} and Arg^{18} are randomized, but position 15 was held constant as Ala. For preparation of the library, plasmid pDW1 #14 was used as the template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 551 and 1003.

1003: GCTGAGACCGGTCCGTGCCGTNNSGCA(NNS)$_3$TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1003 contained four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop, in 32

different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector, which was then digested with *Age*I to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately $5x10^6$ independent clones.

O. Construction of KPI Library 7 with residues 14-18 random.

Library 7 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Met^{15}, Ile^{16}, Ser^{17} and Arg^{18} are randomized. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 551 and 1179.

1179: GCTGAGACCGGTCCGTGCCGT(NNS)$_5$TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1179 contains five randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encoded all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector, which was then digested with *Age*I to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately $1x10^7$ independent clones.

P. Selection of Libraries 6 & 7 with human factor XIIa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor XIIa (Enzyme Research Laboratories, South Bend, IN), was biotinylated as follows. Factor XIIa (0.5 mg) in 5mM sodium acetate pH 8.3 was incubated with Biotin Ester (Zymed) at room temperature for 1.5 h, then buffer-exchanged into assay buffer (AB). Approximately $1x10^{10}$ phage particles of each amplified Library 6 or 7 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were incubated with 50 pmoles of active biotinylated human factor XIIa in a total volume of 200 µl. Phage were allowed to bind for 2 h at room temperature, with rocking. Following the binding period, 100 µl Strepavidin Magnetic Particles (Boehringer Mannheim) were added to the mixture and incubated at room temperature for 30 minutes. Separation of magnetic particles from the supernatant and wash/elution buffers was carried out using MPC-E-1 Neodymium-iron-boron permanent magnets (Dynal). Unbound phage were removed by washing the magnetically bound biotinylated XIIa-phage complexes three times with 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After 3 or 4 rounds of selection with factor XIIa, phagemid DNA was isolated from individual colonies and subjected to DNA sequence analysis.

Sequences in the randomized regions were compared with one another to identify consensus sequences appearing more than once. From Library 6 a phagemid was identified which encoded M15L, S17Y, R18H. From Library 7 a phagemid was identified which encoded M15A, S17Y, R18H.

Q. Construction of KPI Library P48 with residue 48 random.

Library P48 was constructed to for expression of KPI (M15A, S17Y, R18H) IN WHICH AMINO ACID n which amino acid position Tyr^{48} is randomized. Construction of Library P48 is detailed in Figure 55. For preparation of the library, plasmid pDW1-L6-16, encoding the pBG022 KPI peptide as a fusion with the m13 gIII protein, was used as template in a PCR amplification of the KPI region extending from the *Rsr*II site to the *Bam*HI site. The oligonucleotide primers used were 1663 and 1945.

1663: GCTTTACTGTTTACCCCGGTGACCAAAGCCGAGGTGTGC

1945: ATTAGCGGATCCGCACACTGCCATGCASNNCTCTTCAGTGTCAAAG

Oligonucleotide primer 1945 contains a single randomized codon of the sequence SNN, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Following the procedure delineated *supra*, PCR amplification from the wild-type KPI gene resulted in the production of a mixture of *Rsr*II-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with *Rsr*II and *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pBG022 was digested with *Rsr*II and *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector. The vector containing the insert was purified by agarose gel electrophoresis and recircularized.

R. Construction of pBG015 KPI (-4→57; M15L, S17Y, R18H), pBG022 (-4→57; M15A, S17Y, R18H, having spontaneous mutation Y48H)

The sequences encoding KPI (155; M15L, S17Y, R18H) and KPI (155; M17A, S17Y, R18H) were moved from the phagemid vectors to the yeast expression vector so that the KPI variant could be purified and tested. Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of the phagemid vectors were isolated and ligated into the yeast vector to yield plasmids pBG015 and pBG022, encoding yeast ∝-factor fused to KPI (-4→57; M15L, S17Y, R18H), and KPI (-4→57; M15A, S17Y, R18H, having spontaneous mutation Y48H), respectively. Figure 20 shows the synthetic gene for the ∝-factor fusion with KPI variant (-4→57; M15A, S17Y, R18H, having spontaneous mutation Y48H). Figure 37 shows the amino acid sequence of KPI variant (-4→57; M15A, S17Y, R18H, having spontaneous mutation Y48H).

S. Construction of pBG033 KPI (-4→57; T9V, M15A, S17Y, R18H)

Plasmid pBG022 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously. The annealed

oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG022, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG033, encodes the 445 bp synthetic gene for the yeast ∝-factor-KPI (-4→57; T9V, M15A, S17F, R18H) fusion. Figure 21 shows the synthetic gene for the ∝-factor fusion with KPI variant (-4→57; T9V, M15A, S17Y, R18H, Y48H).). Figure 38 shows the amino acid sequence of KPI variant (-4→57; T9V, M15A, S17Y, R18H, Y48H).

T. Construction of pBG048 KPI (-4→57; Y48H)

Figure 52 outlines the construction of pBG048 KPI (-4→57; Y48H). Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Aat*II and *Bam*HI and the 92 bp *Aat*II-*Bam*HI fragment was discarded. Plasmid pBG022 encoding KPI (-4→57; M15L, S17Y, R18H, Y48H) was digested with *Aat*II and *Bam*HI. The resulting 92 bp *Aat*II-*Bam*HI fragment was isolated and ligated into the yeast vector to yield plasmid pBG048, encoding yeast ∝-factor fused to KPI (-4→57; Y48H). Figure 22 shows the synthetic gene for the ∝-factor fusion with KPI variant (-4→57; Y48H). Figure 39 shows the amino acid sequence of KPI variant (-4→57; 48H).

U. Construction of pBG049 KPI (-4→57; M15A, S17Y, R18H)

Figure 53 outlines the construction of pBG049 KPI (-4→57; M15A, S17Y, R18H). Plasmid pBG022 encoding KPI (-4→57; M15A, S17Y, R18H, Y48H) was digested with *Aat*II and *Bam*HI and the 92 bp *Aat*II-*Bam*HI fragment was discarded. Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Aat*II and *Bam*HI. The resulting 92 bp *Aat*II-*Bam*HI fragment was isolated and ligated into the yeast vector to yield plasmid pBG048, encoding yeast ∝-factor fused to KPI (-4→57; M15A, S17Y, R18H).). Figure 23 shows the synthetic gene for the ∝-factor fusion with KPI variant (-4→57; M15A, S17Y, R18H). Figure 40 shows the amino acid sequence of KPI variant (-4→57; M15A, S17Y, R18H).

V. Construction of pBG050 KPI (-4→57; T9V, M15A, S17Y, R18H)

Figure 54 outlines the construction of pBG050 KPI (-4→57; T9V, M15A, S17Y, R18H). Plasmid pBG033 encoding KPI (-4→57; T9V, M15A, R18H, Y48H) was digested with *Aat*II and *Bam*HI and the 92 bp *Aat*II-*Bam*HI fragment was discarded.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Aat*II and *Bam*HI. The resulting 92 bp *Aat*II-*Bam*HI fragment was isolated and ligated into the yeast vector to yield plasmid pBG050, encoding yeast ∝-factor fused to KPI (-4→57; T9V, M15A, S17Y, R18H).). Figure 24 shows the synthetic gene for the ∝-factor fusion with KPI variant (-4→57; T9V, M15A, S17Y, R18H). Figure 41 shows the amino acid sequence of KPI variant (-4→57; T9V, M15A, S17Y, R18H).

W. Construction of pBG029 KPI (-4→57, T9V, M15L, S17Y, R18H)

Plasmid pBG015 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously.

1593:

CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCTG
AGGTTG

1642:

GACCAACCTCAGCTTGTTCAGAGCACACCTCTCTAACAA
CCTCTCTTTTAT

The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG015, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG029, encodes the 445 bp synthetic gene for the yeast ∝-factor-KPI (-4→57; T9V, M15L, S17F, R18H) fusion.

X. Selection of Library 16-19 with human factor Xa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor Xa (Haematologic Technologies, Inc., Essex Junction, VT) was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized Xa resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, $CaCl_2$, $MgCl_2$, 0.1% gelatin, and 0.05% Triton X-100). Approximately $4x10^{10}$ phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin,

were bound to 50 µl Xa resin in a total volume of 250 µl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the Xa resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on Xa-Sepharose, phagemid DNA was isolated and subjected to DNA sequence analysis.

Sequences in the randomized Ala^{14}-Ser^{17} region were compared with one another to identify consensus sequences appearing more than once. A phagemid was identified which encoded KPI (155; M15L, I16F, S17K).

Y. Construction of pDD131 KPI (-4→57; M15L, I16F, S17K)

The sequences encoding KPI (155; M15L, I16F, S17K) were moved from the phagemid vector to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of the phagemid vector was isolated and ligated into the yeast vector to yield plasmid pDD131, encoding yeast ∝-factor fused to KPI (-4→57; M15L, I16F, S17K).

Z. Construction of pDD134 KPI (-4→57; M15L, I16F, S17K, G37Y)

Plasmid pDD131 was digested with *Aat*I and *Bam*HI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (738 + 739) was phosphorylated, annealed and gel-purified as described previously.

738:

CACTGAAGGTAAGTGCGCTCCATTCTTTTACGGCGGTTGCTACGGCA
ACCGT

AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739:

GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACG
GTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGAC
GT

The annealed oligonucleotides were ligated into the *Aat*I and *Bam*HI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD134 encodes the 445 bp synthetic gene for the yeast ∝-factor-KPI (-4→57; M15L, I16F, S17K, G37Y) fusion.

AA. Construction of pDD135 KPI (-4→57; M15L, I16F, S17K, G37L)

Plasmid pDD131 was digested with *Aat*II and *Bam*HI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (738 + 739) was phosphorylated, annealed and gel-purified as described previously.

738:

CACTGAAGGTAAGTGCGCTCCATTCTTTTACGGCGGTTGCTACGGCA
ACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739:

GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACG
GTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGAC
GT

The annealed oligonucleotides were ligated into the *Aat*II and *Bam*HI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD135 encodes the 445 bp synthetic gene for the yeast ∝-factor-KPI (-4→57; M15L, I16F, S17K, G37L) fusion.

Example 4. Kinetic analysis of KPI(-4→57) variants

The concentrations of active human plasma kallikrein, factor XIIa, and trypsin were determined by titration with p-nitrophenyl p'-guanidinobenzoate as described by

Bender *et al., supra*, and Chase *et al., Biochem. Biophys. Res. Commun.* 29:508 (1967). Accurate concentrations of active KPI(-4→57) inhibitors were determined by titration of the activity of a known amount of active-site-titrated trypsin. For testing against kallikrein and trypsin, each KPI(-4→57) variant (0.5 to 100nM) was incubated with protease in low-binding 96-well microtiter plates at 30°C for 15-25 min, in 100mM Tris-HCl, pH 7.5, with 500mM NaCl, 5mM KCl, 5mM CaCl2, 5mM MgCl2, 0.1% Difco gelatin, and 0.05% Triton X-100. Chromogenic synthetic substrate was then be added, and initial rates at 30°C recorded by the SOFTmax kinetics program via a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA). The substrates used were N-α-benzoyl-L-Arg p-nitroanilide (1mM) for trypsin (20nM), and N-benzoyl-Pro-Phe-Arg p-nitroanilide (0.3mM) for plasma kallikrein (1nM). The Enzfitter (Elsevier) program was used both to plot fractional activity (*i.e.*, activity with inhibitor, divided by activity without inhibitor), a, versus total concentration of inhibitor, I_t, and to calculate the dissociation constant of the inhibitor (K_i) by fitting the curve to the following equation:

$$a = 1 - \frac{[E]_t + [I]_t + K_i - \sqrt{([E]_t + [I]_t + K_i)^2 - 4[E]_t[I]_t}}{2[E]_t}$$

The K_is determined for purified KPI variants are shown in Figures 63 and 69. The most potent variant, KPI (-4→57; M15A, S17F) DD185 is 115-fold more potent as a human kallikrein inhibitor than wild-type KPI (-4→57). The least potent variant, KPI (-4→57; I16H, S17W) TW6185 is still 35-fold more potent than wild-type KPI. Replacement of arginine at position 18 of the native KPI peptide with histidine (R18H) in combination with one or more additional substitutions at residues 9, 15 and 17 of the native KPI peptide also exhibited potent and specific serine protease inhibition toward selected serine proteases of interest than the native KPI peptide. In particular, the specific substitutions T9V, M15A, S17Y and M15A ,S17Y in the context of the R18H substitution exhibited such potent serine protease inhibition. *See* Figures 63 and 64. Substituting the native tyrosine at position 48 in these R18H substituted peptides with histidine (pBG022, 50D4, 50B6; Y48H), glutamine (50B6, 50L1, 50M1; Y48Q), alanine (50P5, 50C4; Y48A) or aspartic acid (50N1; Y48D) produced a significant

increase in their level of expression in comparison to the R18H substituted peptides without the position 48 substitution. *See* Figures 69B, E and F.

For testing against factor XIIa, essentially the same reaction conditions were used, except that the substrate was N-benzoyl-Ile-Glu-Gly-Arg p-nitroaniline hydrochloride and its methyl ester (obtained from Pharmacia Hepar, Franklin, OH), and corn trypsin inhibitor (Enzyme Research Laboratories, South Bend, IN) was used as a control inhibitor. Factor XIIa was also obtained from Enzyme Research Laboratories.

Various data for inhibition of the serine proteases of interest kallikrein, plasmin, and factors Xa, XIa, and XIIa by a series of KPI variants are given in Figure 64. The results indicate that KPI variants can be produced that can bind to and preferably inhibit the activity of serine proteases. The results also indicate that the peptides of the invention may exhibit the preferable more potent and specific inhibition of one or more serine proteases of interest.

Example 5. Effect of KPI variant KPI185-1 on postoperative bleeding

A randomized, double-blinded study using an acute porcine cardiopulmonary bypass (CPB) model was used to investigate the effect of KPI185-1 on postoperative bleeding. Sixteen pigs (55-65 kg) underwent 60 minutes of hypothermic (28°C) open-chest CPB with 30 minutes of cardioplegic cardiac arrest. Pigs were randomized against a control solution of physiological saline (NS; n=8) or KPI-185 (n=8) groups. During aortic cross-clamping, the tricuspid valve was inspected through an atriotomy which was subsequently repaired. Following reversal of heparin with protamine, dilateral thoracostomy tubes were placed and shed blood collected for 3 hours. Shed blood volume and hemoglobin (Hgb) loss were calculated from total chest tube output and residual intrathoracic blood at time of sacrifice.

Total blood loss was significantly reduced in the KPI185-1 group (245.75 $\pm$ 66.24 ml vs. 344.25 $\pm$ 63.97 ml, p=0.009). In addition, there was a marked reduction in total Hgb loss in the treatment group (13.59 $\pm$ 4.26 gm vs. 23.61 $\pm$ 4.69 gm, p=0.0005). Thoracostomy drainage Hgb was significantly increased at 30 and 60 minutes in the control group [6.89 $\pm$ 1.44 vs. 4.41 $\pm$ 1.45 gm/dl (p=0.004) and 7.6 $\pm$ 1.03 vs. 5.26 $\pm$ 1.04 gm/dl (p=0.0002), respectively]. Preoperative and post-CPB hematocrits were not statistically different between the groups. These results are shown in graphical form in Figures 65-68.

Example 6. Effect of KPI Variant KPI-BG022 on Transplant Rejection

KPI-BG022 was tested for its ability to delay transplant rejection in a rat model of acute xenograft rejection. Xenotransplantation of vascularized organs between discordant species results in hyperacute graft rejection within minutes to hours after graft reperfusion. Cardiac xenografts from male Hartley guinea pigs were heterotopically grafted into male rats that were complement deficient. Experimental animals received 5 mg/kg KPI-BG022 IV prior to reperfusion, and control animals received saline placebo. The data in Figure 70 demonstrate that a single KPI-BG022 dose significantly prolongs survival of guinea pig hearts grafted into complement-deficient rats.

Example 7. Effect of KPI Variant KPI-BG022 on Ulcerative Colitis

KPI-BG022 was tested in a rat model of TNBS (trinitrobenzene sulfonic acid) induced colitis. Animals were subjected to intracolonic instillation of TNBS to induce inflammation and ulceration. Tail-vein injection of KPI or vehicle was begun at the time of TNBS infusion and continued with three different dosing regimens: twice daily injections for 7 days; once daily injections for 7 days; and, two injections only in the day following injury. In each treatment group, half of the animals were sacrificed and scored for colonic injury 8 days following injury, and the remaining animals were sacrificed at 14 days. There were no significant differences in damage scores between saline or KPI treated animals sacrificed 8 days following injury. As shown in Figure 71, in all three dosing groups there was a significant reduction in damage in KPI-treated animals at 14 days after injury. Even the animals receiving only three doses of KPI in the 24 hours following injury showed significant reduction in colonic damage two weeks after the TNBS instillation.

Example 8. Effect of KPI Variant KPI-BG022 on Postoperative Bleeding

KPI-BG022 will be tested in an ovine model of cardiopulmonary bypass-associated pulmonary pathophysiology and blood loss and conducted as described in Friedman, M., Sellke, F.W., Wang, S.Y., Weintraub, R.M., and Johnson, R.G. (1994) *Circulation 90*: II262-II268; Friedman, M., Wang, S.Y., Sellke, F.W., Cohn, W.E., Weintraub, R.M., and Johnson, R.G. (1996) *J. Thorac. Cardiovasc. Surg. 111:* 460-468) with modifications as follows:

Surgical procedures:

Dorset-Rambouillet sheep (n=10 in each group) weighing 25-30 kg each will be anesthetized with intravenous 80 mg/kg alpha-chlorarose and 500 mg/kg urethane. Animals will be intubated and mechanically ventilated (Harvard Apparatus). Arterial blood gas and pH measurements will be performed during the procedure (pH blood gas analyzer 1306, Instruments Lab, Lexington, MA) and alpha-stat pH management will be used during CPB. Systemic arterial pressure will be continuously monitored after direct cannulation of the femoral artery, and a separate femoral artery cannula will be used for blood collection. A jugular vein cannula will be used for drug administration. Lymph fluid will be collected from the lungs as follows: the efferent duct of the caudal mediastinal lymph node will be cannulated through a right thoracotomy in the fifth intercostal space using a silicone, heparin-coated catheter.

CPB preparation:

A midline sternotomy will be performed and the pulmonary artery (PA) isolated and surrounded with an ultrasonic flowmeter (Transonic System, Ithaca, NY). Animals will be heparinized to achieve an activated clotting time (ACT) > 750 seconds as monitored using a Hemochron device. At the end of CPB the heparin will be reversed with protamine sulphate to baseline ACT. A catheter will be inserted into the left atrium (LA) for blood withdrawl and pressure recording, and the PA will be cannulated for continuous pressure monitoring. Venous drainage will be provided by a cannula in the right atrium (RA) and an aortic perfusion catheter will be placed in the aorta. The extracorporeal circuit will consist of a roller pump (Cardiovascular Instruments, Wakefield, MA) and bubble oxygenator (Bently Bio-2, Baxter Health Care). The circuit will be primed with 1 l lactated Ringer's solution.

Myocardial protection will be provided by antegrade cold blood cardioplegia at 4°C using a 4:1 ratio of autologous blood to crystalloid cardioplegia (KCl 60 meq, mannitol 12.5 g, citrate-phosphate-dextrose solution 50 mL, THAM 10 meq, 5% dextrose and saline 0.225% QS). Iced slush will be used for topicial cooling to augment the cardioplegia. Immediately after application of the aortic cross-clamp cardioplegia will be given until arrest of the heart and then reinfused every 20

minutes. With institution of CPB all animals will be cooled to a core temperature of 27°C. After a mean time of 50 minutes, rewarming will be commenced approximately 10 minutes before removal of aortic cross-clamp to achieve a core temperature of 37°C at the termination of bypass. Flow will be maintained to keep aortic mean pressure not less than 40 mm/Hg. Norepinephrine bitartrate injection will be given through the CVP line to all animals after termination of CPB with an incrementally decreasing infusion rate until the infusion is stopped one hour post-CPB.

Physiologic and biochemical determinations:

Hemodyamic measurements will be made before institution of CPB (baseline), every 30 minutes during bypass and every 15 minutes for the first hour after termination of CPB. Thereafter measurements will be made every 30 minutes for 3 hours. Cardiac output will be determined as pulmonary artery flow (Qpa in L/min) or, during CPB, as pump flow. Cardiac index (CI), systemic vascular index (SVRI), pulmonary vascular resistance index (PVRI), will be calculated by standard equations. Simultaneous with the hemodynamic measurements, 2 ml blood samples will be collected from left and right artria and placed into ice-cooled EDTA tubes. Hematocrit, blood gases, and oxygen content will be measured for each sample. After blood is centrifuged, supernatant platelet counts and white blood cell counts will be performed.

Lymph collection and measurements:

Lymph volume will be measured and the protein content determined. Lymph protein clearance will be calculated as milliliters lung lymph flow per 30 minutes x lymph:plasma protein ratio. Protein clearance is considered reflective of the degree to which larger molecules leak into the lymph, as an indication of damage greater than that seen with lymph fluid flow alone.

Study protocol:

A double-blind study will be performed. Sheep will be randomized to 3 groups of 10 animals each: Group 1 = saline control; Group 2 = KPI-BG022 dose 1; and, Group 3 = KPI-BG022 dose 2. Vehicle and KPI-BG022 will be formulated and aliquoted into coded tubes such that after anesthesia each animal will receive a

loading dose of 100 ml, a 100 ml pump prime and a 25 ml/hr infusion during the course of CPB. We propose to test two total doses of the KPI-BG022 variant: 5 mg/kg, and 0.5 mg/kg. Therefore, Group 2 will receive a 70 mg loading dose of KPI-BG022, a 70 mg pump prime and 18 mg/hr infusion. Group 3 will receive a 7 mg loading dose, a 7 mg pump prime and 1.8 mg/hr infusion..

Total, non-pulsatile hypothermic CPB will be continued for 90 minutes with a cross-clamp time of 1 hour. Rewarming will start 10 minutes before removal of the cross-clamp and will be continued until a core temperature of 37°C is attained. CPB will be terminated when core temperature has stabilized at 37°C. Post-CPB monitoring will continue for 3 hours. Protamine will be given in the first 30 minutes post-CPB, and when ACT has been reduced to baseline levels the chest will be closed with a large-bore thoracostomy tube left in place for drainage.

Blood and hemoglobin loss measurements:

The thoracostomy tube will be connected to a drainage system and suction applied at a force of 10 kPa. Drain losses will be collected for a total of two hours post-CPB, and then the sternotomy wound will be reopened and all shed blood will be aspirated from the thorax and pericardium. The volume of blood loss and hemoglobin will be measured and used to calculate the total hemoglobin loss in grams.

Based on previous experience with this (Friedman *et al.*, 1994; Friedman *et al.*, 1996) model the control group should demonstrate several parameters of pulmonary injury, including increases in: pulmonary vascular resistance (PVR) (170% increase reported), pulmonary lymph flow (233% reported), and lung water (15% reported). An increase in sequestration of WBCs and platelets in the lung should be seen in the control group. Arterial oxygenation (PaO_2) should fall significantly upon cessation of CPB with a gradual recovery in the post-bypass period.

With respect to blood and hemoglobin loss in the post-bypass period, our experience with KPI-wt in another sheep model of CPB (Ohri *et al*, *Ann. Thorac. Surg.* 1996 Apr;61(4):1223-1230) leads us to anticipate collection of 200-400 ml blood in chest drains in the control group, containing 10-20 g hemoglobin. In that study, recombinant KPI was assessed in an ovine model of CPB as a hemostatic agent after CPB. Sheep (n = 22) underwent CPB for 90 minutes. Two thoracic drains were sited and drain losses collected for a period of 3 hours after CPB. Wounds were

subjectively assessed before closure for "dryness" using a visual analogue scale. Sheep were randomized to control (n = 8), aprotinin (n = 8), and rKPI (n = 6) groups. Control animals had a drain loss of 409.4 +/- 39.4 mL/3 h, compared with 131.3 +/- 20.3 mL/3 h for the aprotinin group and 163.7 +/- 34.3 mL/3 h for the rKPI group ($p = 0.16$). Hemoglobin loss was 11.6 +/- 3.6, 6.02 +/- 2.1, and 4.6 +/- 1.2 g/3 h for the control, rKPI, and aprotinin groups respectively ($p = 0.25$). The subjective analysis of the wounds at the end of CPB found aprotinin (1.25 +/- 0.16; $p < 0.05$) and rKPI (1.17 +/- 0.17; $p < 0.05$) animals to score significantly lower than control animals (2.63 +/- 0.42).

Indeed, significant reductions in blood and hemoglobin loss in the KPI-BG022 treated groups are expected. Measureable reductions in one or more of the other parameters of post-bypass pulmonary injury are also expected in the KPI-BG022 treated group. Positive results in this regard would include smaller increases or no increases in PVR, pulmonary lymph flow or lung water content in the KPI-treated groups, as well as reduced WBCs and platelets sequestered in the lungs. One significant indication of improved pulmonary function in the KPI groups would be improved arterial oxygenation in the immediate post-bypass period.

The invention has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof.

What Is Claimed Is:

1. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^{13}-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, and Glu;

X^2 is selected from Thr, Val, Ile and Ser;

X^3 is selected from Pro and Ala;

X^4 is selected from Arg, Ala, Leu, Gly, and Met;

X^5 is selected from Ile, His, Leu, Lys, Ala, and Phe;

X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, and Glu;

X^7 is selected from Arg, His, and Ala;

X^8 is selected from Phe, Val, Leu, and Gly;

X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, and Tyr;

X^{10} is selected from Ala, Arg, and Gly;

X^{11} is selected from Lys, Ala, and Asn;

X^{12} is selected from Ser, Ala, and Arg;

X^{13} is selected from His, Gln, Ala, and Asp.

2. A protease inhibitor according to claim 1, wherein X^1 is Asp-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Met, X^5 is Ile, X^6 is Ser or Tyr, X^7 is His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg.

3. A protease inhibitor according to claim 1, wherein X^1 is Asp-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Ala, X^5 is Ile, X^6 is Phe, X^7 is Arg, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg.

4. A protease inhibitor according to claim 2, wherein X^2 is Thr or Val.

5. A protease inhibitor according to claim 4, wherein X^2 is Thr.

6. A protease inhibitor according to claim 4, wherein X^2 is Val.

7. A protease inhibitor according to claim 2, wherein X^2 is Thr or Val, and X^4 is Ala.

8. A protease inhibitor according to claim 2, wherein X^2 is Thr or Val, and X^4 is Met.

9. A protease inhibitor according to claim 2, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is His.

10. A protease inhibitor according to claim 2, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Gln.

11. A protease inhibitor according to claim 2, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Ala.

12. A protease inhibitor according to claim 2, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Asp.

13. A protease inhibitor according to claim 2, wherein X^2 is Thr, X^4 is Met, X^6 is Ser, and X^{13} is selected from His, Ala, or Gln.

14. A protease inhibitor according to claim 2, wherein X^2 is Val, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln.

15. A protease inhibitor according to claim 2, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln.

16. A protease inhibitor according to claim 14, wherein X^{13} is selected from His or Ala.

17. A protease inhibitor according to claim 15, wherein X^{13} is selected from His or Ala.

18. A protease inhibitor according to claim 16, wherein X^{13} is His.

19. A protease inhibitor according to claim 16, wherein X^{13} is Ala.

20. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1.

21. An isolated DNA molecule according to claim 20, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

22. An isolated DNA molecule according to claim 21, further comprising a DNA sequence encoding a secretory signal peptide.

23. An isolated DNA molecule according to claim 22, wherein said secretory signal peptide comprises the signal sequence of yeast ∝-mating factor.

24. A host cell transformed with a DNA molecule according to claim 20.

25. A host cell according to claim 24, wherein said host cell is *E. coli* or a yeast cell.

26. A host cell according to claim 25, wherein said host cell is a yeast cell.

27. A host cell according to claim 26, wherein said yeast cell is *Saccharomyces cerevisiae.*

28. A host cell according to claim 26, wherein said yeast cell is *Pichia pastoris.*

29. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 24 and isolating and purifying said protease inhibitor.

30. A pharmaceutical composition, comprising a protease inhibitor according to claim 1, together with a pharmaceutically acceptable sterile vehicle.

31. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 30.

32. The method of treatment of claim 31, wherein said clinical condition is blood loss during surgery.

33. A method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition according to claim 30.

34. The method of claim 33, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

35. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-Ala-Ile-Tyr-His-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^3-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, or Glu;

X^2 is selected from Thr and Val;

X^3 is selected from His, Gln, Ala, or Asp.

36. A protease inhibitor according to claim 35, wherein X^1 is Glu-Val-Val-Arg-Glu.

37. A protease inhibitor according to claim 36, wherein X^2 is Thr.

38. A protease inhibitor according to claim 36, wherein X^2 is Val.

39. A protease inhibitor according to claim 38, wherein X^3 is His.

40. A protease inhibitor according to claim 38, wherein X^3 is Gln.

41. A protease inhibitor according to claim 38, wherein X^3 is Ala.

42. A protease inhibitor according to claim 38, wherein X^3 is Asp.

43. A protease inhibitor according to claim 35, wherein X^1 is Asp-Val-Val-Arg-Glu.

44. A protease inhibitor according to claim 43, wherein X^2 is Thr.

45. A protease inhibitor according to claim 43, wherein X^2 is Val.

46. A protease inhibitor according to claim 45, wherein X^3 is His.

47. A protease inhibitor according to claim 45, wherein X^3 is Gln.

48. A protease inhibitor according to claim 45, wherein X^3 is Ala.

49. A protease inhibitor according to claim 45, wherein X^3 is Asp.

50. A protease inhibitor according to claim 35, wherein X^1 is Glu.

51. A protease inhibitor according to claim 50, wherein X^2 is Thr.

52. A protease inhibitor according to claim 50, wherein X^2 is Val.

53. A protease inhibitor according to claim 52, wherein X^3 is His.

54. A protease inhibitor according to claim 52, wherein X^3 is Gln.

55. A protease inhibitor according to claim 52, wherein X^3 is Ala.

56. A protease inhibitor according to claim 52, wherein X^3 is Asp.

57. A protease inhibitor according to claim 35, wherein X^1 is Asp.

58. A protease inhibitor according to claim 57, wherein X^2 is Thr.

59. A protease inhibitor according to claim 57, wherein X^2 is Val.

60. A protease inhibitor according to claim 59, wherein X^3 is His.

61. A protease inhibitor according to claim 59, wherein X^3 is Gln.

62. A protease inhibitor according to claim 59, wherein X^3 is Ala.

63. A protease inhibitor according to claim 59, wherein X^3 is Asp.

64. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Met, X^5 is Ile, X^6 is Ser or Tyr, X^7 is His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg.

65. A protease inhibitor according to claim 64, wherein X^2 is Thr or Val.

66. A protease inhibitor according to claim 65, wherein X^2 is Thr.

67. A protease inhibitor according to claim 65, wherein X^2 is Val.

68. A protease inhibitor according to claim 64, wherein X^2 is Thr or Val, and X^4 is Ala.

69. A protease inhibitor according to claim 64, wherein X^2 is Thr or Val, and X^4 is Met.

70. A protease inhibitor according to claim 64, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is His.

71. A protease inhibitor according to claim 64, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Gln.

72. A protease inhibitor according to claim 64, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Ala.

73. A protease inhibitor according to claim 64, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is Asp.

74. A protease inhibitor according to claim 64, wherein X^2 is Thr, X^4 is Met, X^6 is Ser, and X^{13} is selected from His, Ala, or Gln.

75. A protease inhibitor according to claim 64, wherein X^2 is Val, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln.

76. A protease inhibitor according to claim 64, wherein X^2 is Thr, X^4 is Ala, X^6 is Tyr, and X^{13} is selected from His, Ala, or Gln.

77. A protease inhibitor according to claim 75, wherein X^{13} is selected from His or Ala.

78. A protease inhibitor according to claim 76, wherein X^{13} is selected from His or Ala.

79. A protease inhibitor according to claim 77, wherein X^{13} is His.

80. A protease inhibitor according to claim 77, wherein X^{13} is Ala.

81. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 18.

82. An isolated DNA molecule according to claim 81, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

83. An isolated DNA molecule according to claim 82, further comprising a DNA sequence encoding a secretory signal peptide.

84. An isolated DNA molecule according to claim 83, wherein said secretory signal peptide comprises the signal sequence of yeast ∝-mating factor.

85. A host cell transformed with a DNA molecule according to claim 81.

86. A host cell according to claim 85, wherein said host cell is *E. coli* or a yeast cell.

87. A host cell according to claim 86, wherein said host cell is a yeast cell.

88. A host cell according to claim 87, wherein said yeast cell is *Saccharomyces cerevisiae.*

89. A host cell according to claim 87, wherein said yeast cell is *Pichia pastoris.*

90. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 85 and isolating and purifying said protease inhibitor.

91. A pharmaceutical composition, comprising a protease inhibitor according to claim 18, together with a pharmaceutically acceptable sterile vehicle.

92. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 91.

93. The method of treatment of claim 92, wherein said clinical condition is blood loss during surgery.

94. A method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition according to claim 91.

95. The method of claim 94, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

96. A method for increasing the expression levels of recombinant protease inhibitors comprising the step of culturing a host cell transformed with an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1.

97. The method according to claim 96, wherein said host cell is *E. coli* or a yeast cell.

98. The method according to claim 97, wherein said host cell is a yeast cell.

99. The method according to claim 98, wherein said yeast cell is *Saccharomyces cerevisiae.*

100. The method according to claim 98, wherein said yeast cell is *Pichia pastoris.*

101. A method for increasing the yield of recombinant protease inhibitors comprising the step of culturing a host cell transformed with an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1, wherein X^1 is Asp-Val-Val-Arg-Glu-, and isolating and purifying said protease inhibitor.

102. The method according to claim 101, wherein said host cell is a yeast cell.

103. The method according to claim 102, wherein said yeast cell is *Saccharomyces cerevisiae.*

104. The method according to claim 102, wherein said yeast cell is *Pichia pastoris.*

105. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-X^3-Ile-X^4-X^5-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^6-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, or Glu;

X^2 is selected from Thr or Val;

X^3 is selected from Arg and Met;

X^4 is selected from Ser and Tyr;

X^5 is selected from Arg, His, or Ala; and

X^6 is selected from His, Gln, Ala or Asp.

106. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-Leu-Phe-Lys-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Leu-Gly-Asn-

Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-X^2-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp-Val-Val-Arg-Glu-, Asp, and Glu;

X^2 is selected from His, Gln, Ala, and Asp.

107. A protease inhibitor according to claim 106, wherein X^1 is Asp-Val-Val-Arg-Glu.

108. A protease inhibitor according to claim 107, wherein X^2 is His.

109. A protease inhibitor according to claim 107, wherein X^2 is Gln.

110. A protease inhibitor according to claim 107, wherein X^2 is Ala.

111. A protease inhibitor according to claim 107, wherein X^2 is Asp.

112. A protease inhibitor according to claim 106, wherein X^1 is Glu-Val-Val-Arg-Glu.

113. A protease inhibitor according to claim 112, wherein X^2 is His.

114. A protease inhibitor according to claim 112, wherein X^2 is Gln.

115. A protease inhibitor according to claim 112, wherein X^2 is Ala.

116. A protease inhibitor according to claim 112, wherein X^2 is Asp.

117. A protease inhibitor according to claim 106, wherein X^1 is Asp.

118. A protease inhibitor according to claim 117, wherein X^2 is His.

119. A protease inhibitor according to claim 117, wherein X^2 is Gln.

120. A protease inhibitor according to claim 117, wherein X^2 is Ala.

121. A protease inhibitor according to claim 117, wherein X^2 is Asp.

122. A protease inhibitor according to claim 106, wherein X^1 is Glu.

123. A protease inhibitor according to claim 122, wherein X^2 is His.

124. A protease inhibitor according to claim 122, wherein X^2 is Gln.

125. A protease inhibitor according to claim 122, wherein X^2 is Ala.

126. A protease inhibitor according to claim 122, wherein X^2 is Asp.

127. A protease inhibitor comprising the sequence:

Asp-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-Leu-Phe-Lys-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Leu-Gly-Asp-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile.

Figure 1

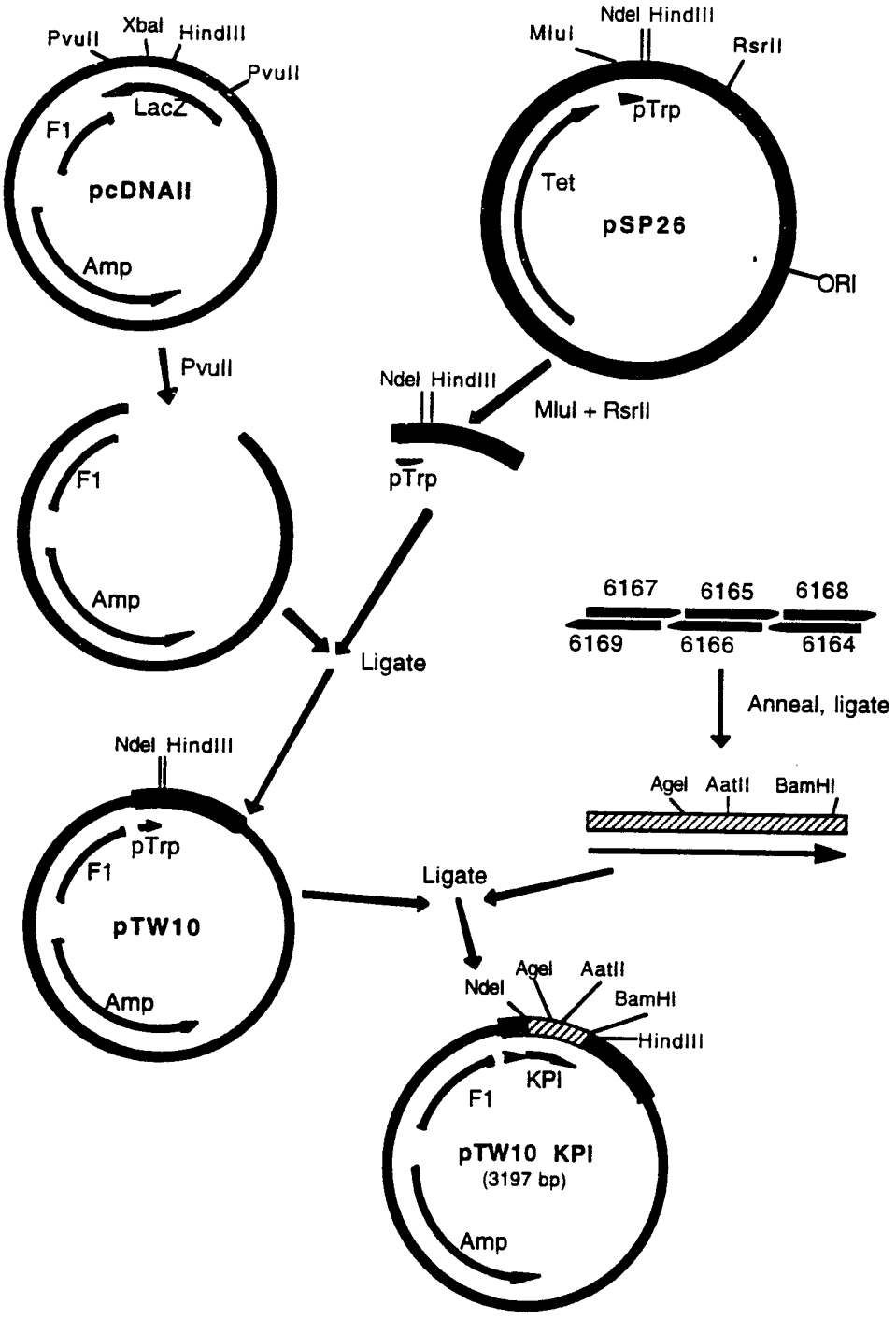

PvuII
XbaI
HindIII
PvuII
LacZ
F1
pcDNAII
Amp
PvuII
F1
Amp
NdeI HindIII
MluI
RsrII
pTrp
Tet
pSP26
ORI
NdeI HindIII
MluI + RsrII
pTrp
Ligate
6167
6165
6168
6169
6166
6164
Anneal, ligate
AgeI AatII BamHI
NdeI HindIII
pTrp
F1
pTW10
Amp
Ligate
NdeI
AgeI
AatII
BamHI
HindIII
KPI
F1
pTW10 KPI
(3197 bp)
Amp

Figure 2

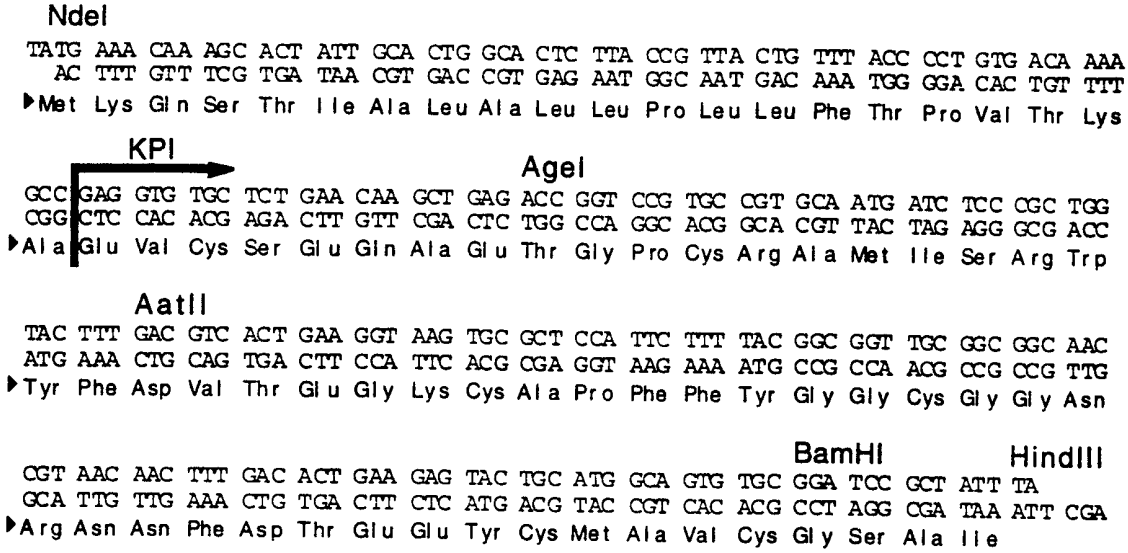

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AC TTT GTT TCG TGA TAA CGT GAC CGT GAG AAT GGC AAT GAC AAA TGG GGA CAC TGT TTT
Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys
KPI
AgeI
GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
CGG CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC
Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp
AatII
TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
ATG AAA CTG CAG TGA CTT CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG
Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn
BamHI
HindIII
CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 3

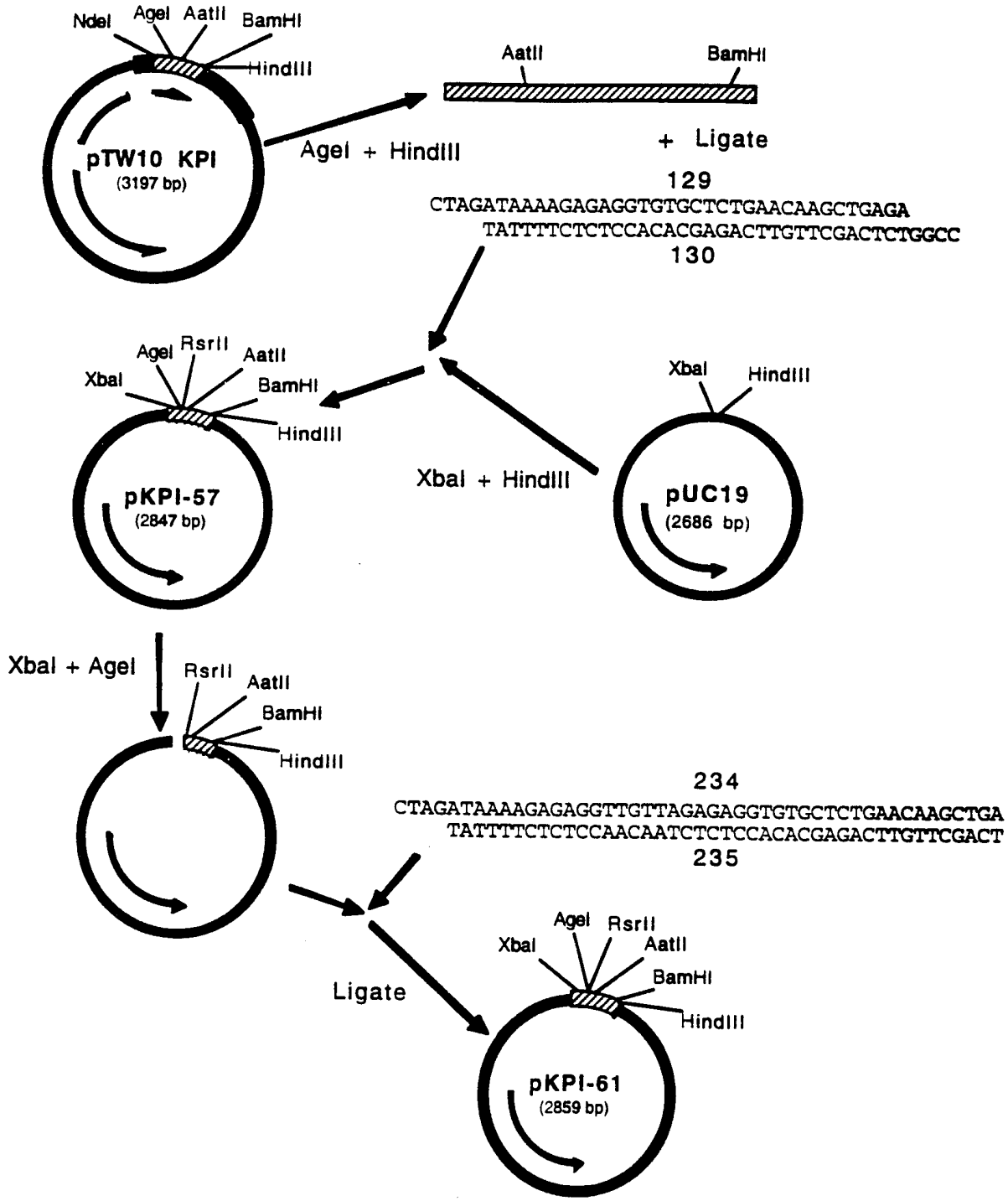

NdeI
AgeI
AatII
BamHI
HindIII
pTW10 KPI
(3197 bp)
AgeI + HindIII
AatII
BamHI
+ Ligate
129
CTAGATAAAAGAGAGGTGTGCTCTGAACAAGCTGAGA
TATTTTCTCTCCACACGAGACTTGTTCGACTCTGGCC
130
AgeI
RsrII
AatII
XbaI
BamHI
HindIII
pKPI-57
(2847 bp)
XbaI + HindIII
XbaI
HindIII
pUC19
(2686 bp)
XbaI + AgeI
RsrII
AatII
BamHI
HindIII
234
CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCTGA
TATTTTCTCTCCAACAATCTCTCCACACGAGACTTGTTCGACT
235
Ligate
AgeI
RsrII
XbaI
AatII
BamHI
HindIII
pKPI-61
(2859 bp)

Figure 4

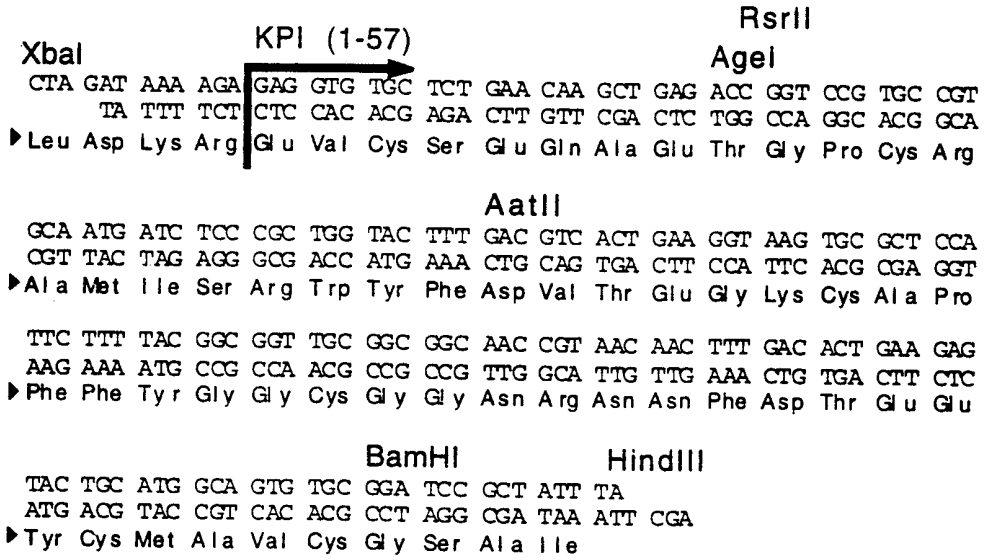

RsrII
KPI (1-57)
XbaI
AgeI
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TA TTT TCT CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA
Leu Asp Lys Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg
AatII
GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA
CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT CCA TTC ACG CGA GGT
Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro
TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA GAG
AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG TGA CTT CTC
Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu
BamHI
HindIII
TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 5

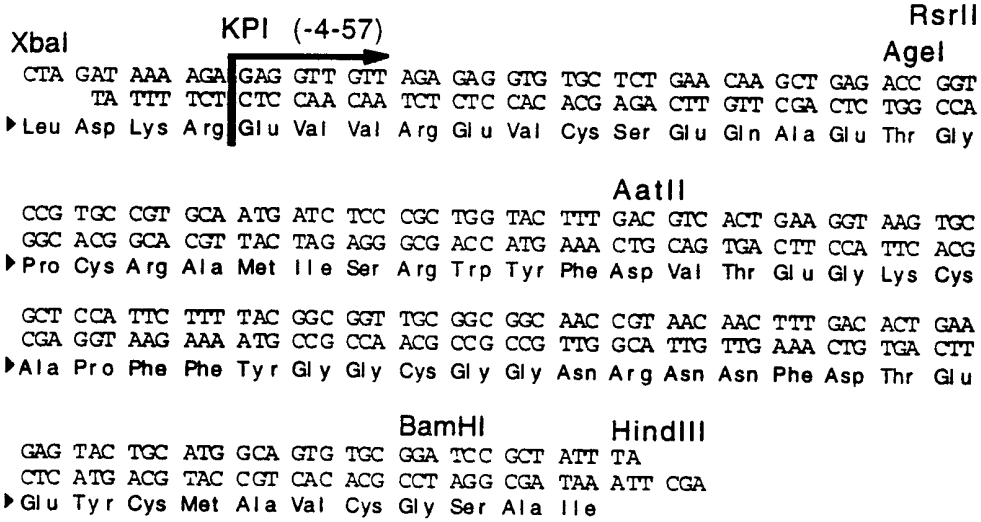

RsrII
KPI (-4-57)
XbaI
AgeI
CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT
TA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA
Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly
AatII
CCG TGC CGT GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC
GGC ACG GCA CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT CCA TTC ACG
Pro Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys
GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG TGA CTT
Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu
BamHI
HindIII
GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 6

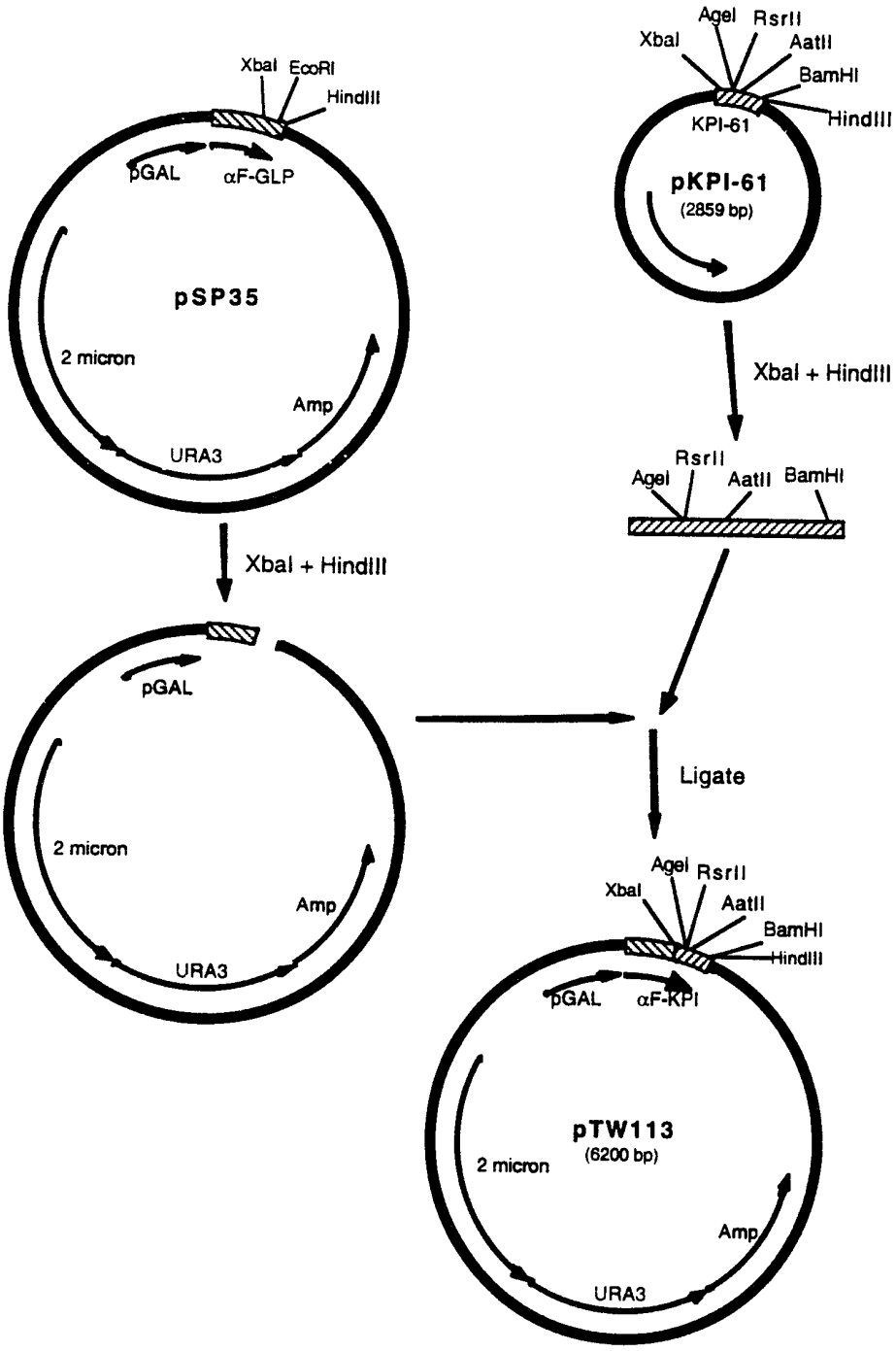

XbaI
EcoRI
HindIII
pGAL
αF-GLP
pSP35
2 micron
Amp
URA3
XbaI + HindIII
pGAL
2 micron
Amp
URA3
AgeI
RsrII
XbaI
AatII
BamHI
HindIII
KPI-61
pKPI-61
(2859 bp)
XbaI + HindIII
RsrII
AgeI
AatII
BamHI
Ligate
AgeI
RsrII
XbaI
AatII
BamHI
HindIII
pGAL
αF-KPI
pTW113
(6200 bp)
2 micron
Amp
URA3

Figure 7

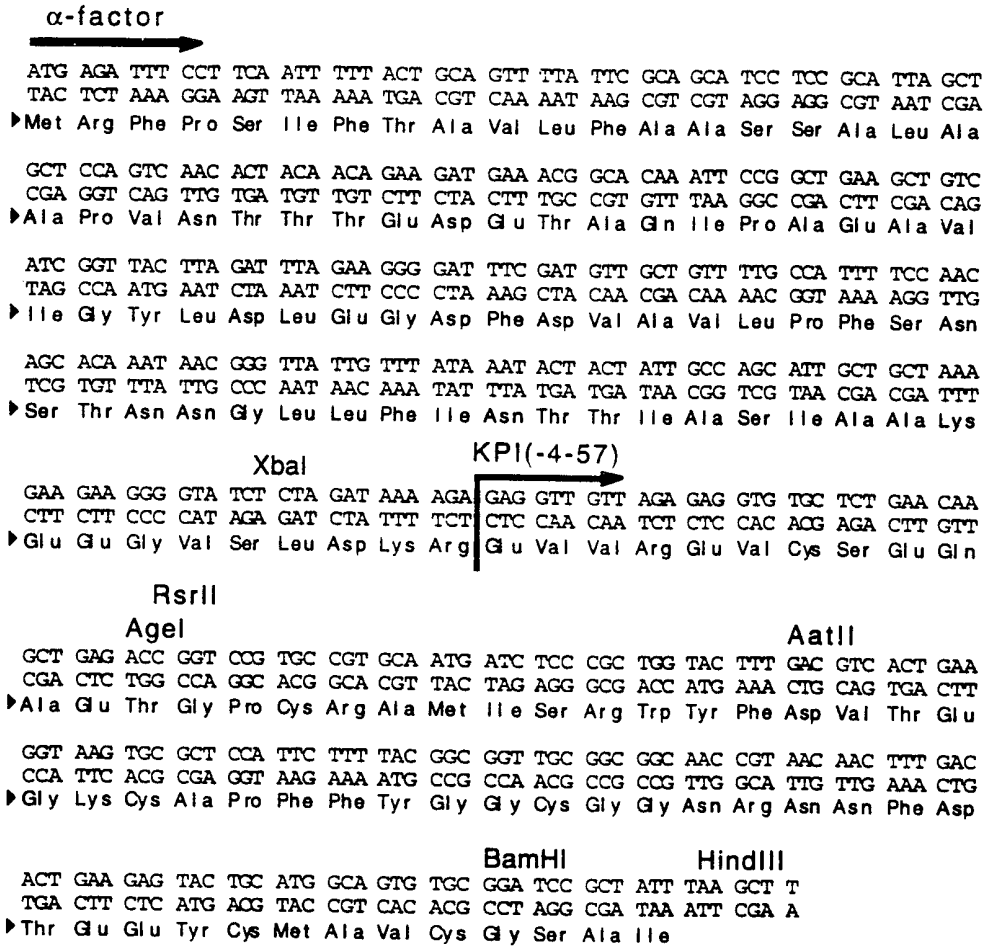

α-factor
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TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure8

KPI(-4-57)

Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7

Glu	Thr	Gly	Pro	Cys	Arg	Ala	Met	Ile	Ser	Arg
8	9	10	11	12	13	14	15	16	17	18

Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys	Ala
19	20	21	22	23	24	25	26	27	28	29

Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg
30	31	32	33	34	35	36	37	38	39	40

Asn	Asn	Phe	Asp	Thr	Glu	Glu	Tyr	Cys	Met	Ala
41	42	43	44	45	46	47	48	49	50	51

Val	Cys	Gly	Ser	Ala	Ile
52	53	54	55	56	57

Figure 9

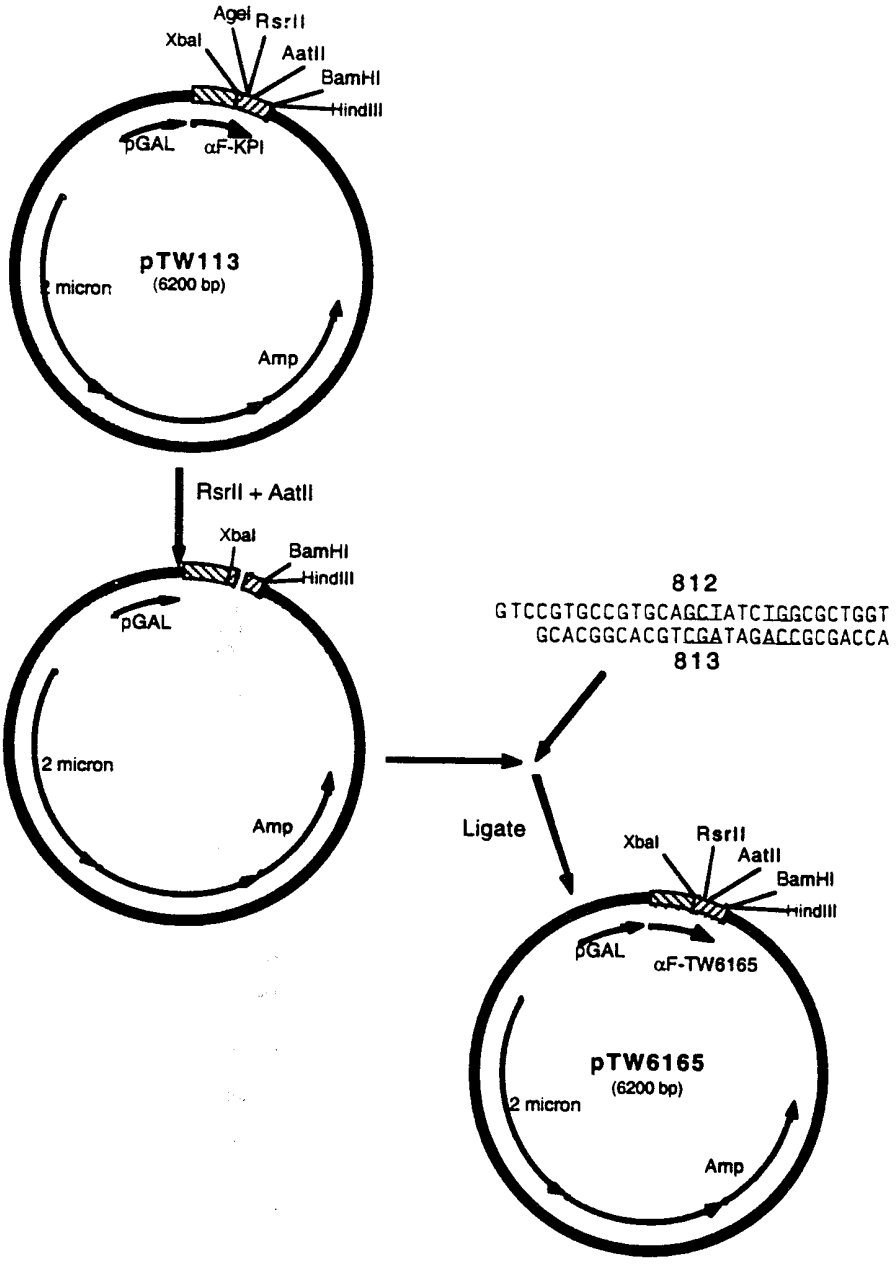

AgeI
RsrII
XbaI
AatII
BamHI
HindIII
pGAL
αF-KPI
pTW113
(6200 bp)
2 micron
Amp
RsrII + AatII
XbaI
BamHI
HindIII
pGAL
2 micron
Amp
812
GTCCGTGCCGTGCAGCIATCIGGCGCTGGT
GCACGGCACGTCGATAGACCGCGACCA
813
Ligate
XbaI
RsrII
AatII
BamHI
HindIII
pGAL
αF-TW6165
pTW6165
(6200 bp)
2 micron
Amp

Figure 10

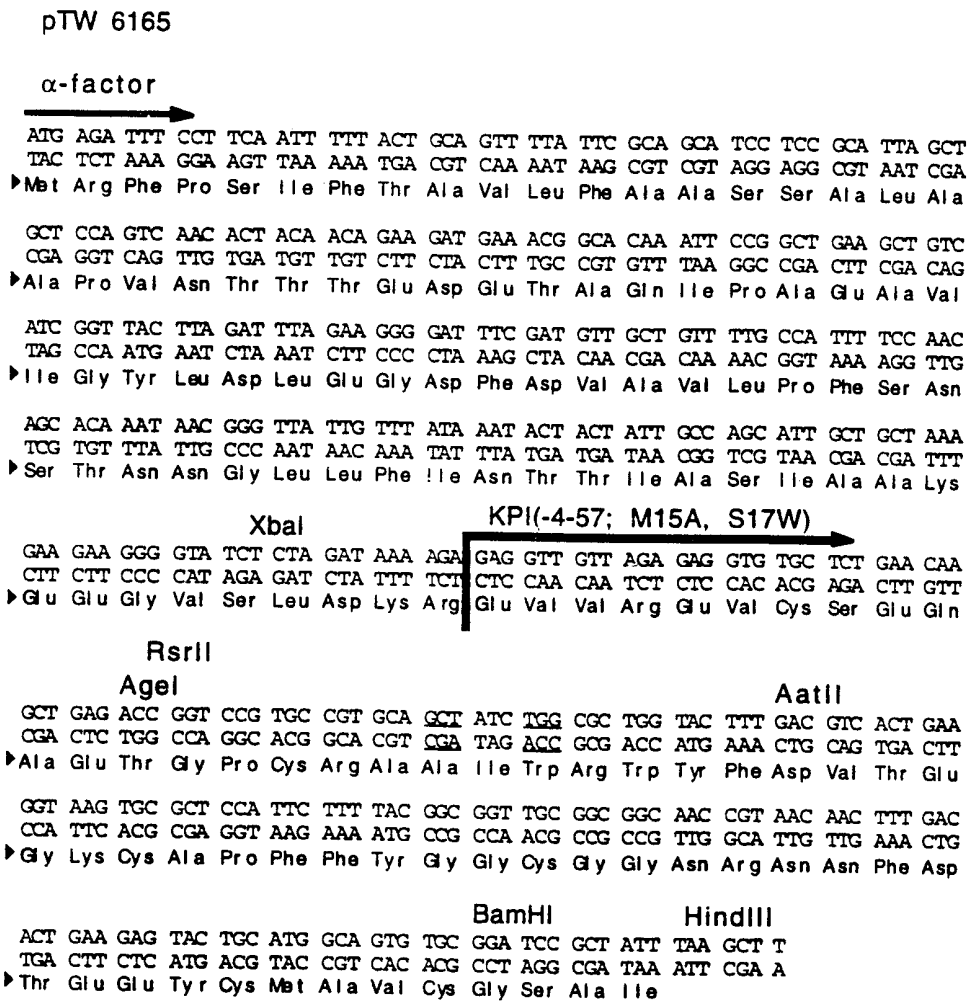

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Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; M15A, S17W)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TGG CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ACC GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Trp Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
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Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 11

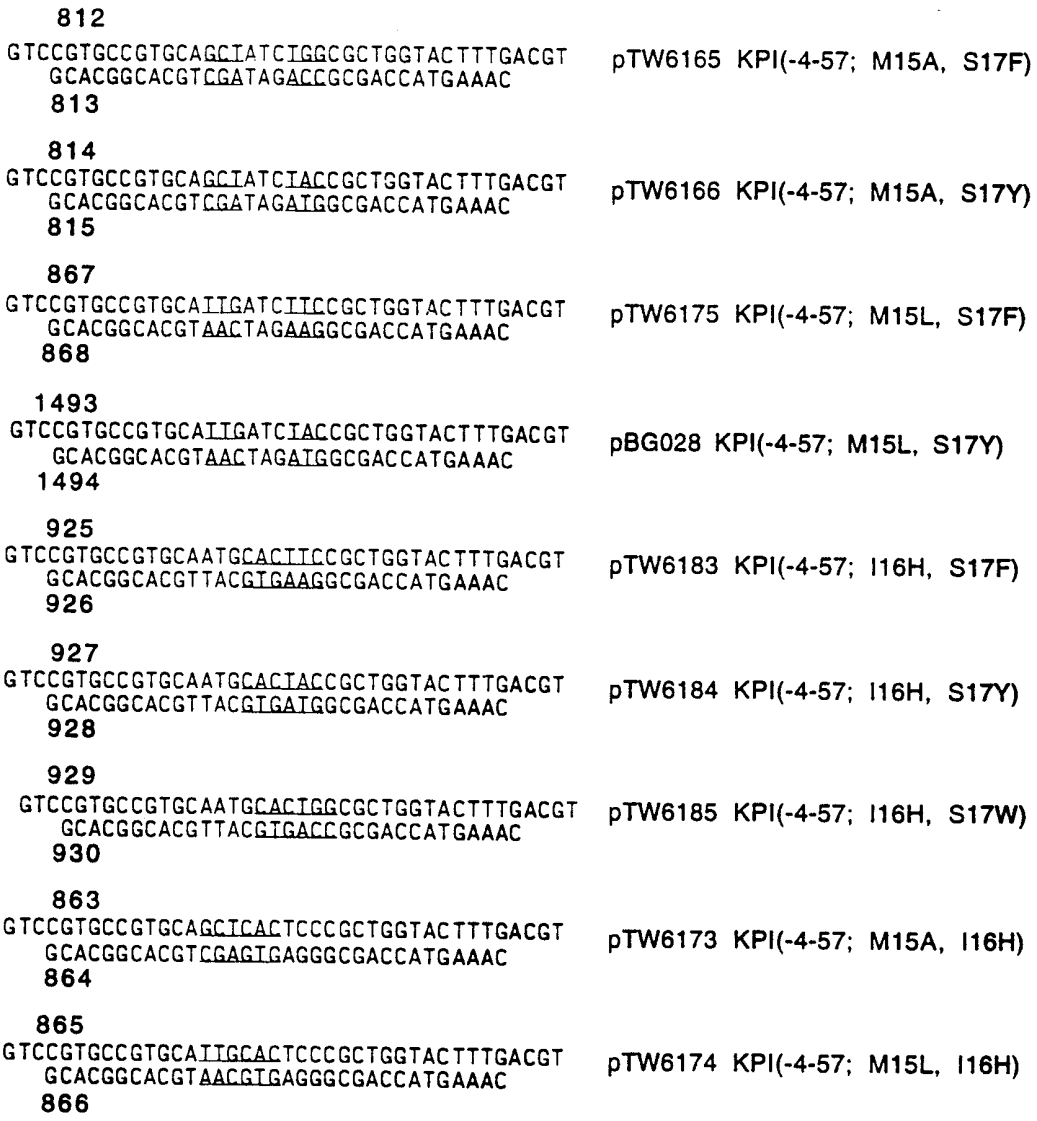

812
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813
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814
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815
pTW6166 KPI(-4-57; M15A, S17Y)
867
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868
pTW6175 KPI(-4-57; M15L, S17F)
1493
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1494
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925
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926
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927
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928
pTW6184 KPI(-4-57; I16H, S17Y)
929
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GCACGGCACGTTACGTGACCGCGACCATGAAAC
930
pTW6185 KPI(-4-57; I16H, S17W)
863
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864
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Figure 12

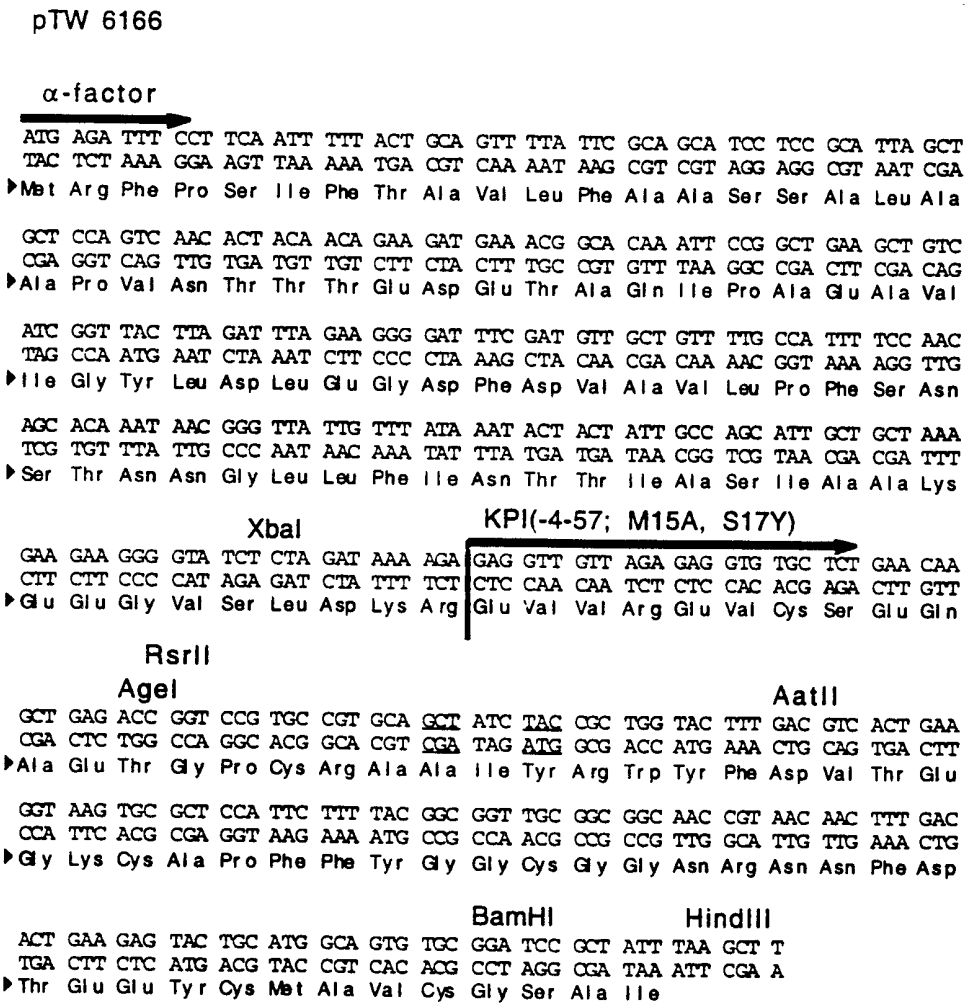

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α-factor
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Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; M15A, S17Y)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 13

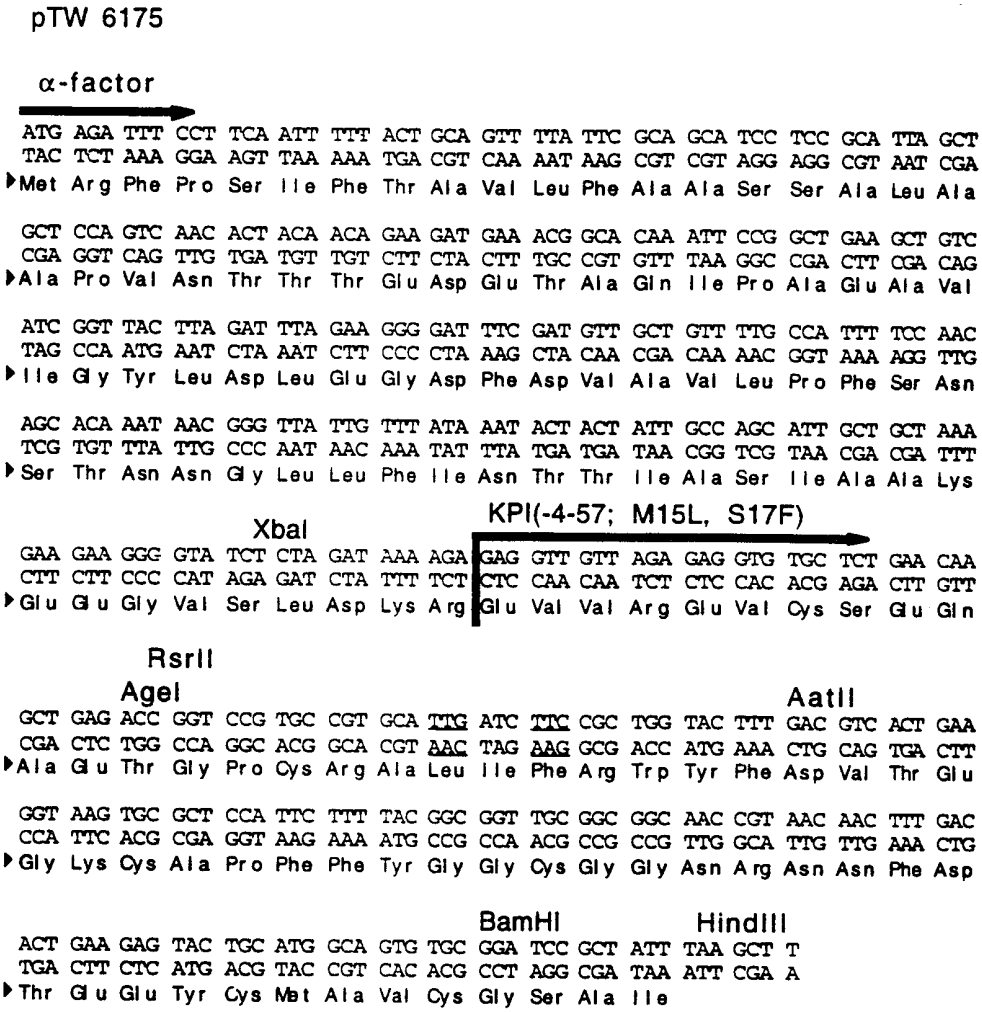

pTW 6175
α-factor
KPI(-4-57; M15L, S17F)
XbaI
RsrII
AgeI
AatII
BamHI
HindIII

Figure 14

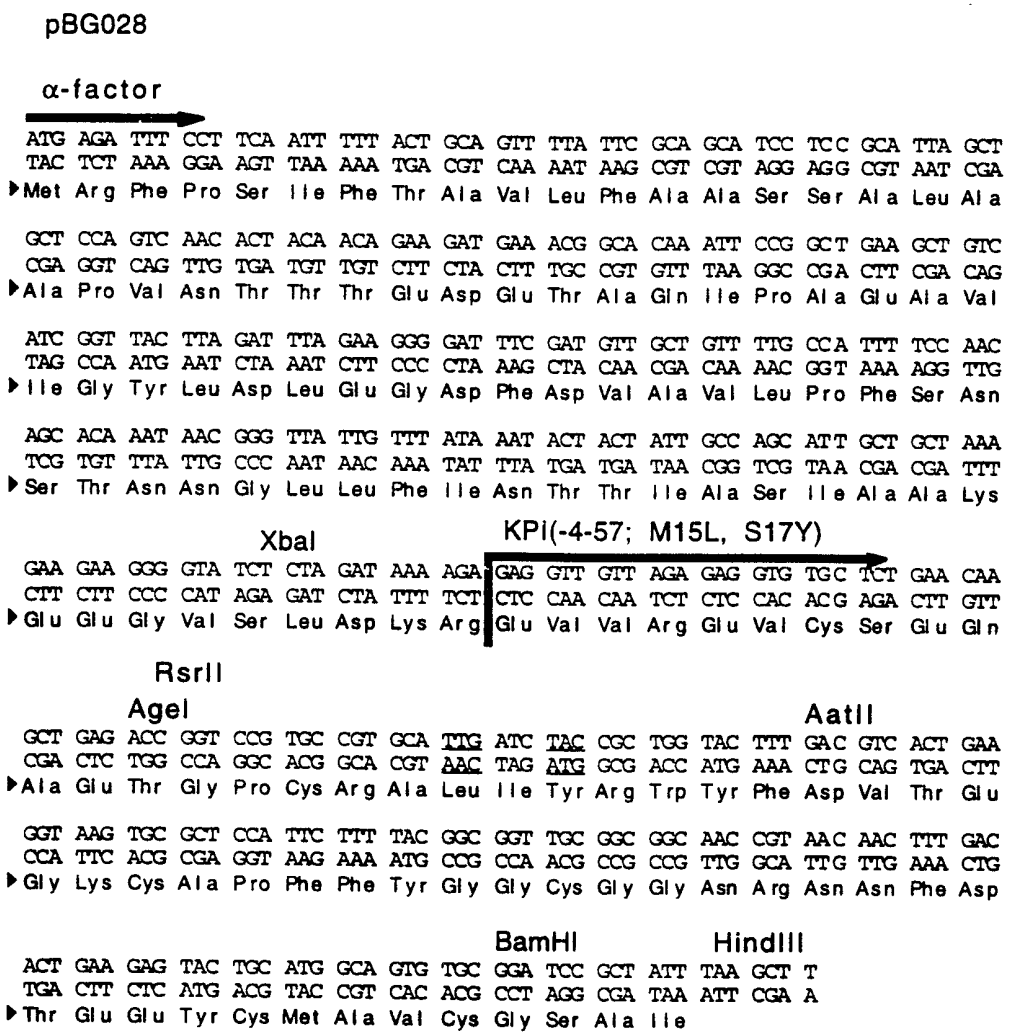

pBG028
α-factor
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TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; M15L, S17Y)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 15

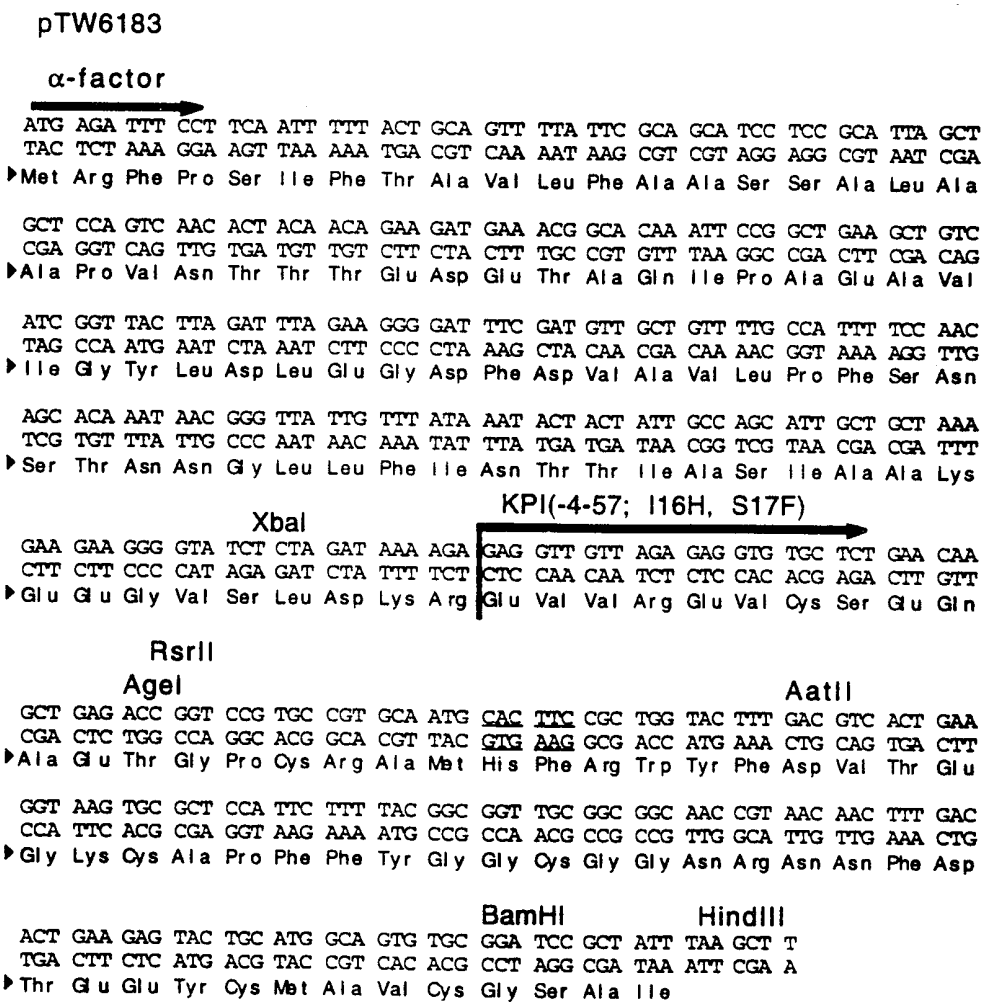

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α-factor
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TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; I16H, S17F)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TTC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG AAG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Met His Phe Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 16

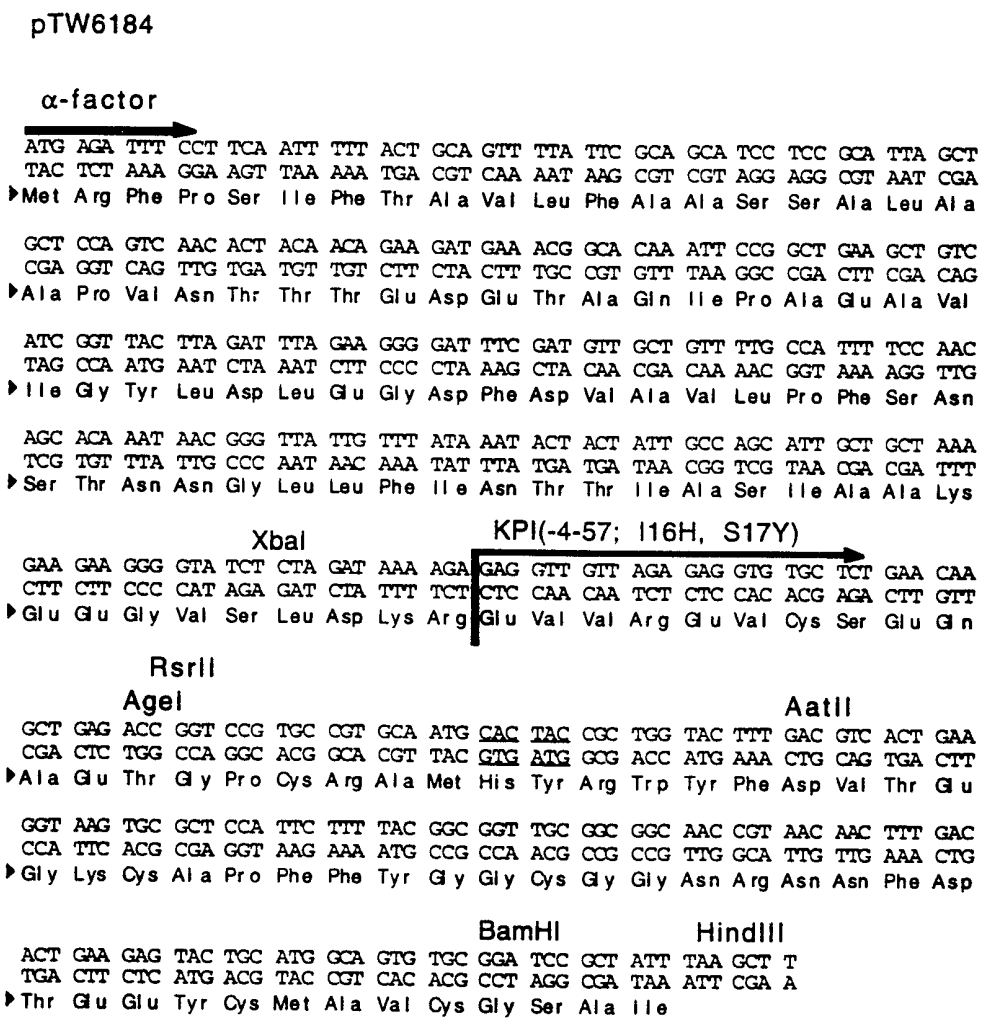

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α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; I16H, S17Y)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ATG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Met His Tyr Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 17

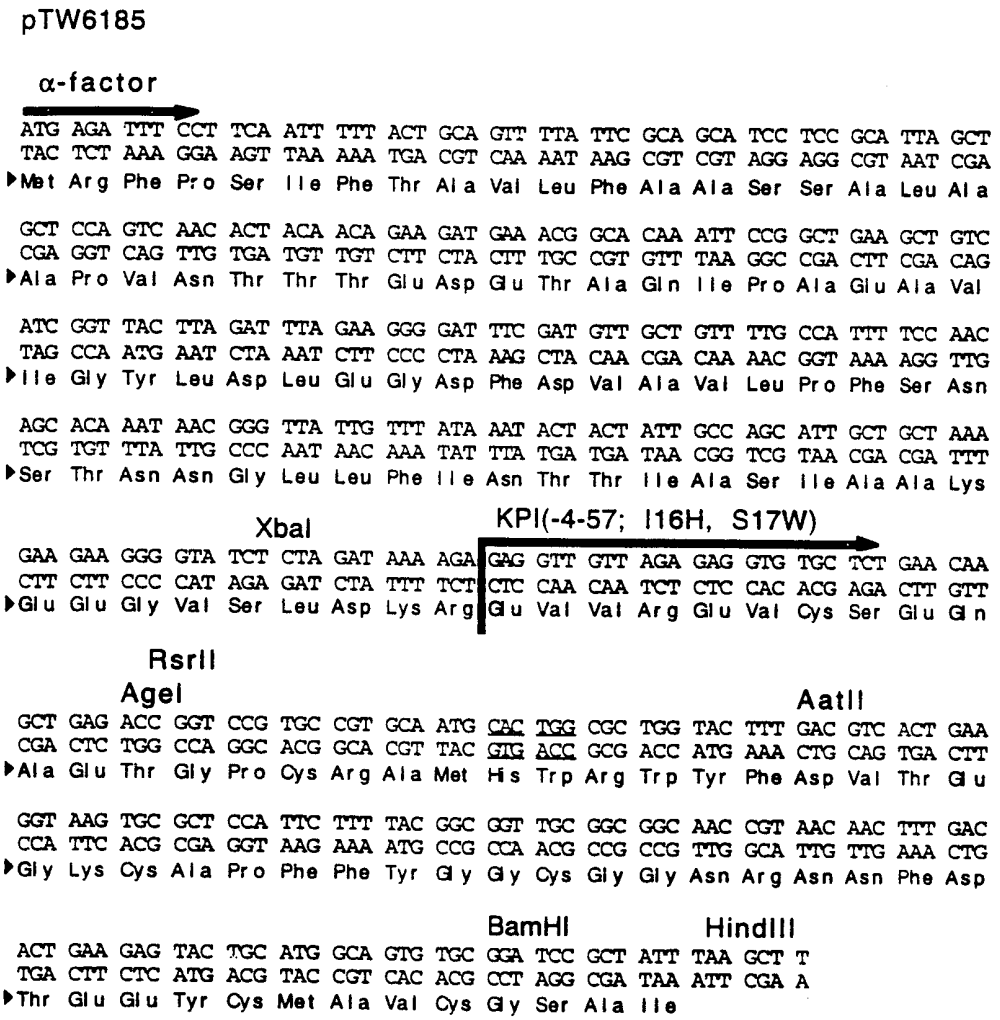

pTW6185
α-factor
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TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; I16H, S17W)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TGG CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ACC GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Met His Trp Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

Figure 18

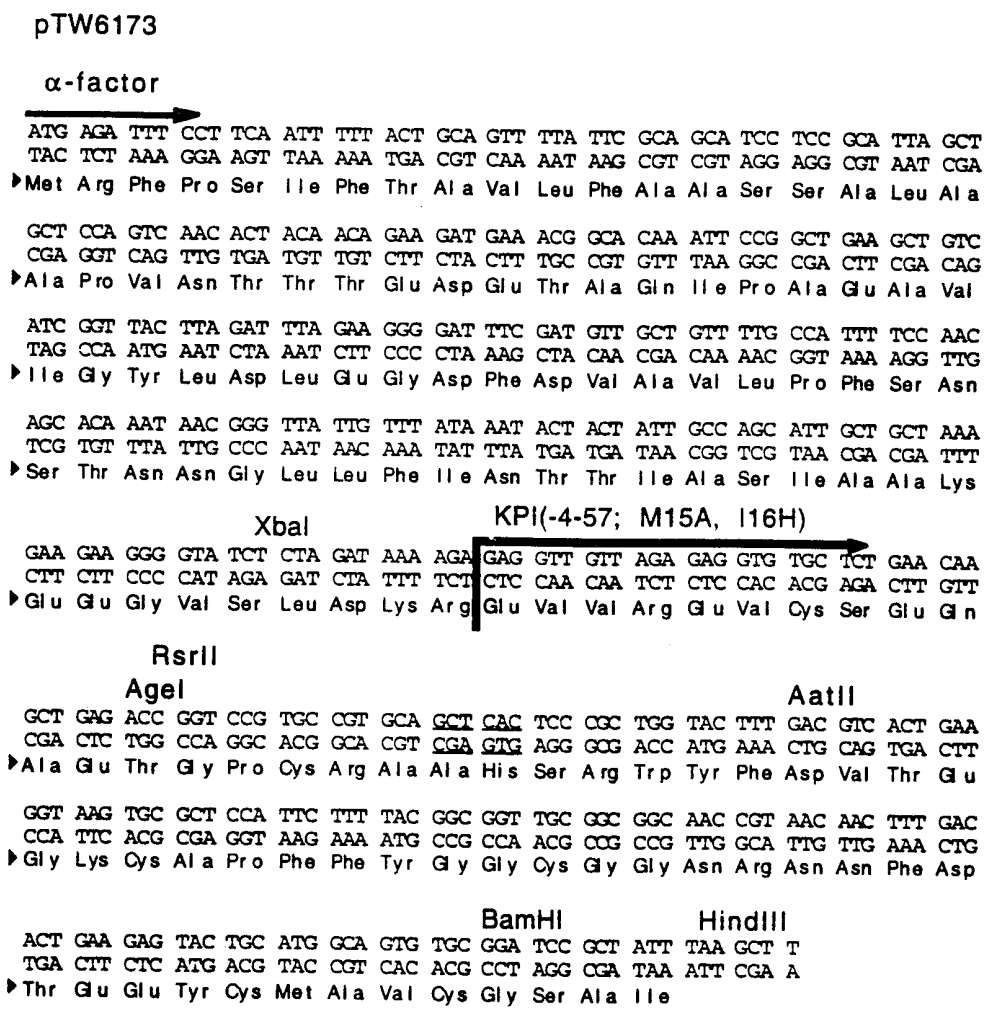

pTW6173
α-factor
XbaI
KPI(-4-57; M15A, I16H)
RsrII
AgeI
AatII
BamHI
HindIII

Figure 19

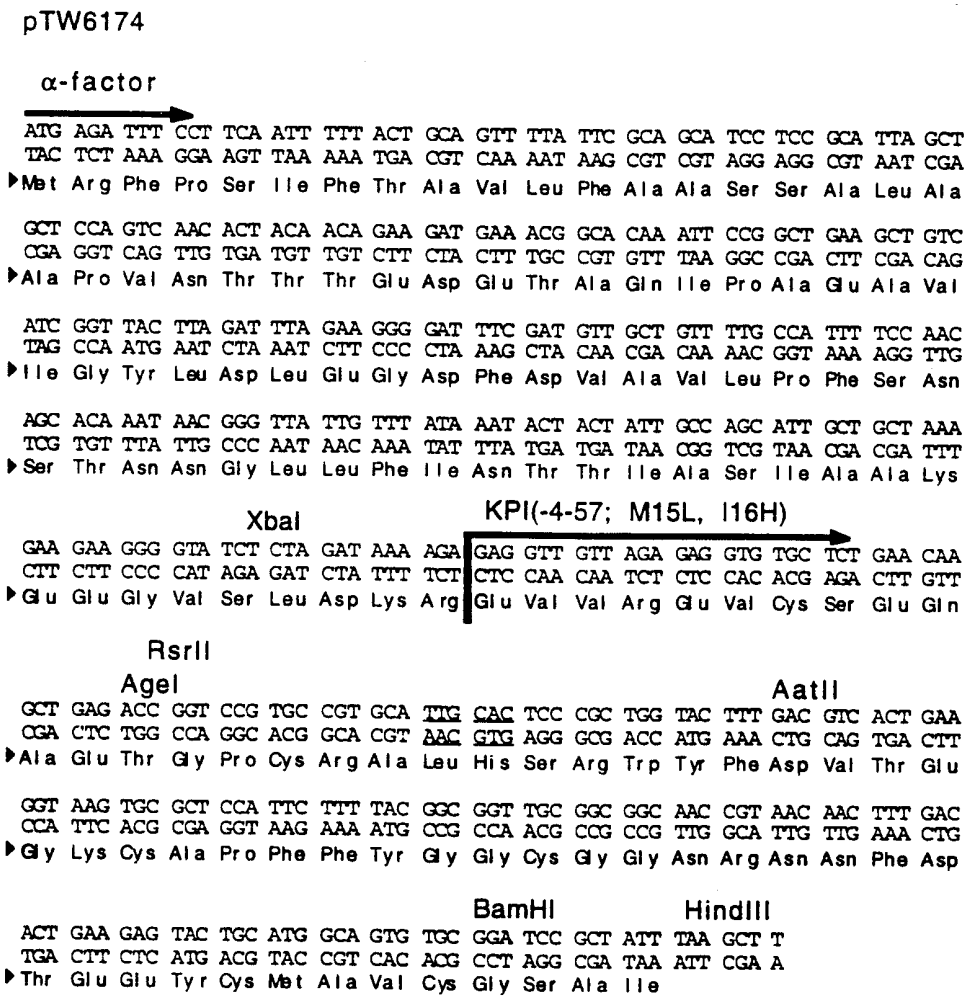

pTW6174
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; M15L, I16H)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA TTG CAC TCC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC GTG AGG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Leu His Ser Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

FIGURE 20

α-factor:KPI-BG022 (M15A,S17Y,R18H,Y48H)

α-factor ⟶

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser

TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr

GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu

GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn

AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT
Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala

KPI-BG022 ⟶

AAA GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu

GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCG GCA ATC
Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala *Ala* Ile

TAC CAC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC
Tyr *His* Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe

TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

GAG CAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCTT
Glu *His* Cys Met Ala Val Cys Gly Ser Ala Ile

FIGURE 21

α-factor:KPI-BG033 (T9V,M15A,S17Y,R18H,Y48H)

α-factor ——————→

```
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser

TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr

GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu

GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn

AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT
Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala

                                        KPI-BG033 ——————→
AAA GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu

GTG TGC TCT GAA CAA GCT GAG GTT GGT CCG TGC CGT GCG GCA ATC
Val Cys Ser Glu Gln Ala Glu Val Gly Pro Cys Arg Ala Ala Ile

TAC CAC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC
Tyr His Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe

TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

GAG CAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCTT
Glu His Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIGURE 22

α-factor:KPI-BG048 (Y48H)

α-factor ——————→

```
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser

TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr

GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu

GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn

AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT
Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala

                                              KPI-BG048 ——————→
AAA GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu

GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCG ATG ATC
Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile

TCC CGC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC
Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe

TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

GAG CAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCTT
Glu His Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIGURE 23

α-factor:KPI-BG049 (M15A,S17Y,R18H)

α-factor ⟶

```
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser

TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr

GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu

GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn

AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT
Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala

                                                KPI-BG049 ⟶
AAA GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu

GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCG GCA ATC
Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile

TAC CAC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC
Tyr His Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe

TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCTT
Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIGURE 24

α-factor:KPI-BG050 (T9V,M15A,S17Y,R18H)

α-factor ——————→

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser

TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr

GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu

GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn

AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT
Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala

KPI-BG050 ——————→

AAA GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu

GTG TGC TCT GAA CAA GCT GAG GTT GGT CCG TGC CGT GCG GCA ATC
Val Cys Ser Glu Gln Ala Glu *Val* Gly Pro Cys Arg Ala *Ala* Ile

TAC CAC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC
Tyr *His* Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe

TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCTT
Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

FIGURE 25

phoA:KPI (M15A,S17Y,R18H,Y48H):gIII

phoA signal ——→
GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG
Val Lys Gln Ser Thr Ile Ala Leu Ala leu Leu Pro Leu Leu Phe Thr Pro Val

KPI (M15A,S17Y,R18H,Y48H) ——→ RsrII
ACC AAA GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA GCA
Thr Lys Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Ala

ATC TAC CAC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC
Ile Tyr His Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr

GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA GAG CAC TGC ATG GCA
Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu His Cys Met Ala

BamH gIII ——→
GTG TGC GGA TCC GGT GGT GGC TCT GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG
Val Cys Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met

GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC GCG CTA CAG
Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln

TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT ATC
Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile

GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT GCT ACT GGT
Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly

GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT TCA
Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser

CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT
Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys

GAC AAA ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC
Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr

TTT ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA
Phe Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Ler Arg Asn Lys Glu Ser

TAA GCTT

FIGURE 26

α-factor:KPI-P48 Library (M15A,S17Y,R18H,Y48X)

α-factor ——————→

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser

TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr

GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TTA GAT TTA GAA
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Leu Asp Leu Glu

GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn

AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT
Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala

KPI ——————→

AAA GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG
Lys Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu

GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCG GCA ATC
Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala *Ala* Ile

TAC CAC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC
Tyr *His* Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe

TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

GAG NNS TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCTT
Glu *XXX* Cys Met Ala Val Cys Gly Ser Ala Ile

FIGURE 27

KPI(-4-57; M15A, S17W) TW6165

Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu	Thr	Gly	Pro	Cys	Arg	Ala	Ala	Ile	Trp	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn	Asn	Phe	Asp	Thr	Glu	Glu	Tyr	Cys	Met	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val	Cys	Gly	Ser	Ala	Ile					
52	53	54	55	56	57					

FIGURE 28

KPI(-4-57; M15A, S17Y) TW6166

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Ala -	Ile -	Tyr -	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIGURE 29

KPI(-4-57; M15L, S17F) TW6175

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Leu -	Ile -	Phe -	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIGURE 30

KPI(-4-57: M15L, S17Y) BG026

Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu	Thr	Gly	Pro	Cys	Arg	Ala	Leu	Ile	Tyr	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn	Asn	Phe	Asp	Thr	Glu	Glu	Tyr	Cys	Met	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val	Cys	Gly	Ser	Ala	Ile					
52	53	54	55	56	57					

FIGURE 31

KPI(-4-57; I16H, S17F) TW6183

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Met -	His -	Phe -	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIGURE 32

KPI(-4-57; I16H, S17Y) TW6184

Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu	Thr	Gly	Pro	Cys	Arg	Ala	Met	His	Tyr	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn	Asn	Phe	Asp	Thr	Glu	Glu	Tyr	Cys	Met	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val	Cys	Gly	Ser	Ala	Ile					
52	53	54	55	56	57					

FIGURE 33

KPI(-4-57; I16H, S17W) TW6185

Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu	Thr	Gly	Pro	Cys	Arg	Ala	Met	His	Trp	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn	Asn	Phe	Asp	Thr	Glu	Glu	Tyr	Cys	Met	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val	Cys	Gly	Ser	Ala	Ile					
52	53	54	55	56	57					

FIGURE 34

KPI(-4-57; M15A, S17F) DD185

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Ala -	Ile -	Phe -	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIGURE 35

KPI(-4-57; M15A, I16H) TW6173

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Ala -	His -	Ser -	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIGURE 36

KPI(-4-57; M15L, I16H) TW6174

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	**Ala**
-4	-3	-2	-1	1	2	3	4	5	6	**7**
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Leu -	His -	Ser -	**Arg**
8	9	10	11	12	13	14	15	16	17	**18**
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	**Ala**
19	20	21	22	23	24	25	26	27	28	**29**
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	**Arg**
30	31	32	33	34	35	36	37	38	39	**40**
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	**Ala**
41	42	43	44	45	46	47	48	49	50	**51**
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIGURE 37

KPI-BG022 (M15A,S17Y,R18H,Y48H)

```
Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro
 -4                                                      11

Cys Arg Ala Ala Ile Tyr His Trp Tyr Phe Asp Val Thr Glu Gly
             15      17  18                              26

Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn
                                                        41

Asn Phe Asp Thr Glu Glu His Cys Met Ala Val Cys Gly Ser Ala
                          48                            56

Ile
 57
```

FIGURE 38

KPI-BG033 (T9V,M15A,S17Y,R18H,Y48H)

Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu *Val* Gly **Pro**
-4 9 **11**

Cys Arg Ala *Ala* Ile *Tyr* *His* Trp Tyr Phe Asp Val Thr Glu **Gly**
15 17 18 **26**

Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg **Asn**
41

Asn Phe Asp Thr Glu Glu *His* Cys Met Ala Val Cys Gly Ser **Ala**
48 **56**

Ile
57

FIGURE 39

KPI-BG048 (Y48H)

Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro
-4 11

Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly
26

Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn
41

Asn Phe Asp Thr Glu Glu *His* Cys Met Ala Val Cys Gly Ser Ala
48 56

Ile
57

FIGURE 40

KPI-BG049 (M15A,S17Y,R18H)

Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly **Pro**
-4 **11**

Cys Arg Ala *Ala* Ile *Tyr* *His* Trp Tyr Phe Asp Val Thr Glu **Gly**
15 17 18 **26**

Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg **Asn**
41

Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser **Ala**
56

Ile
57

FIGURE 41

KPI-BG050 (T9V,M15A,S17Y,R18H)

Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu *Val* Gly **Pro**
-4 9 **11**

Cys Arg Ala *Ala* Ile *Tyr* *His* Trp Tyr Phe Asp Val Thr Glu **Gly**
15 17 18 **26**

Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg **Asn**
41

Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser **Ala**
56

Ile
57

FIGURE 42

KPI-P48 Library (M15A,S17Y,R18H,Y48X)

Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly **Pro**
-4 **11**

Cys Arg Ala *Ala* Ile *Tyr* *His* Trp Tyr Phe Asp Val Thr Glu **Gly**
15 17 18 **26**

Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg **Asn**
41

Asn Phe Asp Thr Glu Glu *XXX* Cys Met Ala Val Cys Gly Ser **Ala**
48 **56**

Ile
57

Figure 43

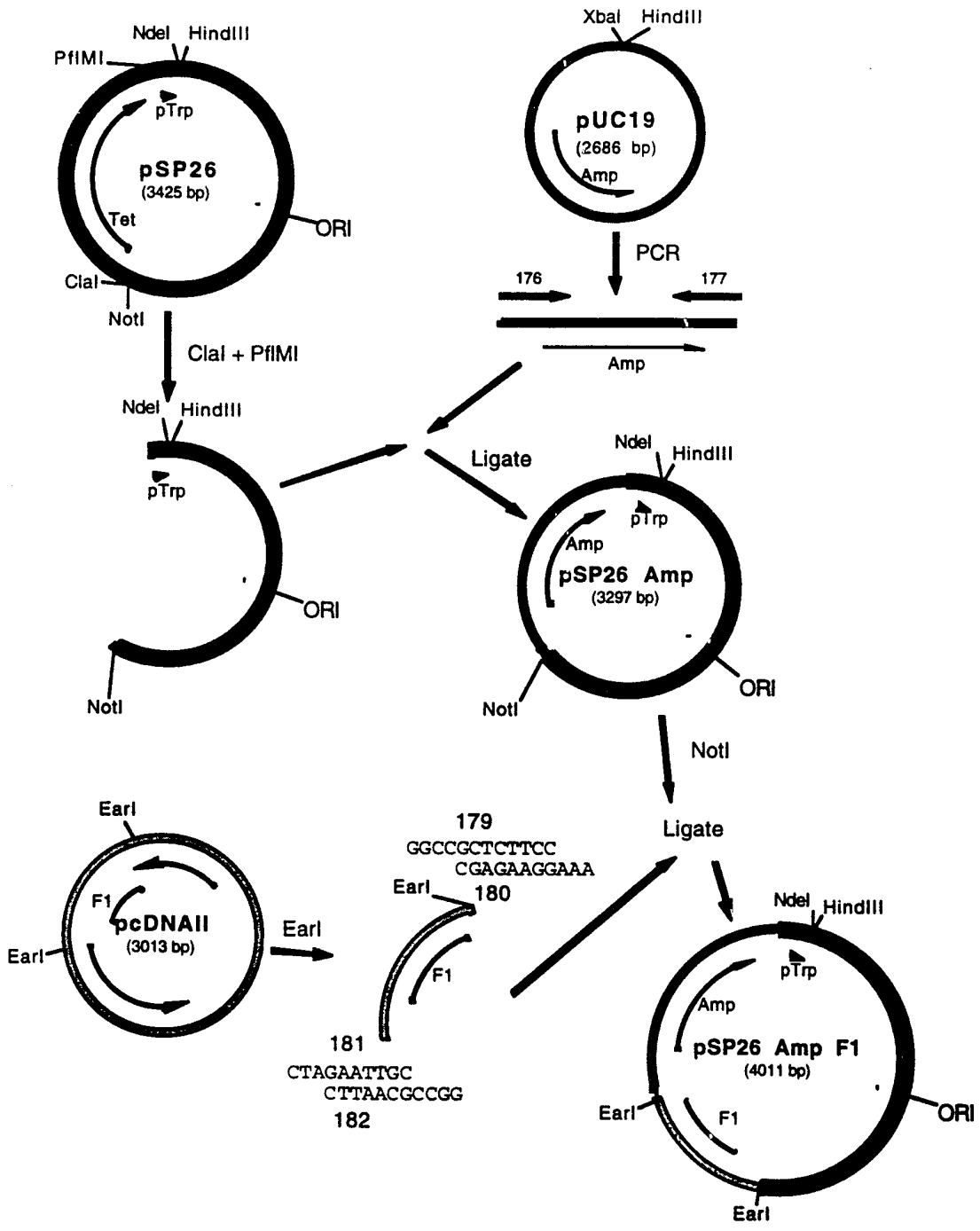

NdeI HindIII
PflMI
pTrp
pSP26
(3425 bp)
Tet
ORI
ClaI
NotI
ClaI + PflMI
XbaI HindIII
pUC19
(2686 bp)
Amp
PCR
176
177
Amp
NdeI HindIII
pTrp
ORI
NotI
Ligate
NdeI HindIII
pTrp
Amp
pSP26 Amp
(3297 bp)
ORI
NotI
NotI
Ligate
EarI
F1
pcDNAII
(3013 bp)
EarI
EarI
179
GGCCGCTCTTCC
CGAGAAGGAAA
EarI
180
F1
181
CTAGAATTGC
CTTAACGCCGG
182
NdeI HindIII
pTrp
Amp
pSP26 Amp F1
(4011 bp)
EarI
F1
ORI
EarI

FIGURE 44

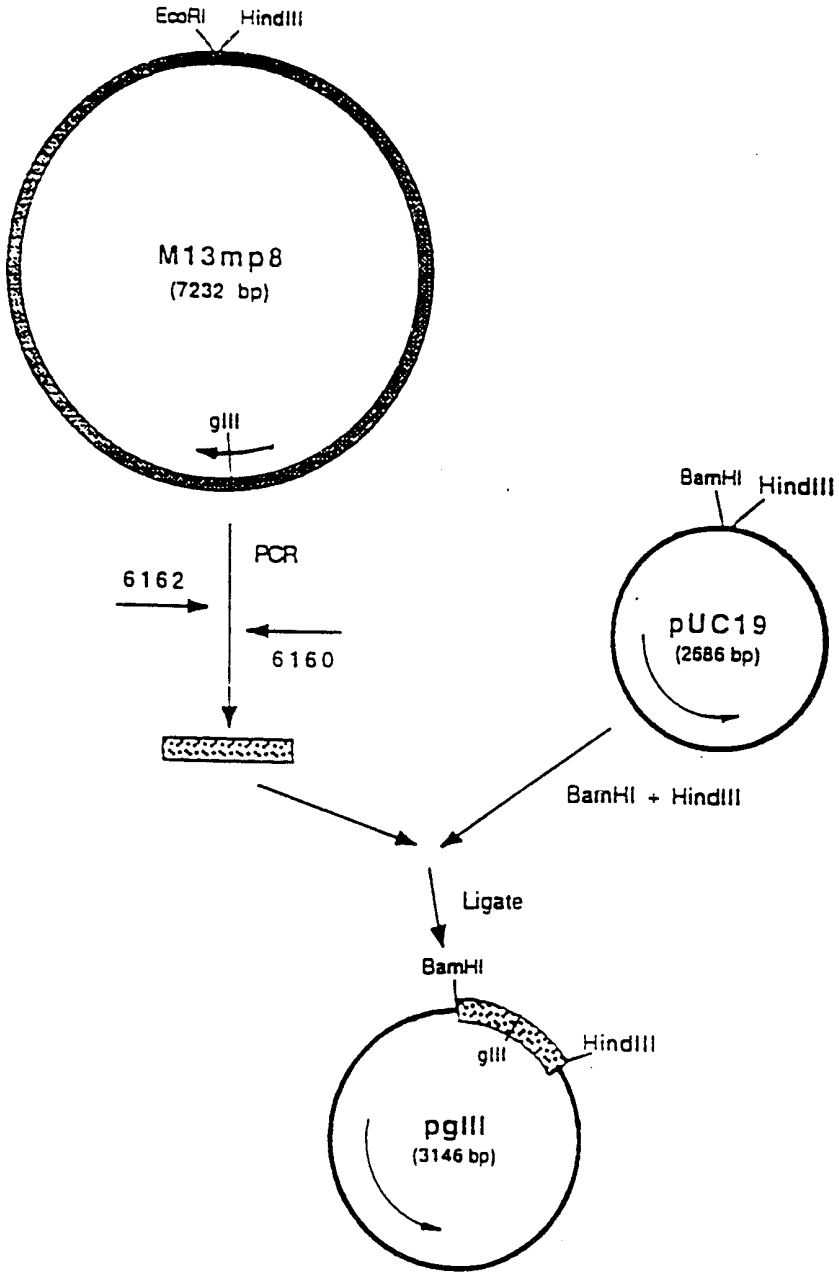

EcoRI
HindIII
M13mp8
(7232 bp)
gIII
PCR
6162
6160
BamHI
HindIII
pUC19
(2686 bp)
BamHI + HindIII
Ligate
BamHI
gIII
HindIII
pgIII
(3146 bp)

FIGURE 45

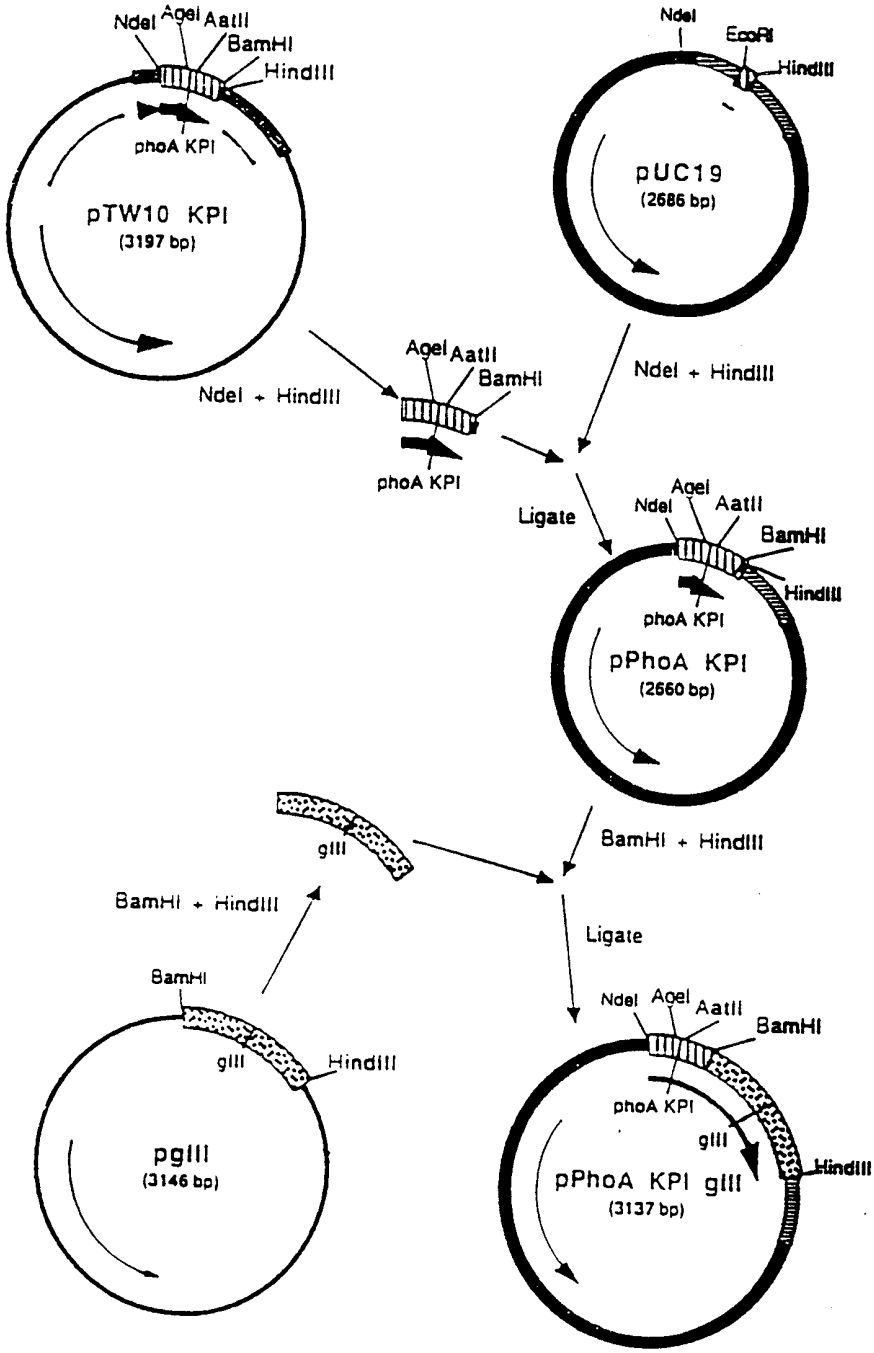

NdeI AgeI AatII
BamHI
HindIII
phoA KPI
pTW10 KPI
(3197 bp)
NdeI + HindIII
AgeI AatII
BamHI
phoA KPI
NdeI
EcoRI
HindIII
pUC19
(2686 bp)
NdeI + HindIII
Ligate
AgeI
NdeI
AatII
BamHI
HindIII
phoA KPI
pPhoA KPI
(2660 bp)
gIII
BamHI + HindIII
BamHI + HindIII
Ligate
BamHI
gIII
HindIII
pgIII
(3146 bp)
NdeI AgeI AatII
BamHI
phoA KPI
gIII
HindIII
pPhoA KPI gIII
(3137 bp)

FIGURE 46

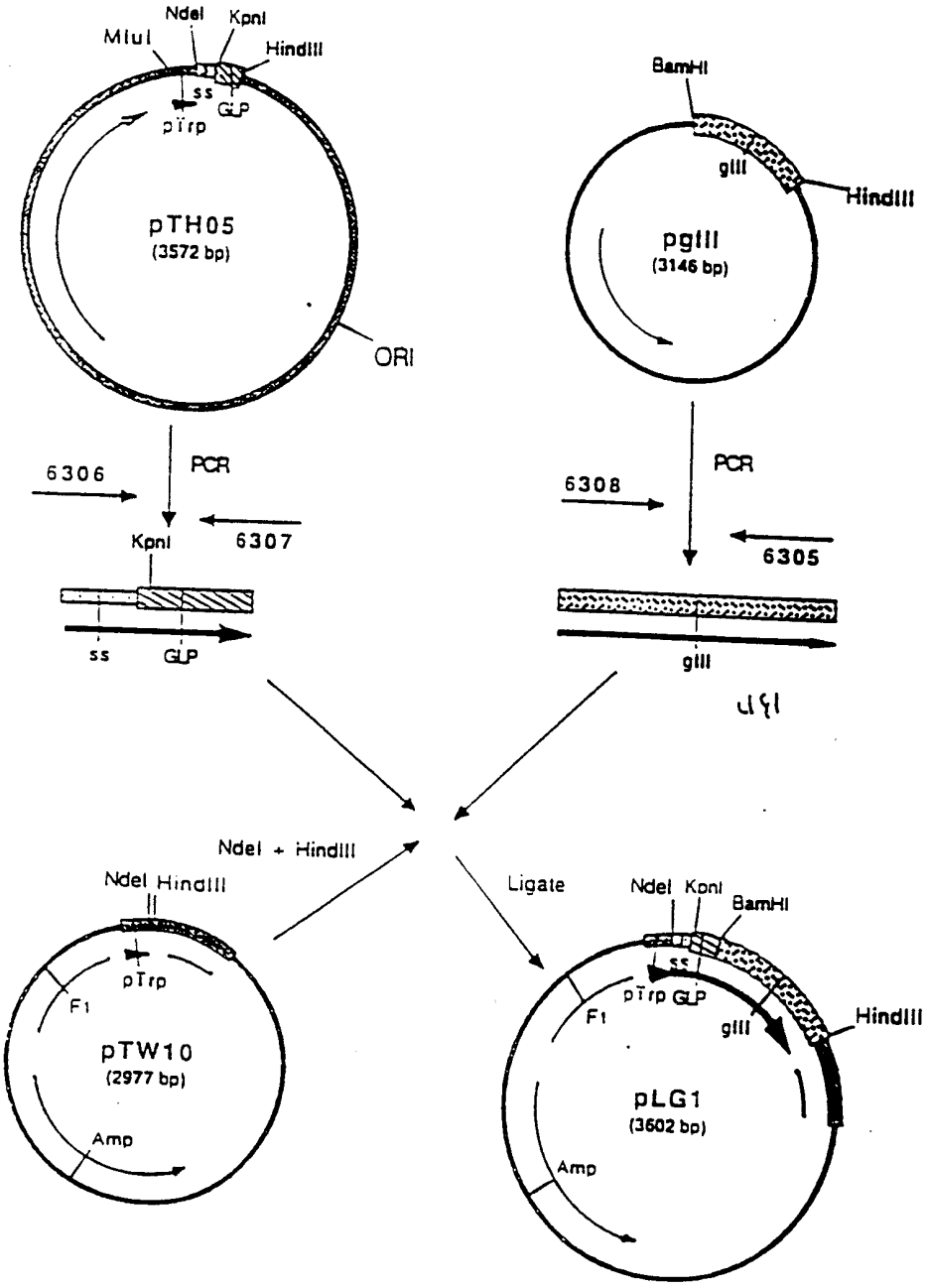

MluI
NdeI
KpnI
HindIII
ss
GLP
pTrp
pTH05
(3572 bp)
ORI
BamHI
gIII
HindIII
pgIII
(3146 bp)
6306
PCR
KpnI
6307
ss
GLP
6308
PCR
6305
gIII
NdeI + HindIII
NdeI HindIII
pTrp
F1
pTW10
(2977 bp)
Amp
Ligate
NdeI
KpnI
BamHI
ss
pTrp
GLP
F1
gIII
HindIII
pLG1
(3602 bp)
Amp

FIGURE 47

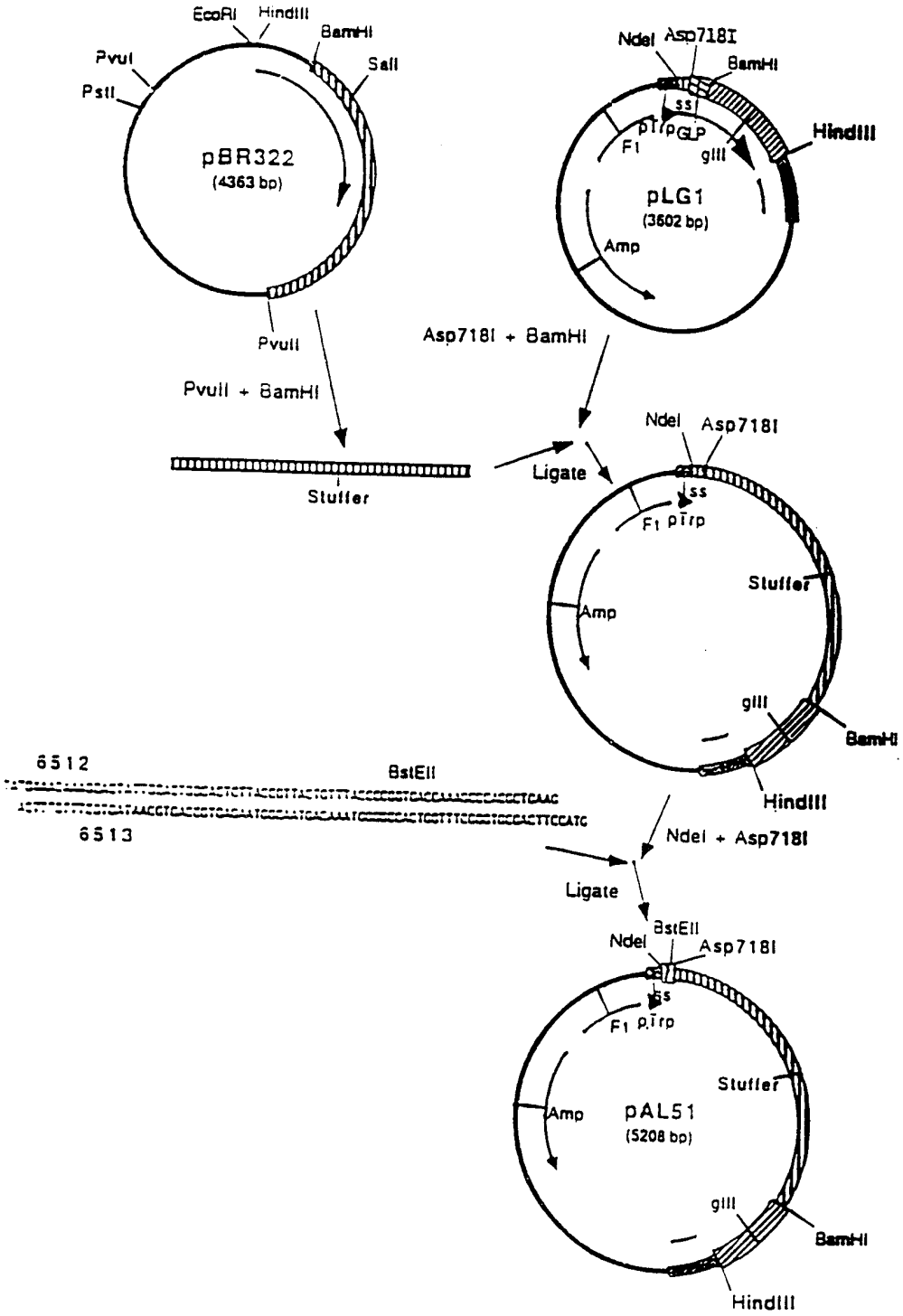

EcoRI
HindIII
BamHI
SalI
PstI
PvuI
pBR322
(4363 bp)
PvuII
PvuII + BamHI
Stuffer
NdeI
Asp718I
BamHI
ss
pTrp
GLP
F1
gIII
HindIII
pLG1
(3602 bp)
Amp
Asp718I + BamHI
Ligate
NdeI
Asp718I
ss
F1
pTrp
Stuffer
Amp
gIII
BamHI
HindIII
6512
BstEII
6513
NdeI + Asp718I
Ligate
BstEII
NdeI
Asp718I
ss
F1
pTrp
Stuffer
Amp
pAL51
(5208 bp)
gIII
BamHI
HindIII

FIGURE 48

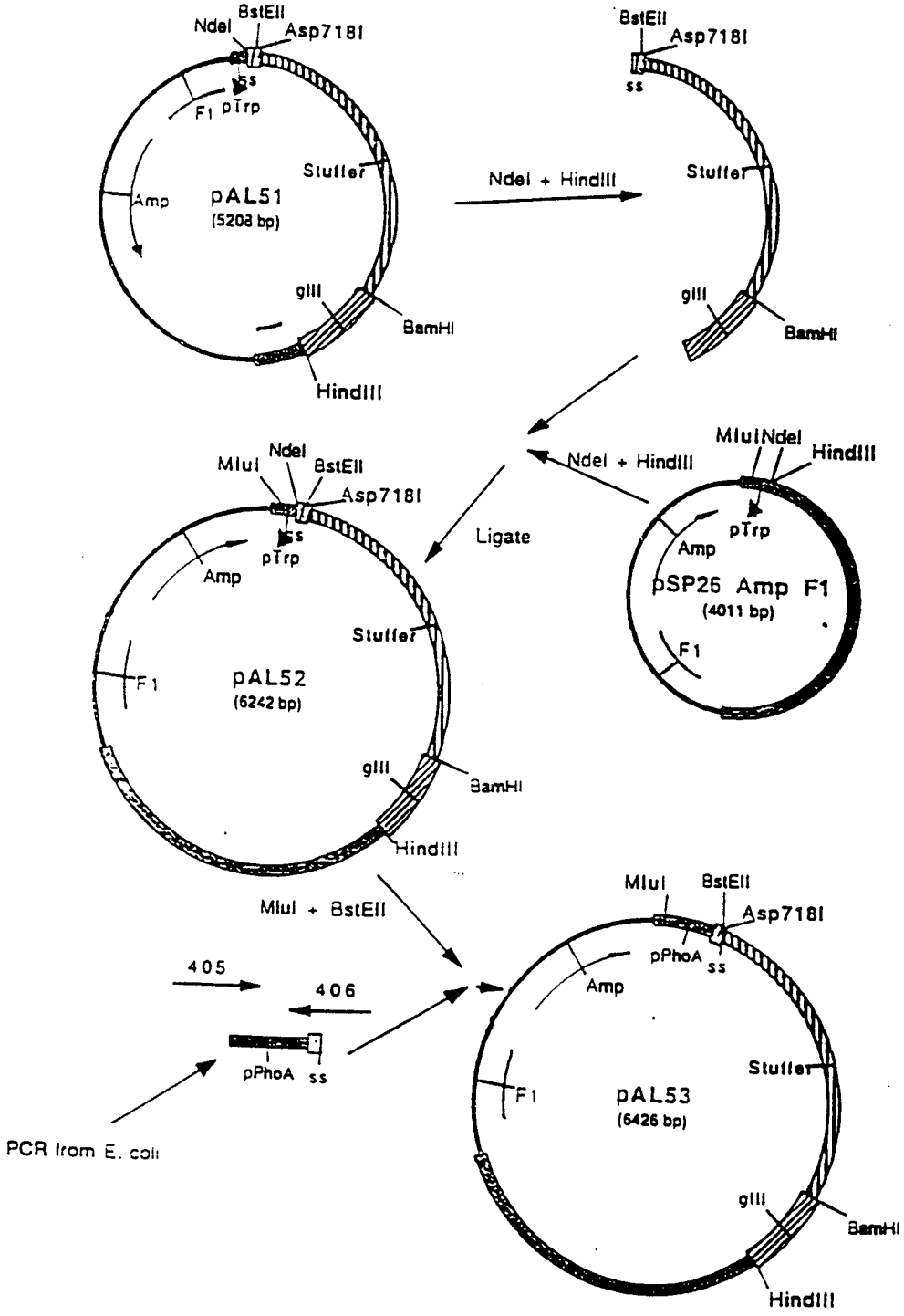

NdeI
BstEII
Asp718I
ss
F1 pTrp
Stuffer
Amp
pAL51
(5208 bp)
gIII
BamHI
HindIII
NdeI + HindIII
BstEII
Asp718I
ss
Stuffer
gIII
BamHI
MluI
NdeI
BstEII
Asp718I
ss
pTrp
Amp
Stuffer
F1
pAL52
(6242 bp)
gIII
BamHI
HindIII
Ligate
MluINdeI
HindIII
pTrp
Amp
pSP26 Amp F1
(4011 bp)
F1
MluI + BstEII
405
406
pPhoA
ss
PCR from E. coli
MluI
BstEII
Asp718I
pPhoA
ss
Amp
Stuffer
F1
pAL53
(5426 bp)
gIII
BamHI
HindIII

FIGURE 49

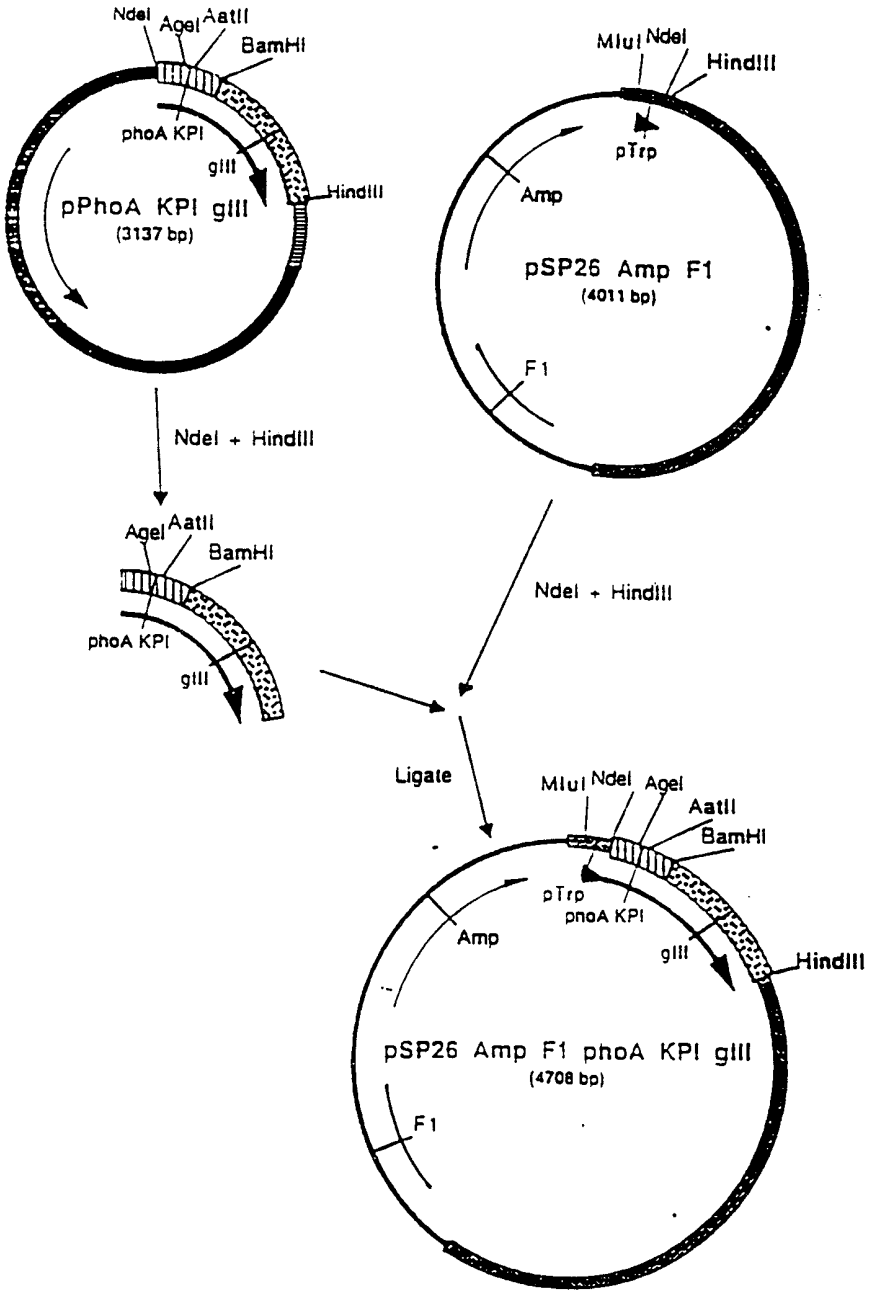

NdeI
AgeI
AatII
BamHI
phoA KPI
gIII
HindIII
pPhoA KPI gIII
(3137 bp)
MluI
NdeI
HindIII
pTrp
Amp
pSP26 Amp F1
(4011 bp)
F1
NdeI + HindIII
AgeI
AatII
BamHI
phoA KPI
gIII
NdeI + HindIII
Ligate
MluI
NdeI
AgeI
AatII
BamHI
pTrp
phoA KPI
Amp
gIII
HindIII
pSP26 Amp F1 phoA KPI gIII
(4708 bp)
F1

FIGURE 50

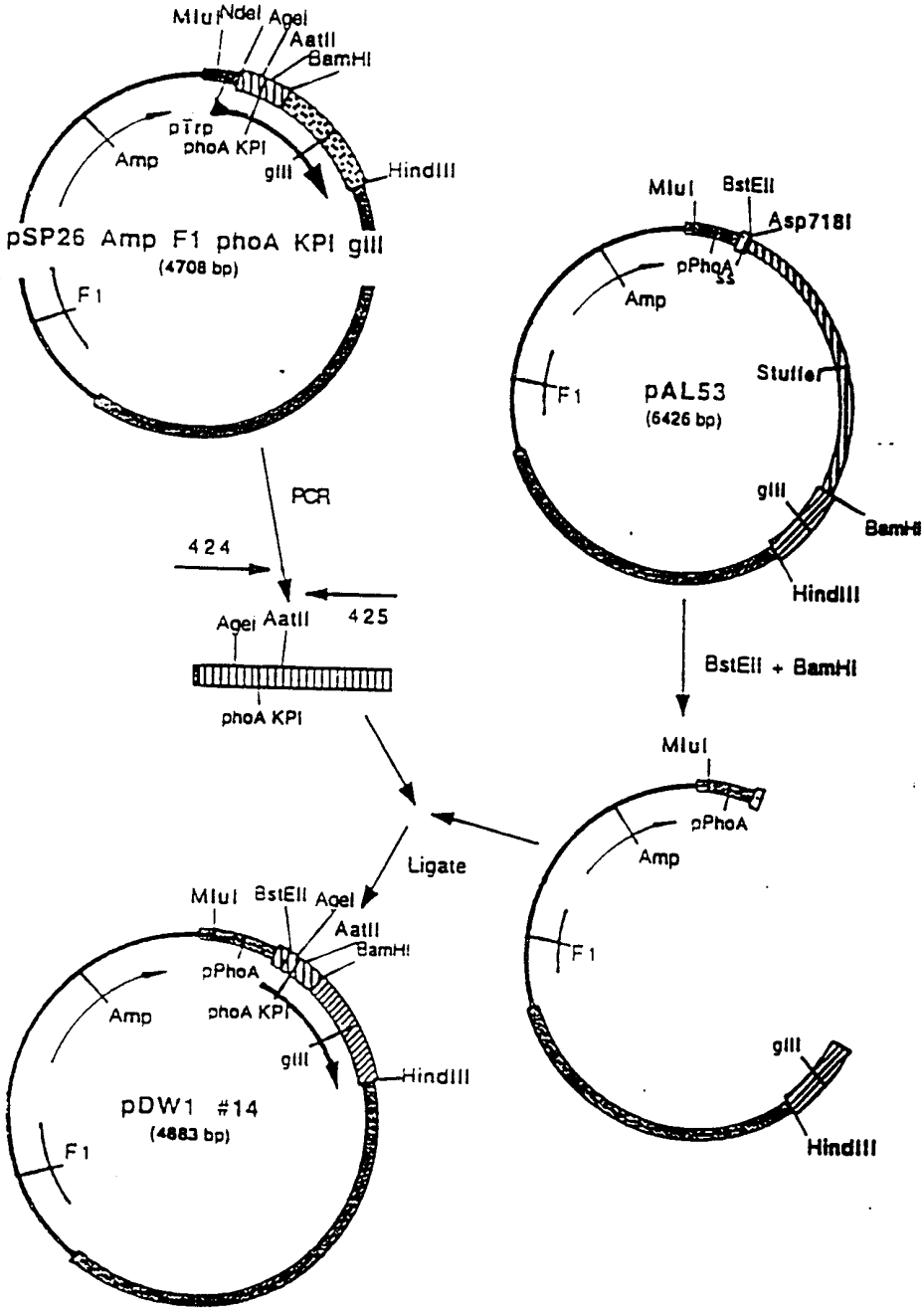

MluI
NdeI
AgeI
AatII
BamHI
pTrp
phoA KPI
Amp
gIII
HindIII
pSP26 Amp F1 phoA KPI gIII
(4708 bp)
F1
PCR
424
425
AgeI
AatII
phoA KPI
MluI
BstEII
Asp718I
pPhoA
SS
Amp
F1
pAL53
(6426 bp)
StuffeI
gIII
BamHI
HindIII
BstEII + BamHI
MluI
pPhoA
Amp
F1
gIII
HindIII
Ligate
MluI
BstEII
AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 #14
(4883 bp)
F1

Figure 51

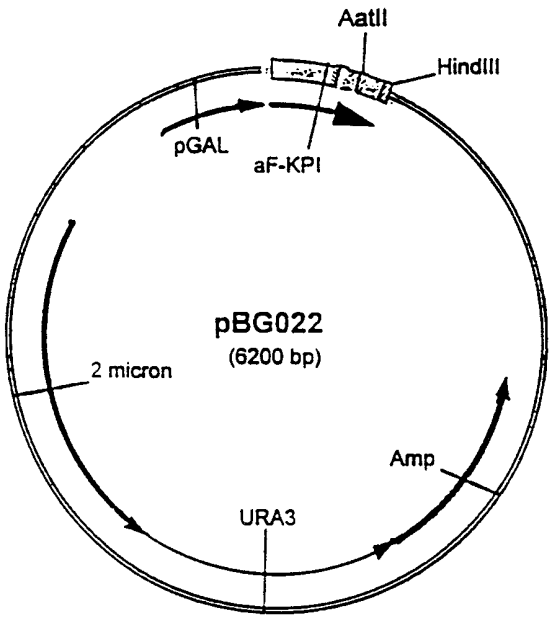


Figure 52

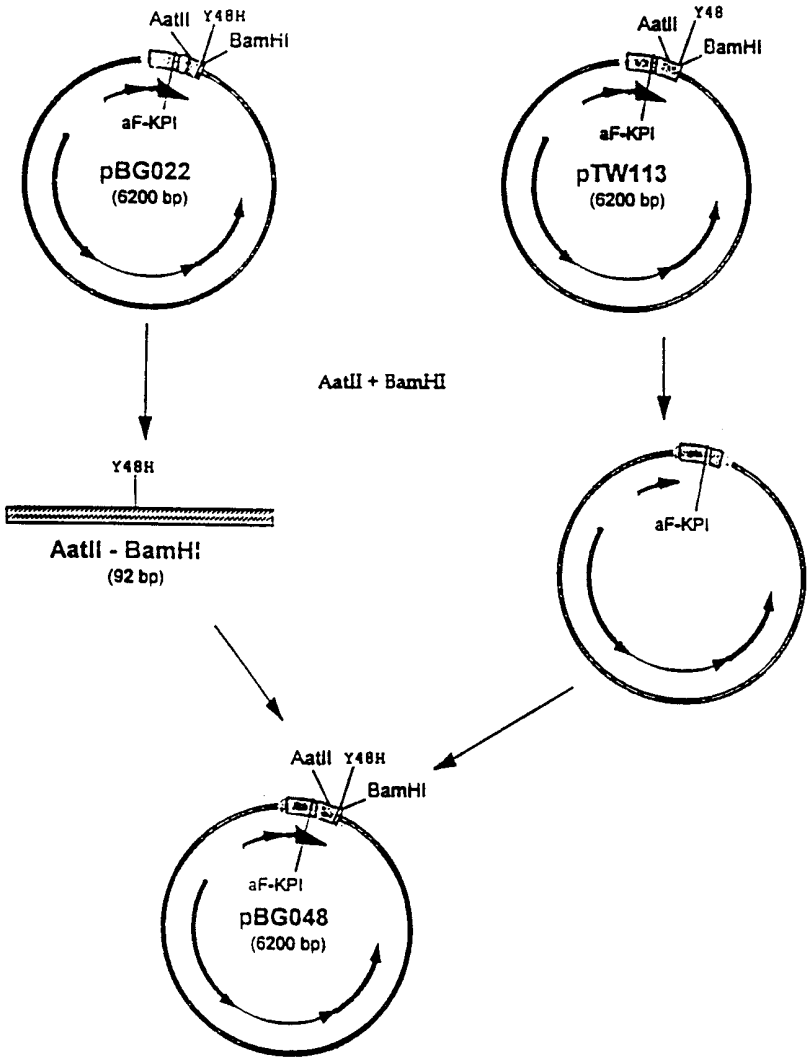

AatII
Y48H
BamHI
aF-KPI
pBG022
(6200 bp)
AatII
Y48
BamHI
aF-KPI
pTW113
(6200 bp)
AatII + BamHI
Y48H
AatII - BamHI
(92 bp)
aF-KPI
AatII
Y48H
BamHI
aF-KPI
pBG048
(6200 bp)

Figure 53

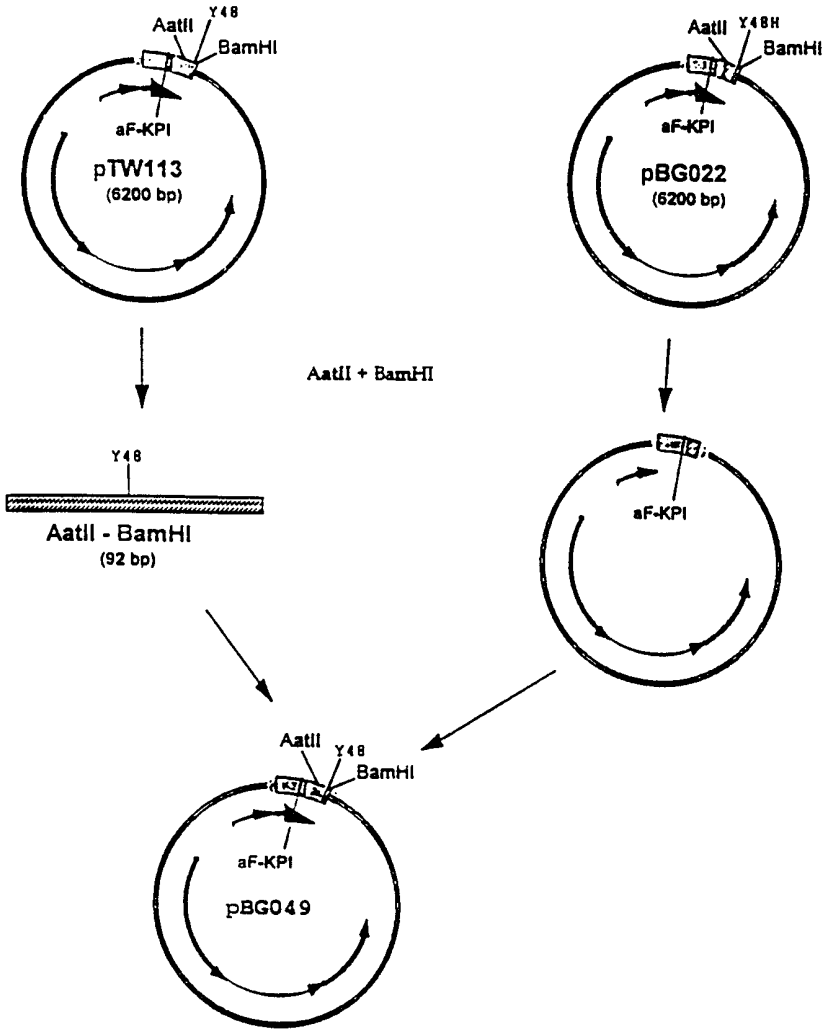

AatII
Y48
BamHI
aF-KPI
pTW113
(6200 bp)
AatII
Y48H
BamHI
aF-KPI
pBG022
(6200 bp)
AatII + BamHI
Y48
AatII - BamHI
(92 bp)
aF-KPI
AatII
Y48
BamHI
aF-KPI
pBG049

Figure 54

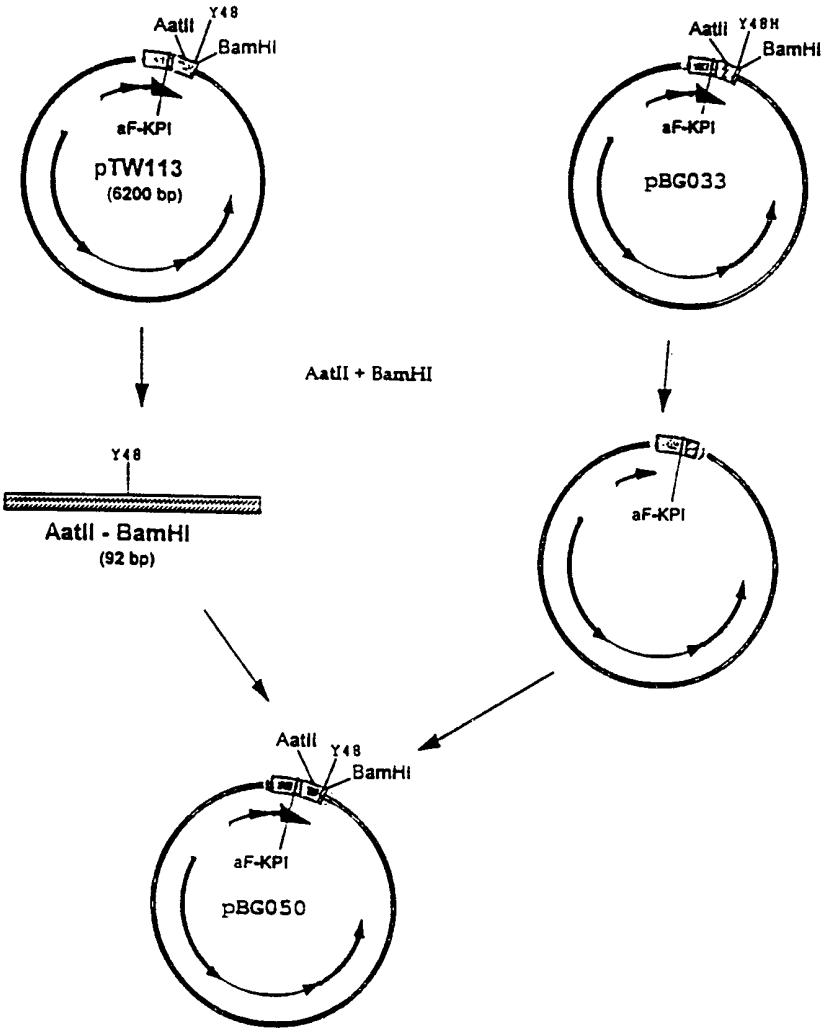

AatII
Y48
BamHI
aF-KPI
pTW113
(6200 bp)
AatII + BamHI
Y48
AatII - BamHI
(92 bp)
AatII
Y48H
BamHI
aF-KPI
pBG033
aF-KPI
AatII
Y48
BamHI
aF-KPI
pBG050

Figure 55

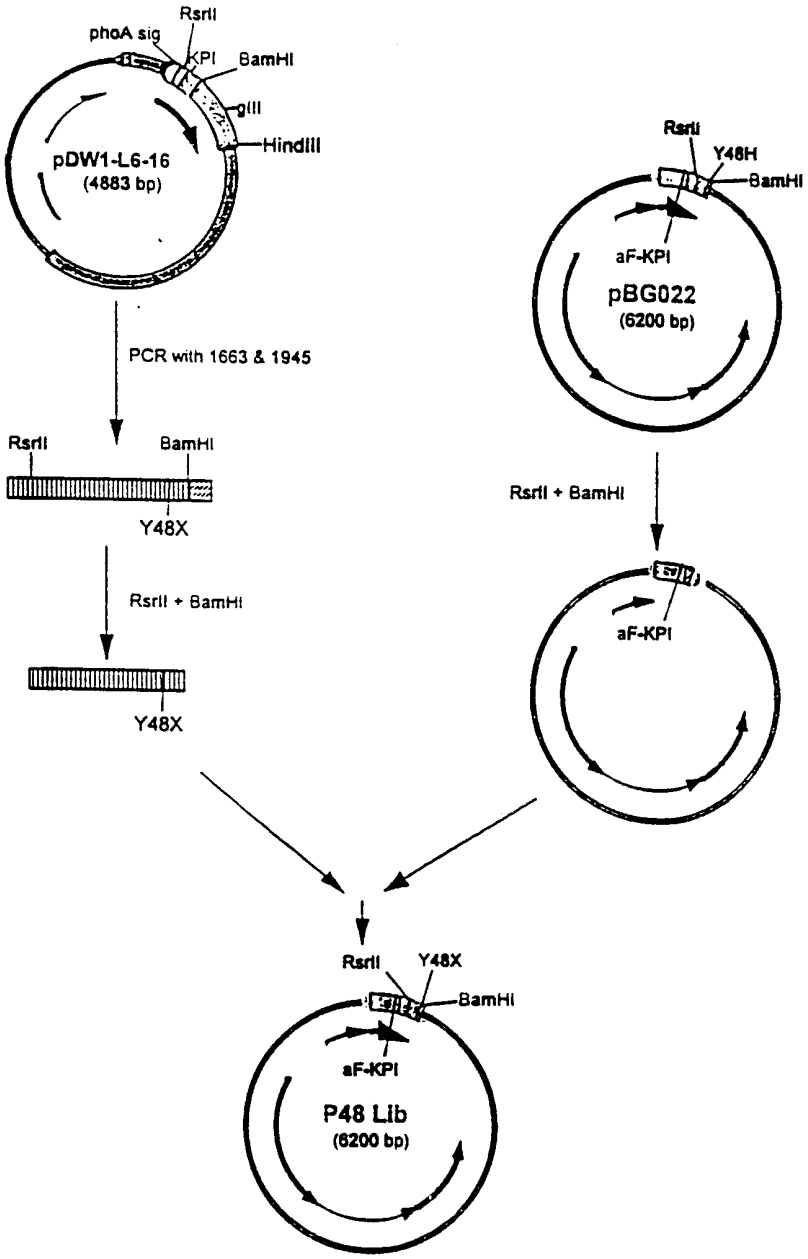
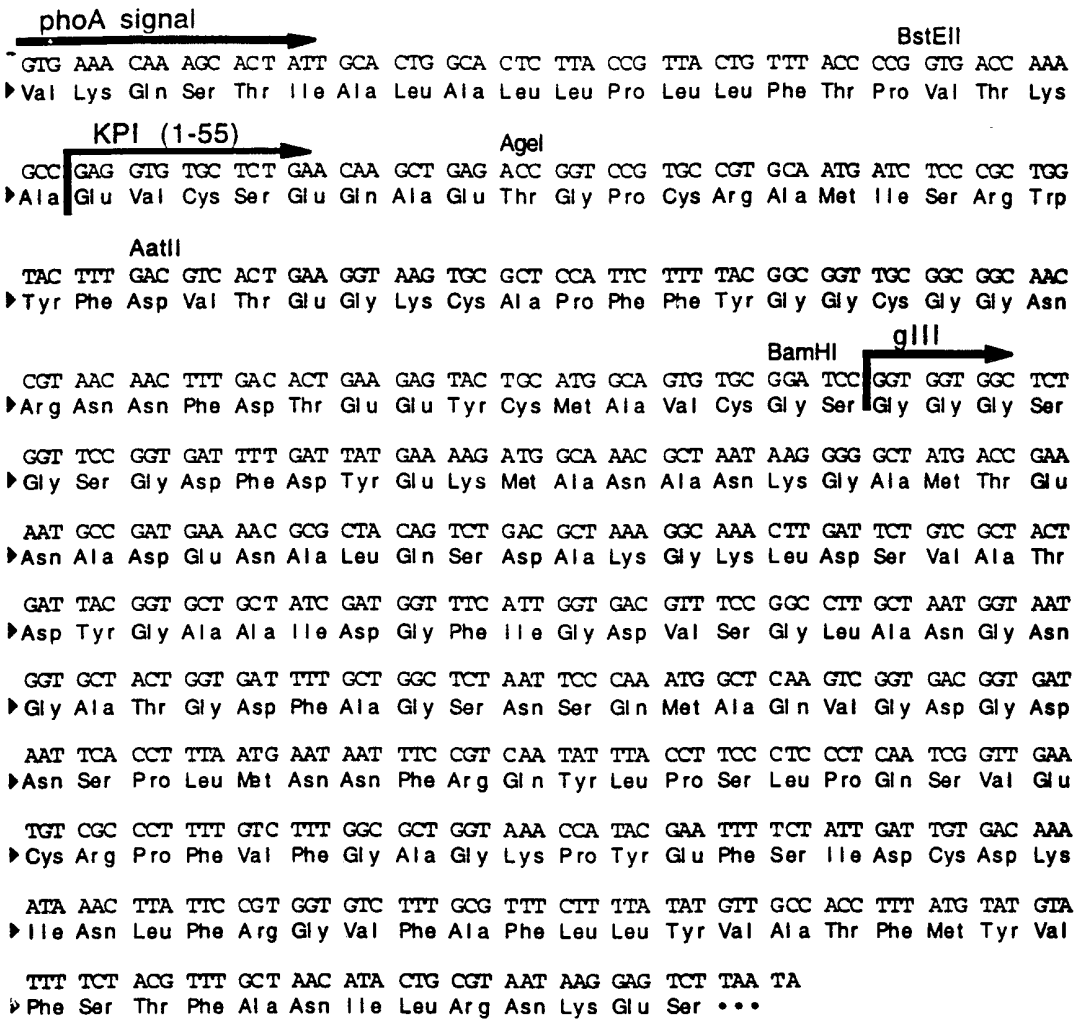

RsrII
phoA sig
KPI
BamHI
gIII
HindIII
pDW1-L6-16
(4883 bp)
PCR with 1663 & 1945
RsrII
BamHI
Y48X
RsrII + BamHI
Y48X
RsrII
Y48H
BamHI
aF-KPI
pBG022
(6200 bp)
RsrII + BamHI
aF-KPI
RsrII
Y48X
BamHI
aF-KPI
P48 Lib
(6200 bp)

Figure 56




```
                                                                  BstEII
GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

        KPI (1-55)                        AgeI
GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

        AatII
TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

                                                  BamHI          gIII
CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT
Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser

GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA
Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser •••
```

Figure 57

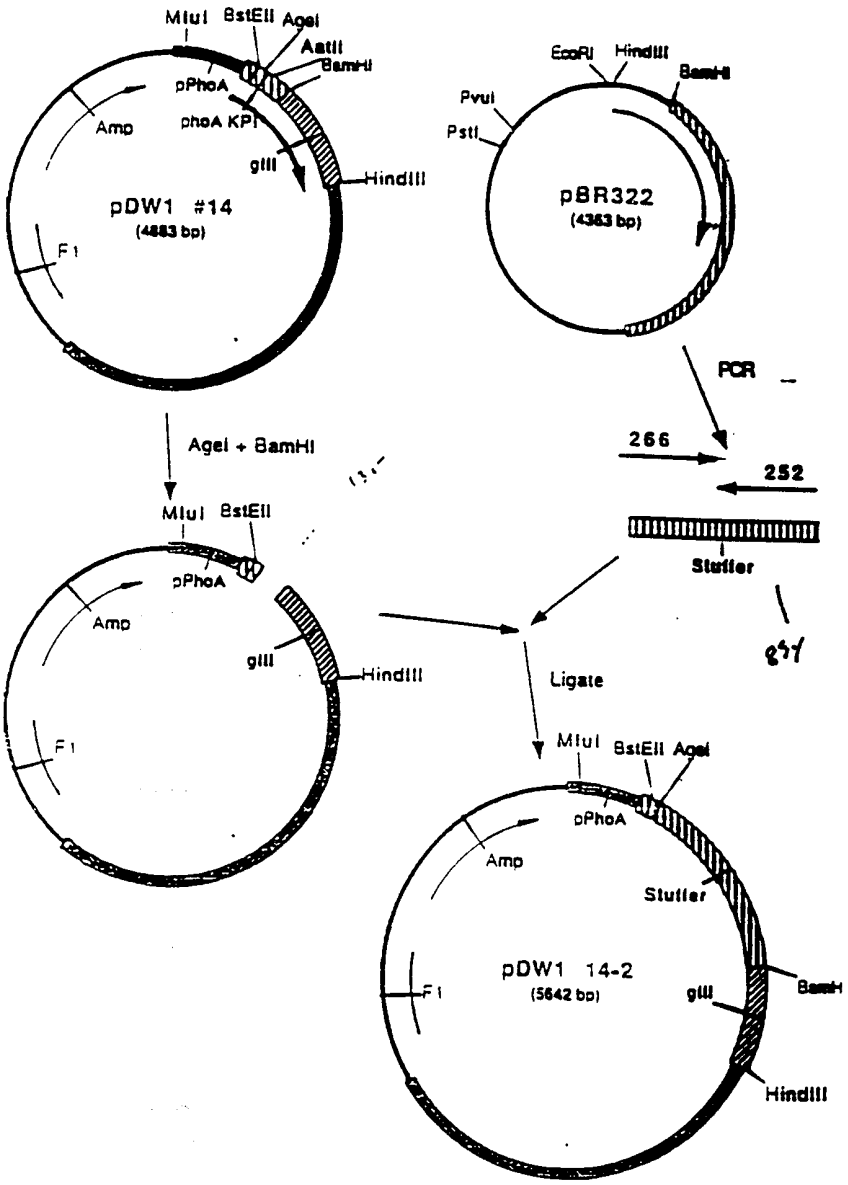

MluI BstEII AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 #14
(4883 bp)
F1
EcoRI HindIII
BamHI
PvuI
PstI
pBR322
(4363 bp)
PCR
AgeI + BamHI
266
252
Stuffer
MluI BstEII
pPhoA
Amp
gIII
HindIII
F1
Ligate
MluI BstEII AgeI
pPhoA
Amp
Stuffer
pDW1 14-2
(5642 bp)
F1
gIII
BamH
HindIII

Figure 58

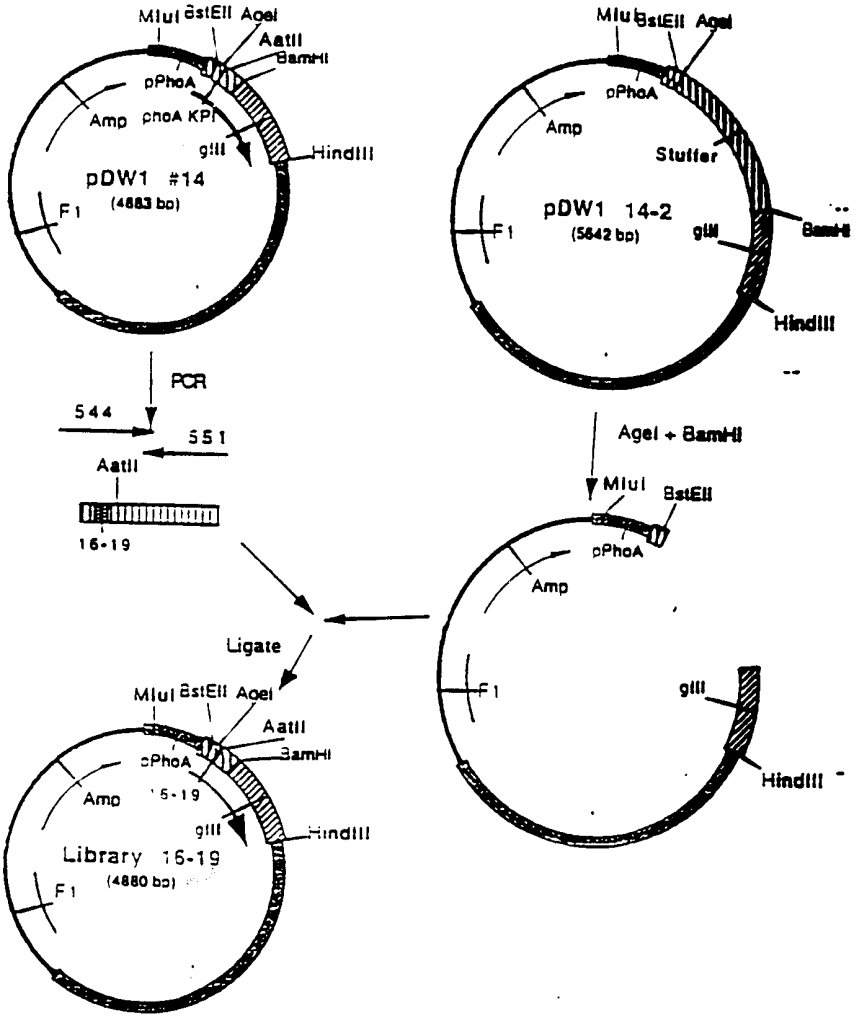
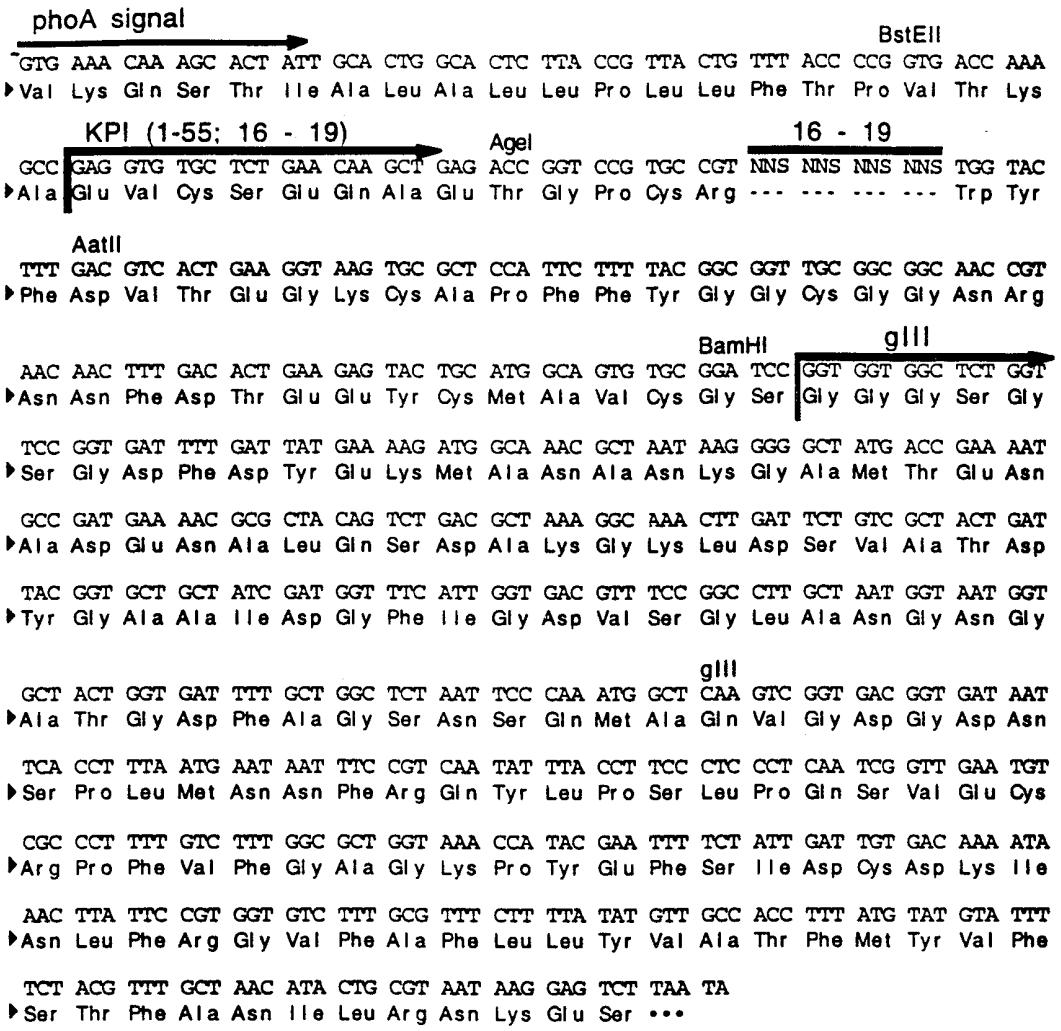

MluI BstEII AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 #14
(4883 bp)
F1
PCR
544
551
AatII
16-19
Ligate
Library 16-19
(4880 bp)
MluI BstEII AgeI
pDW1 14-2
(5642 bp)
Stuffer
AgeI + BamHI

Figure 59




```
                                                                                                                BstEII
GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

      KPI (1-55; 16 - 19)                                  AgeI                                  16 - 19
GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT NNS NNS NNS NNS TGG TAC
Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg --- --- --- --- Trp Tyr

   AatII
TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT
Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg

                                                                                BamHI          gIII
AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT GGT
Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser Gly

TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT
Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn

GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT
Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly

                                                                                gIII
GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT
Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn

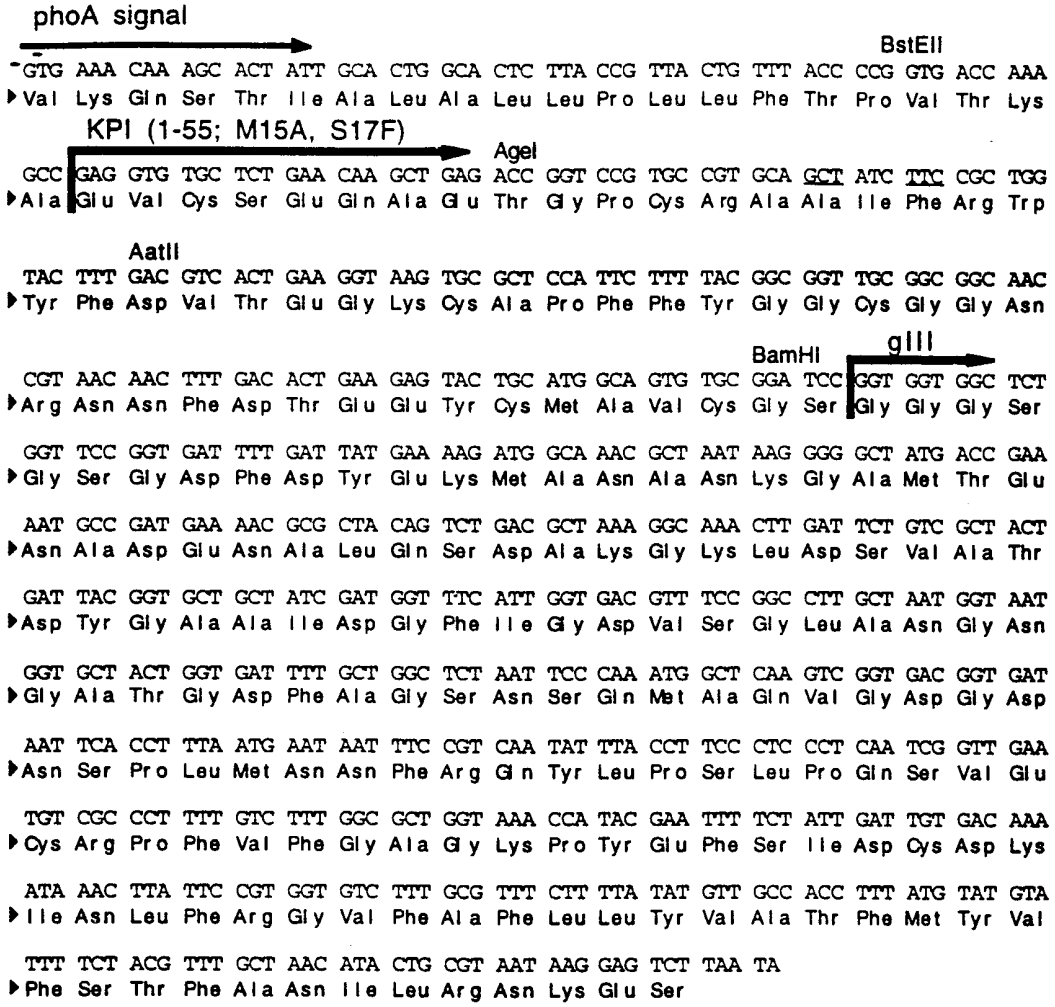
TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT
Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys

CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA ATA
Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile

AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT
Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe

TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser •••
```

Figure 60




```
                                                                                                                BstEII
-GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
 Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

                KPI (1-55; M15A, S17F)                                          AgeI
GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TTC CGC TGG
 Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Phe Arg Trp

          AatII
TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
 Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

                                                                        BamHI          gIII
CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT
 Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser

GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
 Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
 Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
 Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
 Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
 Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
 Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA
 Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
 Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
```

Figure 61

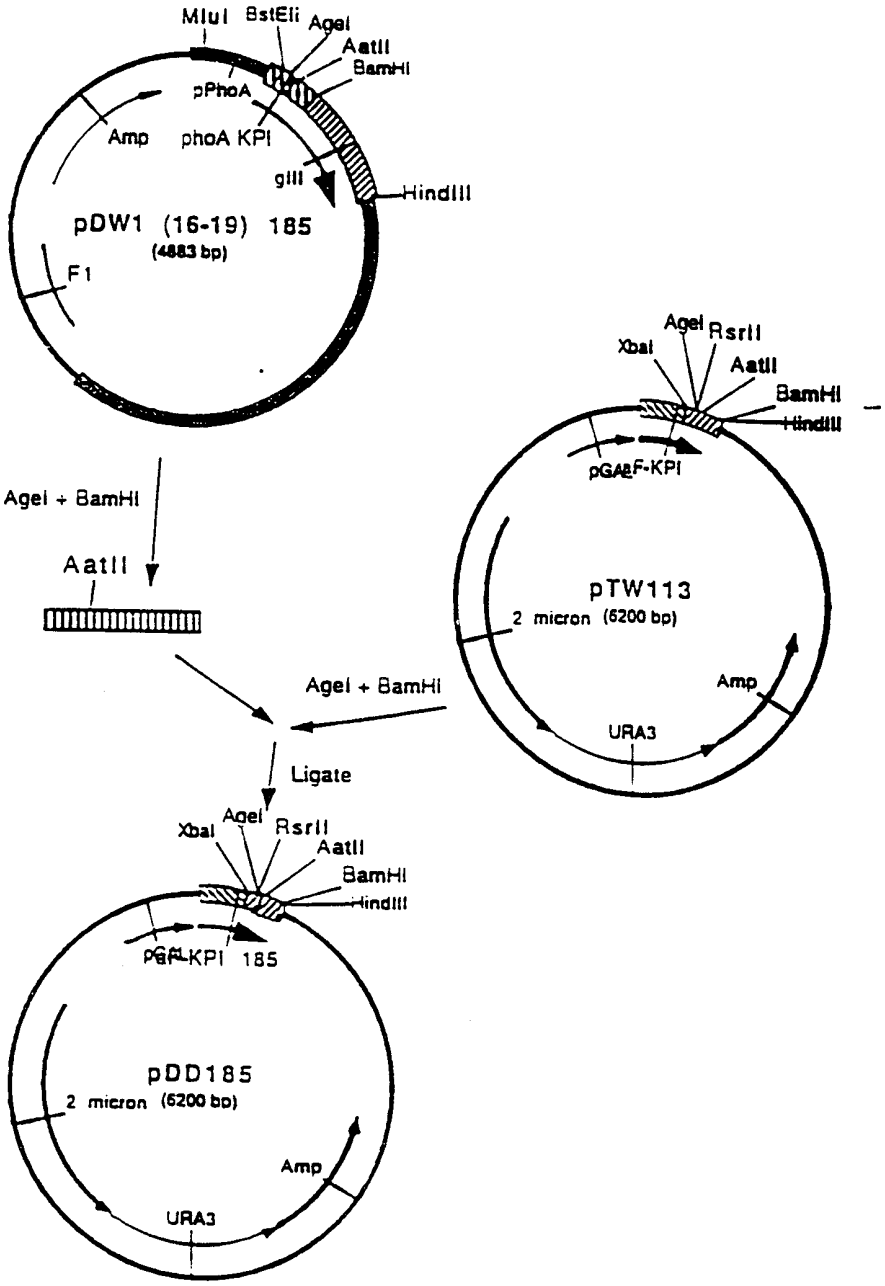

MluI
BstEli
AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 (16-19) 185
(4883 bp)
F1
AgeI + BamHI
AatII
AgeI
XbaI
RsrII
AatII
BamHI
HindIII
pGAP-KPI
pTW113
2 micron (6200 bp)
Amp
URA3
AgeI + BamHI
Ligate
AgeI
XbaI
RsrII
AatII
BamHI
HindIII
pGAP-KPI 185
pDD185
2 micron (6200 bp)
Amp
URA3

Figure 62

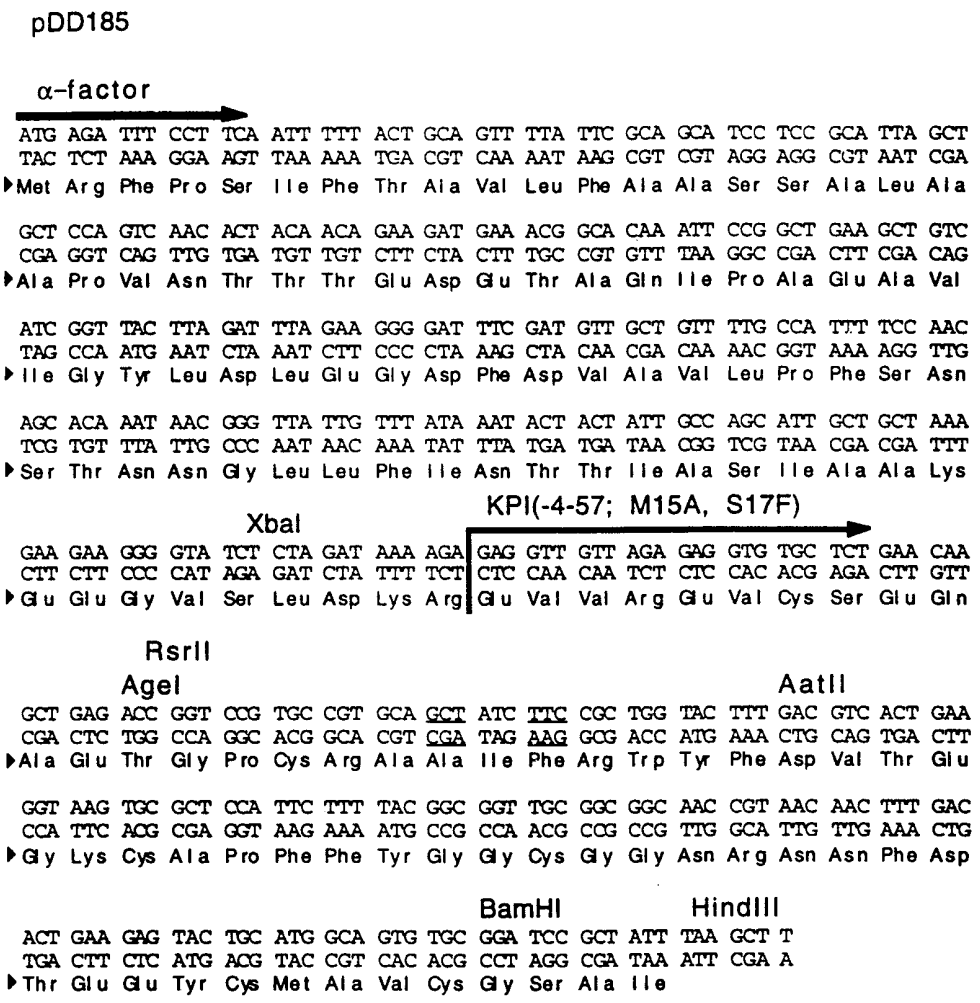

pDD185
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala
GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val
ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn
AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
XbaI
KPI(-4-57; M15A, S17F)
GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln
RsrII
AgeI
AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TTC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG AAG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Phe Arg Trp Tyr Phe Asp Val Thr Glu
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
BamHI
HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

FIGURE 63

Protease inhibition by KPI (-4-57) variants

K_is (nM)

Variant		Substitution						kallikrein	Xla	Xa
		9	15	16	17	18	37			
TW113 KPI (-4-57)								45.00	3718.0	161.0
DD185	KPI (-4-57; M15A, S17F)		A		F			0.39	150.0	196.0
TW6165	KPI (-4-57; M15A, S17W)		A		W			0.65	206.0	nd
TW6166	KPI (-4-57; M15A, S17Y)		A		Y			0.40	73.0	nd
TW6175	KPI (-4-57; M15L, S17F)		L		F			0.50	35.0	56.0
BG028	KPI (-4-57; M15L, S17Y)		L		Y			1.10	93.8	nd
TW6183	KPI (-4-57; I16H, S17F)			H	F			1.20	12440.0	159.0
TW6184	KPI (-4-57; I16H, S17Y)			H	Y			0.91	14000.0	214.0
TW6185	KPI (-4-57; I16H, S17W)			H	W			1.30	388.0	473.0
TW6173	KPI (-4-57; M15A, I16H)		A	H				1.00	1432.0	nd
TW6174	KPI (-4-57; M15L, I16H)		L	H				0.90	2796.0	nd
BG015	KPI (-4-57; M15L, S17Y,R18H)		L		Y	H		6.00	19.4	597.0
BG022	KPI (-4-57; M15A,S17Y,R18H)		A		Y	H		0.64	14.5	nd
BG029	KPI (-4-57; T9V, M15L, S17Y,R18H)	V	L		Y	H		3.20	7.9	nd
BG033	KPI (-4-57; T9V, M15A, S17Y,R18H)	V	A		Y	H		0.75	5.8	nd
DD131	KPI (-4-57; M15L, I16F, S17K)		L	F	K			7.90	1385.0	3.3
DD134	KPI (-4-57; M15L, I16F, S17K, G37Y)		L	F	K		Y	1.10	15640.0	0.6
DD135	KPI (-4-57; M15L, I16F, S17K, G37L)		L	F	K		L	1.30	7473.0	0.9

FIGURE 64

Variant	Sequence	Inhibition Ki (nM) P. kelli	Plasmin	XIa	Xa
Aprotinin	RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA	20.00	0.23	5000.0	
Aprotinin R15, S42	DFCLEPPYTGPCRARIIRYFYNAKAGLCQTFVYGGCRAKSNNFKSAEDCMRTCGGA	0.91	0.17	3983.0	
KPI (-4-57)	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	45.00	34.00	3718.0	161.0
TW6167	EVVREVCSEQAEPGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	61.00		3641.0	288.0
BG031	EVVREVCSEQAEVGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	34.00		498.0	
BG032	EVVREVCSEQAESGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	49.00		731.0	
TW101	EVCSEQAETGPCKAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	2000.00	11.50		
TW6208	EVVREVCSEQAETGPCRGMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			369.0	
TW106	EVCSEQAETGPCRARISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	560.00	3.70		
DD108	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.70	11.20	1600.0	123.0
DD109	EVVREVCSEQAETGPCRAIISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	9.50		1681.0	421.0
DD110	EVVREVCSEQAETGPCRALISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	2.10		624.0	55.0
DD111	EVVREVCSEQAETGPCRASISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	5.60			
DD112	EVVREVCSEQAETGPCRAVISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	6.80		998.0	
TW6179	EVVREVCSEQAETGPCRAGISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	78.00		368.0	
TW6163	EVVREVCSEQAETGPCRAMHSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	4.70	103.58	4532.0	457.0
TW6172	EVVREVCSEQAETGPCRAMASRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	315.00			1463.0
TW6180	EVVREVCSEQAETGPCRAMFSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	70.00		885.0	39.0
TW6181	EVVREVCSEQAETGPCRAMKSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	150.00		1514.0	
BG001	EVVREVCSEQAETGPCRAMLSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	38.00	10.00	489.0	204.0
TW116	EVCSEQAETGPCRAMIIRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	145.00	89.00		806.0
DD102	EVVREVCSEQAETGPCRAMIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	16.00		315.0	
DD103	EVVREVCSEQAETGPCRAMIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	17.00		2128.0	110.0
DD104	EVVREVCSEQAETGPCRAMIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	15.00		237.0	345.0
DD105	EVVREVCSEQAETGPCRAMIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	18.00		198.0	320.0
TW6168	EVVREVCSEQAETGPCRAMILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	25.80		3521.0	395.0

FIGURE 64 (continued)

Variant	Sequence	Inhibition Ki (nM)			
		P. kalli	Plasmin	XIa	Xa
TW6182	EVVREVCSEQAETGPCRAMIHRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	36.00		752.0	
TW6194	EVVREVCSEQAETGPCRAMIERWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	70.83			
TW6210	EVVREVCSEQAETGPCRAMIQRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	54.00		277.0	
CL006	EVVREVCSEQAETGPCRAMISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	110.20		89600.0	133.0
BG012	EVVREVCSEQAETGPCRAMISTWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			40.0	116.0
TW6209	EVVREVCSEQAETGPCRAMISHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	81.00	45.90	184.0	613.0
TW6211	EVVREVCSEQAETGPCRAMISKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	184.00		402.0	
DD128	EVVREVCSEQAETGPCRAMISLWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	44.00			37.0
TW6142	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFVYGGCGGNRNNFDTEEYCMAVCGSAI	18.00	18.00	7972.0	225.0
AL301	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFLYGGCGGNRNNFDTEEYCMAVCGSAI	216.00		1557.0	
AL302	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFGYGGCGGNRNNFDTEEYCMAVCGSAI	39.00			316.0
TW6147	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCAGNRNNFDTEEYCMAVCGSAI	35.00		1090.0	179.0
TW6138	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCKGNRNNFDTEEYCMAVCGSAI	18.00		921.0	309.0
TW6154	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCLGNRNNFDTEEYCMAVCGSAI	11.00		915.0	39.0
TW6155	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCMGNRNNFDTEEYCMAVCGSAI	11.00			27.0
TW6140	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCNGNRNNFDTEEYCMAVCGSAI	35.00		475.0	
TW6156	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCPGNRNNFDTEEYCMAVCGSAI				
TW6141	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCQGNRNNFDTEEYCMAVCGSAI	42.00			
TW118	EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	6.00	24.00	13009.0	68.0
DD100	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCCGNRNNFDTEEYCMAVCGSAI	15.00			
TW6157	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCSGNRNNFDTEEYCMAVCGSAI	40.00		511.0	168.0
TW6158	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCTGNRNNFDTEEYCMAVCGSAI	29.00			
TW6159	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCVGNRNNFDTEEYCMAVCGSAI	17.00			64.0
TW6161	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	7.50	18.00	1507.0	8.7
DD101	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCDGNRNNFDTEEYCMAVCGSAI	64.00		924.0	
TW6151	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCEGNRNNFDTEEYCMAVCGSAI	163.00		1162.0	954.0

FIGURE 64 (continued)

Variant	Sequence	Inhibition Ki (nM) P. kell	Plasmin	XIa	Xa
TW6139	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCHGNRNNFDTEEYCMAVCGSAI	19.00	22.80	152.0	78.0
TW6153	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCIGNRNNFDTEEYCMAVCGSAI	11.20	21.30	65.0	36.0
TW122	EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGANRNNFDTEEYCMAVCGSAI	32.00	27.00		581.0
TW6178	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGRNRNNFDTEEYCMAVCGSAI	16.00		444.0	
TW6148	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGARNNFDTEEYCMAVCGSAI	40.00			
TW124	EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNSNNFDTEEYCMAVCGSAI	64.00	48.00		
TW6149	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNANNFDTEEYCMAVCGSAI	54.00			
TW6173	EVVREVCSEQAETGPCRAAHSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.00	7.24	1432.0	
TW6174	EVVREVCSEQAETGPCRALHSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.90	6.89	2796.0	
BG002	EVVREVCSEQAETGPCRALLSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.98	19.00	403.0	60.0
DD129	EVVREVCSEQAETGPCRALFSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.60		1864.0	6.0
DD185	EVVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.39	8.71	150.0	196.0
TW6165	EVVREVCSEQAETGPCRAAIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.65	16.40	206.0	
TW6166	EVVREVCSEQAETGPCRAAIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.40	10.10	73.0	
BG028	EVVREVCSEQAETGPCRALIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.10	12.10	93.8	
TW6169	EVVREVCSEQAETGPCRALILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.20		619.0	111.0
DD113	EVVREVCSEQAETGPCRALIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.85	12.80	293.0	74.0
TW6175	EVVREVCSEQAETGPCRALIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.50	7.46	35.0	56.0
TW6201	EVVREVCSEQAETGPCRAGIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	34.60		419.0	
TW6202	EVVREVCSEQAETGPCRAGIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	128.50		1237.0	
TW6203	EVVREVCSEQAETGPCRAGIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	31.20		5045.0	
TW6204	EVVREVCSEQAETGPCRAAISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			147.0	87.0
TW6205	EVVREVCSEQAETGPCRALISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			195.0	29.0
DD114	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.70	7.77	224.0	
TW6190	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	0.83	52.20	589.0	1396.0
TW6183	EVVREVCSEQAETGPCRAMHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.20	11.68	12440.0	159.0

FIGURE 54 (continued)

Variant	Sequence	Inhibition Ki (nM) P. kelli	Plasmin	Xla	Xa
TW6184	EVVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.91	11.98	14000.0	214.0
TW6185	EVVREVCSEQAETGPCRAMHWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.30	18.60	388.0	473.0
BG003	EVVREVCSEQAETGPCRAMLHRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	36.00		467.0	
TW6186	EVVREVCSEQAETGPCRAMHSRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	0.48	8.86	186.0	11.0
TW6187	EVVREVCSEQAETGPCRAMIFRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	3.80	15.40	92.0	15.0
TW6188	EVVREVCSEQAETGPCRAMIYRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	4.00		419.0	24.0
TW6189	EVVREVCSEQAETGPCRAMIWRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	4.00			34.0
TW6170	EVVREVCSEQAEPGPCRALILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	2.50			452.0
DD115	EVVREVCSEQAETGPCRGYITRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			213.0	299.0
DD170	EVVREVCSEQAETGPCRALHNRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.99	18.00	550.0	
TW6176	EVVREVCSEQAETGPCRAAHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.50	118.00	56.0	
TW6177	EVVREVCSEQAETGPCRALHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	7.20	32.70	245.0	156.0
BG006	EVVREVCSEQAETGPCRAALFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.30	12.10	80.0	
DD130	EVVREVCSEQAETGPCRALFTRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	5.50			9.5
DD131	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	7.90	2.00	1385.0	3.3
DD132	EVVREVCSEQAETGPCRAFFKRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	112.00			16.8
DD120	EVVREVCSEQAETGPCRAAFSAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	8.30			11.0
DD121	EVVREVCSEQAETGPCRALLSAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	19.00			21.0
BG014	EVVREVCSEQAETGPCRALIWHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	9.20	18.70	18.0	
DD122	EVVREVCSEQAETGPCRALIFAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	15.00			46.0
BG015	EVVREVCSEQAETGPCRALIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	6.00	12.20	19.4	597.0
BG020	EVVREVCSEQAETGPCRAAIHKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.70		106.0	
BG022	EVVREVCSEQAETGPCRAAIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.64	7.26	14.5	
BG023	EVVREVCSEQAETGPCRALIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	23.00		262.0	
BG024	EVVREVCSEQAETGPCRALIYKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	4.10	7.47	38.7	
BG027	EVVREVCSEQAETGPCRAAIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	5.80		144.0	

FIGURE 64 (continued)

Variant	Sequence	Inhibition Ki (nM)			
		P.kell	Plasmin	XIa	Xa
DD116	EVVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.14		583.0	84.0
TW6191	EVVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	0.26		664.0	20.0
DD117	EVVREVCSEQAETGPCRALIPRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.11		1034.0	99.0
BG029	EVVREVCSEQAEVGPCRALIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.20		7.9	
BG030	EVVREVCSEQAESGPCRALIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	4.60		26.1	
BG033	EVVREVCSEQAEVGPCRAAIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.75		5.8	
BG034	EVVREVCSEQAESGPCRAAIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.47		18.5	
BG040	EVVREVCSEQAEIGPCRALIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.40		8.6	
BG016	EVVREVCSEQAETGPCRGAIQIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	160.00		178.0	
BG017	EVVREVCSEQAETGPCRGAIRIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	180.00		200.0	
BG021	EVVREVCSEQAETGPCRGSIRIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	340.00		224.0	
BG025	EVVREVCSEQAETGPCRGLIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	65.00		16.2	
BG026	EVVREVCSEQAETGPCRGAIYIIWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	50.00		34.9	
DD118	EVVREVCSEQAETGPCRALHNRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.53			
DD134	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	1.10	1.05	15640.0	0.6
DD135	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCLGNRNNFDTEEYCMAVCGSAI	1.30		7473.0	0.9
DD136	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCMGNRNNFDTEEYCMAVCGSAI	1.10			1.8

FIGURE 65

VOLUMES

NS	344.25
KPI	245.75

	KPI	NS
	298	366
	266	342
	354	294
	258	385
	168	288
	266	469
	172	338
	184	272
MEAN	245.75	344.25
STDEV	66.2414415	63.97488346
TTEST		0.009094999

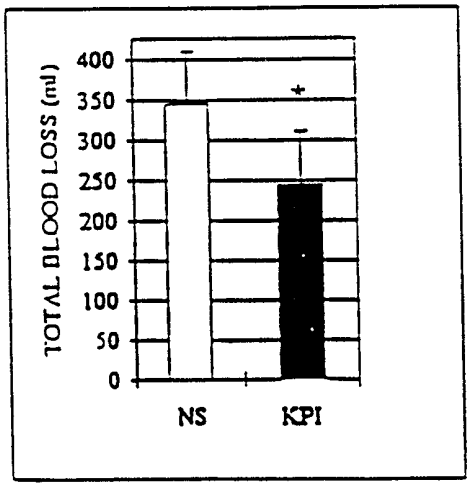


FIGURE 66

HEMOGLOBIN

NS	23.61
KPI	13.59

	KPI	NS
	16.58	24.95
	15.19	24.87
	20.21	20.46
	8.99	27.59
	14.63	18.23
	15.31	31.59
	7.7	23.26
	10.14	17.96
MEAN	13.59375	23.61375
STDEV	4.261438	4.68761
TTEST		0.000536

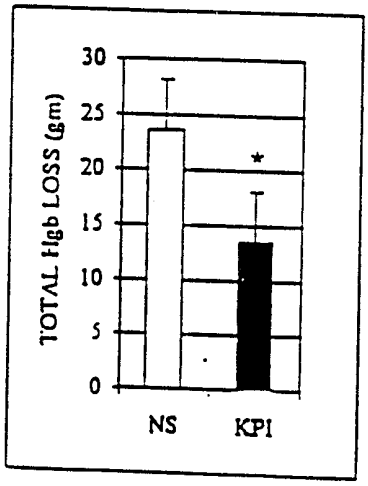


FIGURE 67

PaO2

Baseline PaO2	
KPI	NS
652.2	670.9
654	559.2
596.2	622.9
606.2	689.2
633.1	665.1
646.6	527
563.2	461.7
659.9	508
626.425	588
34.46923	85.50556
p =	*0.268*
N.S.	

End CPB	
KPI	NS
495.7	60.5
444.6	132.2
170.2	93.8
264.2	333.9
567.2	341.7
507.4	226.9
547.1	89.1
416.6	59.7
426.625	167.225
140.4741	117.9931
p =	*0.0014*

Obs 60 min	
KPI	NS
483.7	441.3
330.1	448.7
415.4	85.1
430.2	529.6
613	568.3
564.3	438.1
501	42.6
504.5	405.8
480.275	369.938
88.61879	196.5235
p =	0.17915
N.S.	

Obs 180 min	
KPI	NS
	391.3
264.1	484.6
416.5	81.3
361.9	333.2
90.8	546.6
518.2	485.3
494.2	45.6
452	383.7
371.1	344
150.2774	186.227
p =	0.76

MEAN
STDEV
TTEST

FIGURE 68

Summary of Data

	Total volume loss	Total Hgb Loss	Total Volumes		Serial Chest tube Hgb			
			Chest tube	Sacrifice	0-30min	30-60min	60-120min	120-180min
KPI-1	298	16.58	185	113	3.7	4.3	8.6	6.2
KPI-2	266	15.19	198	68	4.3	6.4	6.7	5.7
KPI-3	354	20.21	142	212	4.1	4.4	7	7.1
KPI-4	258	8.99	190	68	2.8	4	4.4	1.9
KPI-5	168	14.63	96	72	6.3	6.5	7	6.7
KPI-6	266	15.31	188	78	4.1	6.1	5.6	6.3
KPI-7	172	7.7	134	38	3.1	4.6	5.4	4.4
KPI-8	184	10.14	158	26	6.9	5.8	5.4	4.2
MEAN	245.75	13.59			4.41	5.26	6.26	5.3
STDEV	66.24	4.26			1.45	1.04	1.32	1.72
NS-1A	366	24.95	274	92	7.7	8.6	6.1	5.4
NS-2	342	24.87	236	106	7.2	7.4	7.6	7.1
NS-3	294	20.46	253	41	5.4	7.5	7.5	6.5
NS-4	385	27.59	303	82	8.4	7.2	7.1	6.3
NS-5	288	18.23	140	148	7.5	7.2	5.2	5.6
NS-6	469	31.59	261	208	4	7	7.3	7.4
NS-7	338	23.26	218	120	7.5	7.7	5.8	4.2
NS-8	272	17.96	206	66	7.4	8.2	6	5.3
MEAN	344.25	23.61			6.89	7.6	6.58	6.1
STDEV	63.97	4.69			1.44	1.04	0.91	0.85

*p = 0.009 *p = 0.0005 *p = 0.001 *p = 0.0002 NS NS

FIGURE 69

A.

Variant	Sequence changes					Expression mg/ml	kalli Ki (nM)
	9	15	17	18	48		
pTW113 (wt)	T	M	S	R	Y	27	45.00
pBG022		A	Y	H	H	22	0.78
pBG033	V	A	Y	H	H	20	0.82

B.

Variant	Sequence changes					Expression mg/ml	kalli Ki (nM)
	9	15	17	18	48		
pTW113 (wt)	T	M	S	R	Y	27	45.00
pBG022		A	Y	H	H	22	0.78
pBG033	V	A	Y	H	H	20	0.82
pBG048					H	159	44.00
pBG049		A	Y	H		4	0.93
pBG050	V	A	Y	H		4	1.20

C.

Variant	Sequence changes					Expression mg/ml	kalli Ki (nM)
	9	15	17	18	48		
pTW113 (wt)	T	M	S	R	Y	27	45.00
pTW6209				H		3	81.00

FIGURE 70

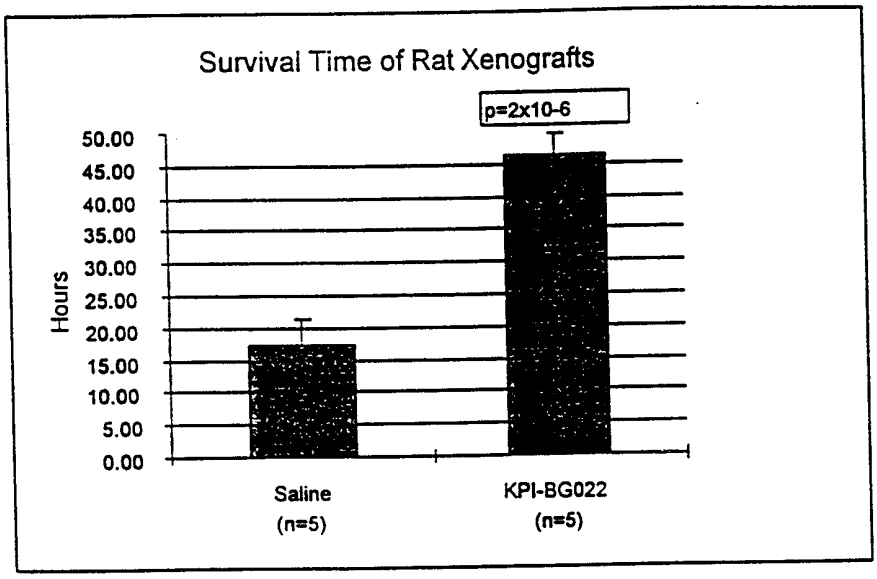

Survival Time of Rat Xenografts
p=2x10-6
Hours
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Saline
(n=5)
KPI-BG022
(n=5)

FIGURE 71

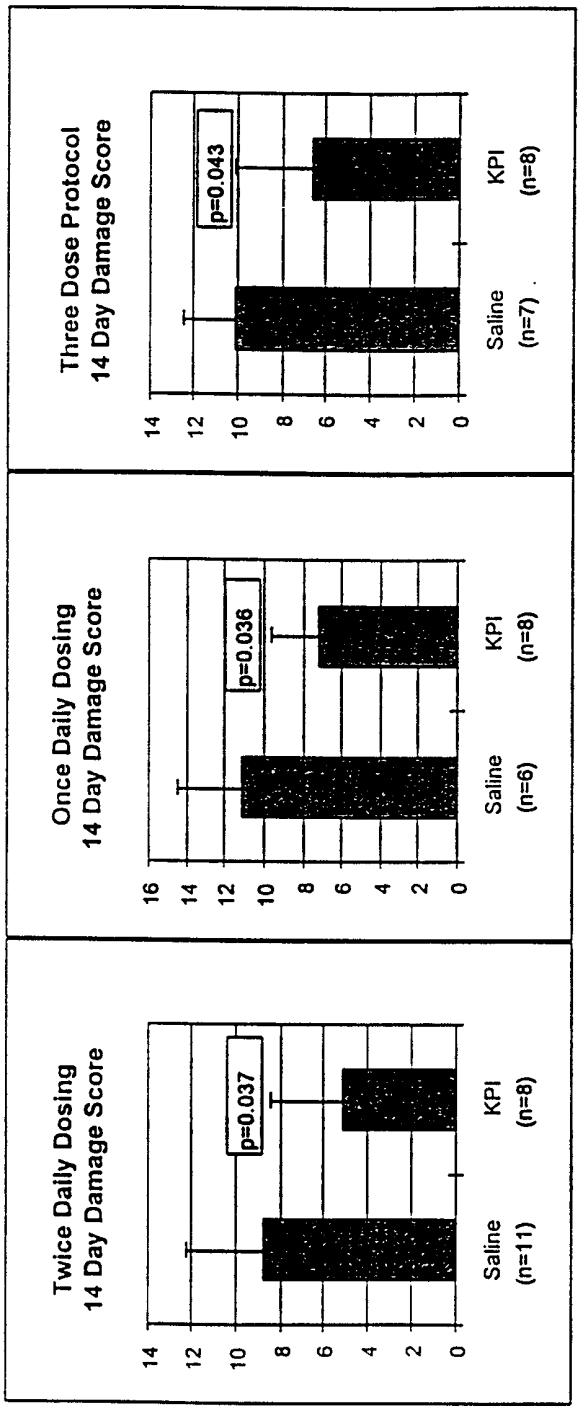
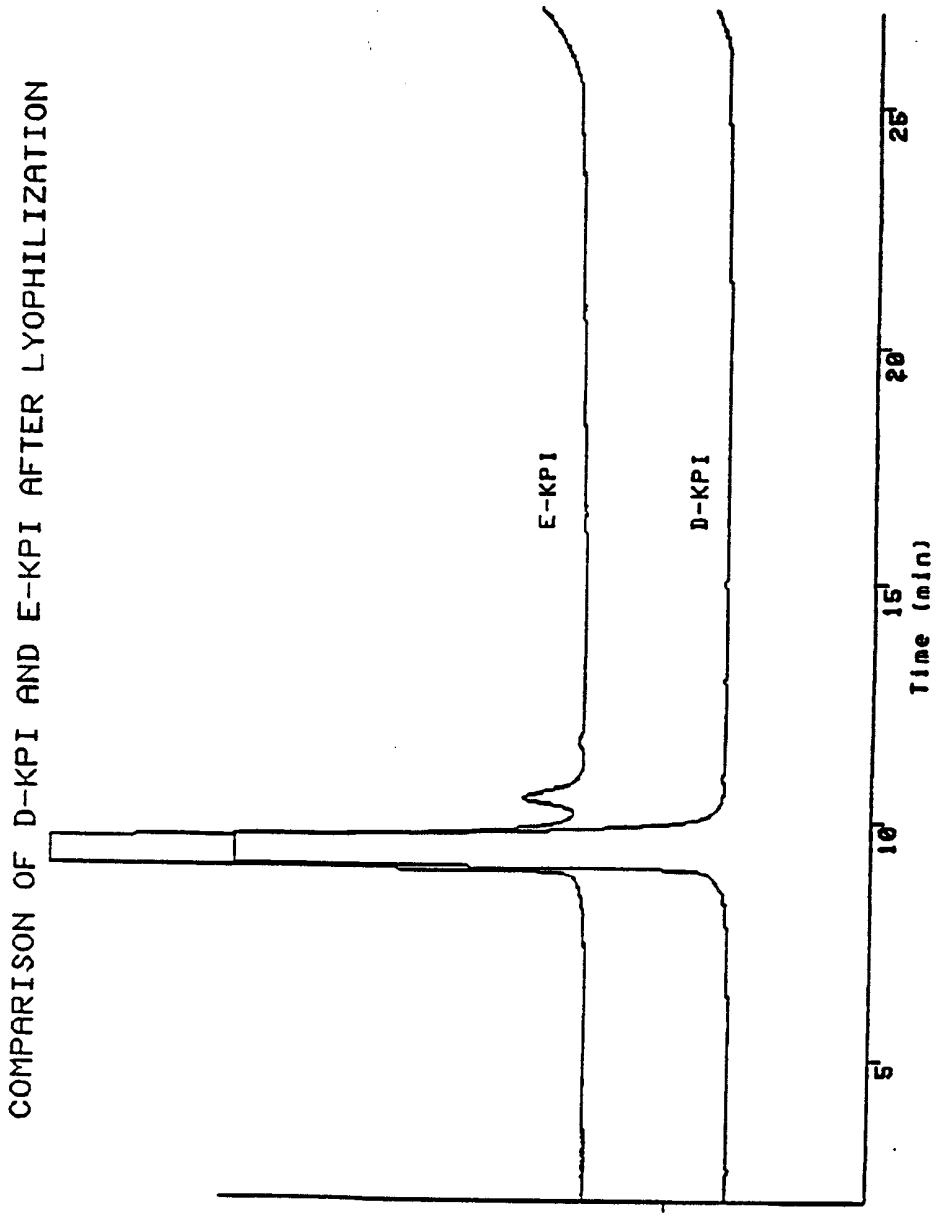

Twice Daily Dosing
14 Day Damage Score
p=0.037
Saline (n=11)
KPI (n=8)
Once Daily Dosing
14 Day Damage Score
p=0.036
Saline (n=6)
KPI (n=8)
Three Dose Protocol
14 Day Damage Score
p=0.043
Saline (n=7)
KPI (n=8)

FIGURE 72


COMPARISON OF D-KPI AND E-KPI AFTER LYOPHILIZATION
E-KPI
D-KPI
5
10
15
20
25
Time (min)