Purification and characterization of recombinant tissue kallikrein from *Escherichia coli* and yeast

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A full-length rat tissue kallikrein cDNA was constructed by oligonucleotide engineering through an extension of RSK1105, a partial cDNA clone containing 534 bp of the 3' end of tissue kallikrein, followed by site-directed mutagenesis to remove the vector sequence from within the chimaeric coding sequence. The cDNA has been cloned both into the plasmid pET3b under the control of the T7 promoter/polymerase system, and into the shuttle vector PYE directed by the α -factor promoter. Expression in *Escherichia coli* was detected by direct radioimmunoassay, and recombinant kallikrein of 36 kDa was identified by Western-blot analysis using both polyclonal and monoclonal antibodies to rat tissue kallikrein, and by autoradiography of ^{14}C -labelled L-amino acid-labelled-protein synthesis in the presence of rifampicin. Expression in yeast was also detected by direct radioimmunoassay, and recombinant kallikrein was identified by Western-blot analysis with a molecular mass of 39 kDa. The recombinant kallikrein from yeast, however, remained mostly inactive. Kallikrein was purified to apparent homogeneity from *E. coli* by DEAE-Sepharose CL-6B and aprotinin-affinity column chromatography and confirmed by the N-terminal ten-amino-acid sequence, which matched the deduced sequence from the cDNA. Both *E. coli* and yeast recombinant kallikreins have Tos-Arg-OMe-esterolytic and kininogenase activities similar to those of purified tissue kallikrein. Comparisons were made between recombinant kallikreins and rat tissue kallikrein with respect to size, charge, substrate specificity, susceptibility to inhibitors and immunological properties. Our results open the way for the study of kallikrein structure–function relationships through protein engineering.

INTRODUCTION

The kallikrein-like enzymes are closely-related serine proteinases encoded by a multigene family (Ashley & MacDonald, 1985; Gerald *et al.*, 1986; Evans *et al.*, 1987). Tissue kallikreins (EC 3.4.21.35) belong to this gene family which, in contrast with other serine proteinases, such as trypsin, chymotrypsin and elastase, is characterized by its high specificity of substrate cleavage. These kallikrein-like proteinases cleave at a very few peptide bonds in their natural substrates, with a preference for positively charged side chains and a strong bias for arginine over lysine (Fiedler & Leysath, 1979; Kettner *et al.*, 1980). The substrate specificity differs among members of the kallikrein family, despite their high sequence identity (Schiller *et al.*, 1976; MacDonald *et al.*, 1988). The most interesting example comes from the comparison between rat tissue kallikrein and tonin. Rat tissue kallikrein is a trypsin-like enzyme which liberates bradykinin, a vasodilative nonapeptide, by the selective cleavage of two peptide bonds (Arg–Arg, Arg–Ala) in both low-molecular-mass and high-molecular-mass kininogens (Kato *et al.*, 1985). Tonin, however, has both trypsin and chymotrypsin-like activities and cleaves either at an Arg–Xaa or a Phe–Xaa bond (Seidah *et al.*, 1979; Chrétien *et al.*, 1980). It converts angiotensinogen directly into the vasoconstrictor angiotensin II by the specific cleavage of a Phe–His bond (Schiller *et al.*, 1976). In addition to 74% amino acid sequence identity, kallikrein and tonin also resemble one another closely in their tertiary structures (Lazure *et al.*, 1987). X-ray-crystallographic data indicate that the differences in the conformations of the two enzymes are very small and are concentrated in several loop regions (Bode *et al.*, 1983; Fujinaga & James, 1986; Lazure *et al.*, 1987). The fact that these structural differences can cause such dramatic changes in their enzymic behaviour has made the kallikrein–tonin system an ideal model for the study of structure–function relationships.

One of the outcomes of recombinant DNA technology is the ability to synthesize large quantities of adventitious proteins, eukaryotic as well as prokaryotic, in *Escherichia coli*, for the study of their basic properties or for commercial exploitation. Significant progress has been made, especially in the past decade, on the expression of heterologous proteins in *E. coli* such that large-scale production is no longer confined to proteins that are difficult to obtain from their natural sources. One of the major advantages of heterologous expression is the convenience with which one can manipulate the protein by site-directed mutagenesis. To begin deciphering the structure–function relationships of the kallikrein-like proteinases, a heterologous expression system becomes necessary to produce large amounts of mutant and wild-type proteins.

The yeast *Saccharomyces cerevisiae* has been a good alternative expression host for many gene products, especially glycosylated proteins. The yeast system has the benefit of: (i) high levels of secretion over mammalian systems; (ii) simple and inexpensive media; (iii) no need for protein refolding and (iv) simple purification (Ratner, 1989). Although the levels of expression in yeast are generally much lower than those in *E. coli*, the potential of yeast to secrete proteins into the medium makes it an attractive system when a quick purification is desired. Previous studies on secretion of yeast proteins have primarily used invertase or acid phosphatase systems which secrete proteins into the periplasmic space or cell wall (Perlman & Halvorson, 1981; Carlson & Botstein, 1982). Recently the α -factor promoter/leader sequence system has been employed very successfully in the secretion of various types of proteins into the culture medium (Bitter *et al.*, 1984; Brake *et al.*, 1984; Zsebo *et al.*, 1986). The system proves attractive in both high-level secretion and simplicity of purification.

Here we describe a different approach in the construction of a full-length rat tissue kallikrein cDNA. Recombinant kallikreins

Abbreviations used: BCA, bicinchoninic acid; DTT, dithiothreitol; NP-40, Nonidet P40; Tos-Arg-OMe, *N*-tosyl-L-arginine methyl ester; LB, Luria–Bertani.

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were cloned and purified from both *E. coli* and yeast systems, followed by characterization of enzymic activities.

EXPERIMENTAL

Materials

The following materials were obtained from commercial sources: restriction enzymes (Boehringer Mannheim and Bethesda Research Laboratories); DEAE-Sepharose CL-6B (Pharmacia); CHAPS, sodium deoxycholate, Triton X-100, Nonidet P40 (NP-40), BSA, bradykinin, Coomassie Blue, rifampicin, β -glucuronidase (Sigma); Affi-Gel 10, low-molecular-mass protein standards, prestained molecular-mass protein standards (Bio-Rad); *N*-tosyl-L-arginine[3 H]methyl ester (Tos-Arg-O[3 H]Me), Na 125 I (Amersham International); acrylamide, *NN'*-methylenebisacrylamide (IBI); trypsin (from bovine pancreas; type IV, twice-crystallized), dithiothreitol (DTT) (Aldrich); nitrocellulose (Schleicher and Schuell; 0.45 μ m pore size); vitamin assay casamino acids, yeast nitrogen base (DIFCO Laboratories). All other reagents used were of analytical grade.

Strains and plasmids

E. coli HB101 [hdsS20, (rB $^-$, mB $^-$) recA13, ara-14, proA2, lacY, galK2, rps (sm r), xyl-5, mtl-1, supE44, λ^- /F $^-$ /] was from Dr. S. Tabor (Harvard Medical School, Boston, MA, U.S.A.; Tabor & Richardson, 1985). *E. coli* BMH71-18 mutL [Δ (lac-proAB), thi, supE; F $^+$ laciq, ZAM15, proA $^+$ B $^+$, MutL::Tn10] (Kramer *et al.*, 1984) was from Dr. D. Menick (Department of Medicine, this Institution). pGP1-2, provided by Dr. S. Tabor, is a derivative of pACYC177 that contains gene 1 of phage T7 under the control of the inducible λ P $_L$ promoter, and the gene for the heat-sensitive λ repressor, cI857. pET3b, a gift from Dr. F. W. Studier (Brookhaven National Laboratory, Upton, NY, U.S.A.; Studier & Maffatt, 1986), was a derivative of pBR322 that contains the T7 promoter (ϕ 10), the gene 10 translation start site (s10), and transcription terminator (T ϕ). *S. cerevisiae* 20B-12 (MAT α , pep4-3, trp1) (Jones, 1977) and vectors p α C2, PYE were gifts from Dr. G. A. Bitter (Amgen, Thousand Oaks, CA, U.S.A.; Bitter *et al.*, 1984). p α C2 was a derivative of pBR322 that contains the α -factor promoter and the prepro- α -factor leader sequence. The shuttle vector PYE contains the yeast 2 μ plasmid and the TRP1 gene for selection in yeast.

Cloning and site-directed mutagenesis

The scheme for the construction of the complete kallikrein cDNA is described in the Results section below. Site-specific mutagenesis was performed essentially as described (Menick *et al.*, 1987). The template was single-stranded M13mp18 or M13mp19 containing the target cDNA. Closed-circular heteroduplex DNA with the desired deletion was synthesized by Klenow enzyme and transfected into the mutator-strain *E. coli* BMH 71-18 mutL to minimize mismatch repair. Phage harbouring the mutation were identified initially by colony-blot hybridization using the 32 P-labelled mutagenic oligonucleotide as a probe (Carter *et al.*, 1984). Phage from positive colonies were plaque purified, and the mutation was verified by dideoxyoligonucleotide sequencing (Sanger *et al.*, 1977). The mutated cDNA, containing the full-length kallikrein cDNA, was subsequently cloned into the plasmid pET3b vector and the recombinant plasmid, pETskbn, was obtained. A reversely oriented clone, pETsk2, and frame-shifted clone, pETsk19, were also constructed. These clones were all transformed into *E. coli* HB101/pGP1-2.

To construct the yeast expression plasmid, two 31-base oligonucleotides (5'-AGCTGTTGTTGGAGGATATAACTGT-GAGATG-3' and 5'-AATTCATCTCACAGTTATATCCTCC-AACAAC-3') were synthesized (Applied Biosystems 380D DNA synthesizer) and annealed as adaptor segment. The adaptor sequence was designed to have a 5' *Hind*III-cohesive end and a 3' *Eco*RI-cohesive end, and it contains the 5' end sequence of the kallikrein cDNA from +1 base to the internal *Eco*RI site. The plasmid pETskbn was digested with *Eco*RI and *Bam*HI. The *Eco*RI-*Bam*HI fragment containing the 3' part of the kallikrein cDNA was inserted into M13mp19 at *Eco*RI, *Bam*HI sites so that the vector *Sal*I site near the 3' end of the cDNA sequence could be used. The cDNA was excised from M13mp19 with *Eco*RI and *Sal*I double digestion. The *Eco*RI-*Sal*I fragment, together with the adaptor sequence, were then ligated into vector p α C2 at *Hind*III/*Sal*I sites by a three-piece ligation. The plasmid obtained, p α C2/k, as confirmed by sequencing, contains the complete kallikrein cDNA sequence in the correct frame and orientation. The kallikrein cDNA with the α -factor promoter and its leader sequence was excised from p α C2 by a partial digestion with *Bam*HI, and the *Bam*HI fragment containing the complete expression construct was inserted into the shuttle vector PYE at *Bam*HI sites. The recombinant plasmid, PYE/k, was examined by restriction mapping and transformed into *S. cerevisiae* 20B-12.

Growth of cells and radioactive labelling

E. coli HB101/pGP1-2 carrying recombinant plasmids were grown with aeration at 30 °C in Luria-Bertani (LB) medium containing ampicillin and kanamycin (50 μ g/ml each). At an A_{590} of 1.5 the cells were induced at 42 °C for 25 min. Rifampicin was then added to a final concentration of 100 μ g/ml. The temperature was reduced to 37 °C for an additional 2 h, and the cells were harvested.

For exclusive labelling of plasmid-encoded proteins, cells were grown in LB medium at 30 °C with ampicillin and kanamycin (50 μ g/ml each). At an A_{590} of 0.5, 2 ml of cells were centrifuged and the pellet was washed with 5 ml of M9 medium and resuspended in 1 ml of M9 medium supplemented with thiamine (20 μ g/ml) and proline and leucine (0.01 % each). The cells were grown with aeration at 30 °C for 30–180 min. The temperature was then increased to 42 °C for 15 min before rifampicin was added to a final concentration of 200 μ g/ml. The cells were incubated at 42 °C for an additional 10 min, at which time the temperature was reduced to 30 °C for 20 min. The cells were then pulsed with 1 μ Ci of 14 C-labelled-L-amino acid mixture for 40 min and were harvested.

Yeast transformation and culture

Yeast cells were transformed by the lithium acetate method (Beggs, 1978). Yeast transformants were selected and cultured in synthetic minimal medium [0.67 % (w/v) yeast nitrogen base without amino acids/2 % dextrose (w/v)] containing 0.5 % (w/v) casamino acids.

Extraction of kallikrein from *E. coli* with various detergents

A 4-litre portion of cells (*E. coli* HB101/pGP1-2 harbouring recombinant plasmid) were grown in enriched medium [50 mM-KH $_2$ PO $_4$ (pH 7.2)/2 % (w/v) tryptone/1 % (w/v) yeast extract/0.5 % NaCl/0.2 % glycerol] containing ampicillin and kanamycin (50 μ g/ml each) at 30 °C to an A_{590} of 1.5. The cells were induced at 42 °C for 25 min. Rifampicin was added to a final concentration of 100 μ g/ml and the temperature was reduced to 37 °C for an additional 2 h. The cells were harvested by centrifugation at 4000 g for 30 min. The pellets were combined, washed

in 10 mM-Tris/HCl, pH 8.0, and re-sedimented at 6000 *g* for 20 min. The pellets were combined in 80 ml of buffer [10 mM-Tris/HCl (pH 8.0)/1 mM-MgCl₂/DNAase I (1 µg/ml)] and lysed by two passages through a French pressure cell (Amicon) at 110.4 MPa (16000 lbf/in²). Unlysed cells and debris were removed by a low-speed centrifugation at 8000 *g* for 15 min. The supernatant was diluted with buffer [50 mM-Tris/HCl (pH 8.0)/1 mM-EDTA] to a protein concentration of 4 mg/ml (Pryde & Philips, 1986). Four different detergents [6 mM-CHAPS, 1% (w/v) deoxycholate, 2% (v/v) Triton X-100 and 0.5% (v/v) NP-40] were examined by incubating them while shaking with aliquots of the supernatant at 30 °C for 2 h. The mixture was centrifuged at 27000 *g* for 1 h and the pellets were resuspended in 0.1 volume (supernatant) of buffer [50 mM-Tris/HCl (pH 8.0)/1 mM-EDTA]. Protein concentrations of each fractions were determined by a bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985).

Yeast-cell fractionation

Yeast transformants were cultured in synthetic minimal medium containing 0.5% casamino acids to an *A*₆₀₀ of 2.0 and the cells collected by centrifugation at 4000 *g* for 30 min. The pellet was resuspended in 0.1 vol. of 1.1 M-sorbitol/0.1 M-sodium citrate/25 mM-EDTA/1 mM-β-mercaptoethanol. The cells were then converted into spheroplasts with 1% (v/v) β-glucuronidase at 30 °C for 1 h. The mixture was centrifuged at 2500 *g* for 1 min. The supernatant was collected and operationally defined as 'periplasmic' proteins. The pellet was resuspended in 0.1 M-sodium citrate (pH 5.8)/25-mM-EDTA, vortex-mixed, and operationally defined as 'cellular' proteins.

SDS/PAGE

SDS/PAGE was performed on a 7.5–15%-(w/v)-polyacrylamide gradient slab gel, using the buffer system of Laemmli (1970). Proteins were stained with 0.2% Coomassie Blue in acetic acid/ethanol/water (2:9:9, by vol.) and destained in acetic acid/methanol/water (2:5:13, by vol.).

Isoelectric focusing on polyacrylamide slab gels

Isoelectric focusing was carried out essentially as previously described (Xiong *et al.*, 1990a) in an LKB-2117 Multiphor electrophoresis system with a pH gradient of 3.5–10.0 formed by Ampholine. The gel was run for 2.5 h at 4 °C with voltage increasing from 150 to 1450 V. After completion of focusing, the edge of the gel was removed and strips of 6 mm width were cut transversely at 4 mm intervals. Each section was placed in a tube containing 1 ml of distilled water and incubated for 24 h, at which time the pH values of the sections were measured and the pH gradient was obtained by plotting the sections' pH value against their original positions on the gel. The remaining gel was fixed in 25% (v/v) trichloroacetic acid overnight, stained in 0.2% Coomassie Blue solution for 15 min and destained in acetic acid/methanol/water (2:5:13, by vol.).

Immunoblotting analysis

Immunoblotting was performed as previously described (Chao *et al.*, 1989). Proteins were resolved on a 7.5–15% linear-gradient polyacrylamide gel containing 0.1% SDS and transferred on to nitrocellulose membrane. All incubations were at room temperature. The nitrocellulose was soaked for 1 h in BLOTTO [0.01 M-sodium phosphate (pH 7.4)/5% (w/v) non-fat dry milk/0.14 M-NaCl/1 µM-*p*-aminodiphenylmethanesulphonyl fluoride/0.1% (w/v) thimerosal/0.02% (w/v) Na₂S₂O₃/0.01% antifoam A]. The blot was then incubated with anti-kallikrein antibodies, mouse anti-(rat tissue kallikrein)mono-

clonal antibody V₄D₁₁ (Woodley *et al.*, 1985) diluted 1:300 or sheep anti-(rat tissue kallikrein)antiserum diluted 1:400 in BLOTTO for 3 h, then washed three times with BLOTTO for 10 min each. Incubation with the second antibodies [¹²⁵I-labelled rabbit anti-mouse IgG diluted in BLOTTO (250000 c.p.m./ml)] in the case of monoclonal antibodies, or [¹²⁵I-labelled kallikrein [diluted in BLOTTO (250000 c.p.m./ml)] in the case of sheep antiserum, was performed for 90 min. The blot was washed three times with BLOTTO for 10 min each and then washed with phosphate-buffered saline [0.01 M-sodium phosphate (pH 7.4)/0.14 M-NaCl]. The blot was air-dried and exposed to X-ray film.

Purification of recombinant kallikreins

E. coli HB101/pGP1-2 harbouring pETskbn and *S. cerevisiae* 20B-12 harbouring PYE/k were cultured as described above. *E. coli* cells were extracted with 2% Triton X-100. The supernatant fraction was dialysed against 0.02 M-Tris/HCl (pH 8.0)/1 mM-EDTA, and then passed through a DEAE-Sepharose CL-6B column (2.2 cm × 20 cm) equilibrated with the same buffer and washed until the *A*₂₈₀ of the effluent decreased to below 0.05. Yeast cells were sedimented at 4000 *g* for 30 min. The culture medium (supernatant) was directly applied to a DEAE-Sepharose CL-6B column. In both cases, the column was developed with a (0–0.6 M) linear gradient of NaCl. Fractions were monitored by measuring esterolytic activity (see under 'Enzymic assays' below) using Tos-Arg-OMe as substrate. The conductivity of the eluate was measured at 25 °C with a Yellow Springs Instruments conductivity bridge (model 31) and a conductivity cell (type 3403). Protein concentrations were determined by the BCA protein assay (Smith *et al.*, 1985). Fractions containing kallikrein-like activity were combined and directly applied to an aprotinin-agarose affinity column equilibrated with 0.02 M-Tris/HCl (pH 8.0)/0.2 M-NaCl/1 mM-EDTA. Proteins absorbed were eluted with 0.1 M-glycine/HCl, pH 2.8, and 3 ml fractions were collected into tubes containing 0.6 ml of 0.5 M-Tris base. The fractions containing Tos-Arg-OMe esterase activity were pooled and dialysed against distilled water.

Enzymic assays

The modified assay described by Beaven *et al.* (1971) was used to measure Tos-Arg-OMe esterase activity, as previously described (Chao & Margolius, 1979). One esterase unit (E.U.) is defined as the amount of enzyme which hydrolyses 1.0 µmol of Tos-Arg-OMe/min at pH 8.0 and 30 °C. The kininogenase assay described by Shimamoto *et al.* (1978) was used to determine the kinin-generating activity of kallikrein, and kinin released was measured by a kinin radioimmunoassay (Shimamoto *et al.*, 1979). Purified bovine low-molecular-mass kininogen (3 µg) and heated (60 °C, 1 h) rat plasma (1:25 dilution; 50 µl) were used as the substrates. Concentrations of recombinant tissue kallikrein was determined by a direct radioimmunoassay (Shimamoto *et al.*, 1979). The pH-dependence of recombinant kallikreins and rat submandibular kallikrein was assayed as previously described (Chao *et al.*, 1979).

Inhibition study

The effects of three proteinase inhibitors (aprotinin, leupeptin and antipain) on the purified recombinant kallikreins were examined as described previously, using Tos-Arg-OMe as the substrate (Chao, 1978). A 10 µl portion of enzyme [(2–4) × 10⁻² E.U./ml] was incubated with 10 µl of inhibitor at a series

of dilutions in 40 μ l of 0.2 M-Tris/HCl, pH 8.0, at 37 °C for 30 min. The solution was left at room temperature for 10 min before Tos-Arg-O[3 H]Me (3.0×10^4 c.p.m./10 μ l) was added. The mixture was incubated at room temperature for another 30 min and stopped with cold Tos-Arg-OMe in 10% (v/v) acetic acid. The [3 H]methanol released was measured in a Beckman LS-35 liquid-scintillation counter.

N-Terminal protein sequencing

Purified recombinant kallikrein from *E. coli* was resolved by SDS/PAGE under reducing conditions and electrotransferred on to poly(vinylidene difluoride) membrane (Millipore). The protein blotted on the membrane was stained with 0.2% (w/v) Ponceau S in 3% trichloroacetic acid for 1 min, destained with 1 M-acetic acid for 2 min. The band was cut out and subjected to N-terminal sequence analysis using a gas-phase protein sequencer (ABI model 470A) by the method described by the manufacturer.

RESULTS

Construction of full-length kallikrein cDNA

Fig. 1 shows the steps in the construction of the full-length tissue-kallikrein cDNA. A partial kallikrein cDNA clone, RSK1105, has previously been isolated in our laboratory (Gerald *et al.*, 1986). The clone is 534 bp in length, with its nucleotide sequence identical with the 3' end of the rat tissue-kallikrein cDNA (Swift *et al.*, 1982). The original partial rat kallikrein cDNA clone, RSK1105/M13mp8, was digested with *Eco*RI and filled with Klenow enzyme. The DNA was then digested with *Bam*HI, and the *Eco*RI (flushed)-*Bam*HI fragment containing the 534 bp RSK1105 sequence was cloned into M13mp19 at *Hinc*II and *Bam*HI sites. The remaining 262 bp sequence of the 5' end of the kallikrein cDNA was completed by the use of four oligonucleotides of 131–135 bases corresponding to the known sequence of the rat pancreatic kallikrein cDNA (Swift *et al.*,

Fig. 1. Construction of full-length tissue kallikrein cDNA

The restriction endonuclease cleavage sites are: E, *Eco*RI; Sm, *Sma*I; Hc, *Hinc*II; P, *Pst*I; H, *Hind*III; B, *Bam*HI; N, *Nde*I; E(x), *Eco*RI site filled by Klenow enzyme. Four oligonucleotides (stippled bars) of 131–135 bases were synthesized as described in the text. The synthetic fragment was obtained by annealing the complementary oligonucleotides, followed by a three-piece ligation. The oligo-piece is represented by the hatched area. The 3' portion of the cDNA, as represented by the solid black area, was derived from a previous clone, RSK1105. The oligo-piece was then excised from the corresponding vector and ligated into M13mp19, 5' to the RSK1105 fragment. Site-directed mutagenesis was performed to remove the vector part from within the coding sequence.

1982). Four oligonucleotides of 131–135 bases were synthesized with an Applied Biosystems 380D DNA synthesizer. The sequences were designed such that the annealed fragments have a complementary six-base cohesive end with which they can be ligated, forming a fragment with a 5' *Nde*I site and a 3' *Pst*I cohesive end. After gel purification, each of the two complementary oligonucleotides was mixed at a 1:1 ratio. The mixtures were denatured at 95 °C and allowed to anneal. The duplexes formed were ligated into M13mp18 at *Hinc*II and *Pst*I sites by a three-piece ligation. The synthetic fragment was then excised with *Sma*I and *Pst*I and cloned into RSK1105/M13mp19 at *Hind*III (flushed) and *Pst*I sites. Deletion of the vector sequence within the coding sequence was carried out by site-directed mutagenesis using a 26-base oligonucleotide. The mutated cDNA was verified by sequencing and shown to contain the continuous full-length sequence of the rat tissue kallikrein.

Expression of tissue kallikrein in *E. coli*

The recombinant plasmid containing the complete rat tissue-kallikrein cDNA, pETskbn, was transformed into *E. coli* HB101pGPI-2, a strain containing the temperature-sensitive λ -repressor, cI857. The expression was analysed by autoradiography of 14 C-labelled L-amino acid-labelled-protein synthesis. Transcription from the ϕ 10 promoter resulted in expression of both the kallikrein cDNA and the β -lactamase gene, which is carried by pET3b. Exclusive expression of these genes was achieved, after heat induction of T7 RNA polymerase, by the addition of rifampicin to shut off *E. coli* RNA polymerase transcription (Chamberlin & Ring, 1973). Fig. 2 shows the expression of kallikrein in *E. coli* analysed by autoradiography. The synthesis of recombinant kallikrein constituted a major band of 36 kDa in the absence of rifampicin (lane 1). The addition of rifampicin resulted in exclusive labelling of plasmid-encoded proteins. After induction, two predominant bands were observed on the autoradiogram of the SDS- containing gel (lane 2). The 28 kDa band represents β -lactamase, whereas the major band of 36 kDa corresponds to recombinant kallikrein. Fig. 3

Fig. 2. Kallikrein expression analysed by autoradiography

E. coli HB101/pGPI-2 harbouring pETskbn were grown in LB medium at 30 °C with 50 μ g each of ampicillin and kanamycin/ml. At an A_{590} of 0.4, the temperature was shifted to 42 °C for 25 min. Rifampicin (100 μ g/ml) was added and, after an additional 10 min at 42 °C, the cells were grown for 20 min at 30 °C. After pulse-labelling with 1 μ Ci of 14 C-labelled L-amino acids, the cells were harvested and subjected to SDS/PAGE and autoradiography. Lane 1, without rifampicin; lane 2, with rifampicin.

Fig. 3. SDS/PAGE and Western-blot analysis of kallikrein in over-producing *E. coli* strains

(a) Electrophoresis was performed on a 7.5–15% linear gradient slab gel and stained with Coomassie Blue. (b) Western blot with 125 I-labelled monoclonal mouse anti-(rat tissue kallikrein)antibody (V_4D_{11}). Lanes 1, purified rat submandibular kallikrein; lanes 2–4, different constructs in *E. coli* strain HB101/pGPI-2 (lanes 2, pETsk2; lanes 3, pETsk19; lanes 4, pETskbn).

shows the high-level production of kallikrein from *E. coli* cells harbouring pETskbn as analysed by protein staining (Fig. 3a) and Western blot (Fig. 3b) of SDS/PAGE gels run under reducing conditions. Lanes 1 are purified rat submandibular (tissue) kallikrein with a molecular mass of 38 kDa as a positive control, whereas lanes 2 and 3 are negative controls with the kallikrein cDNA in reverse orientation (pETsk2) and shifted frame (pETsk19) respectively. On protein staining (Fig. 3a), a major band of 36 kDa was observed in the positive clone pETskbn (lane 4) and not in lanes 2 and 3. The 36 kDa band was recognized by both monoclonal (Fig. 3b, lane 4) and polyclonal (results not shown) anti-kallikrein antibodies in Western-blot analysis. Neither polyclonal nor monoclonal anti-tonin anti-

Fig. 4. SDS/PAGE and Western-blot analysis of kallikrein expression in yeast

(a) SDS/PAGE was performed on a 7.5–15% linear gradient slab gel and stained with Coomassie Blue. (b) Western blot with 125 I-labelled polyclonal sheep anti-(rat tissue kallikrein)antiserum. Lanes 1 are purified rat submandibular kallikrein; lanes 2–4, different fractions of *S. cerevisiae* 20B-12 containing vector PYE (lanes 2, cellular; lanes 3, periplasmic; lanes 4, medium); lanes 5–7, different fractions of 20B-12 containing PYE/k (lane 5, cellular; lanes 6, periplasmic; lanes 7, medium).

bodies cross-reacted with the recombinant tissue kallikrein (results not shown).

Effects of detergents on the release of recombinant kallikrein from *E. coli*

Most of the recombinant kallikreins in *E. coli* was synthesized as insoluble proteins. Four types of detergents (6 mM-CHAPS, 1% deoxycholate, 2% Triton X-100 and 0.5% NP-40) were tested and they displayed similar abilities to solubilize kallikrein. The immunologically reactive kallikrein released into the supernatant after 3 h extraction averaged 2.4 mg/litre of culture medium, measured by direct radioimmunoassay for rat tissue kallikrein, representing 6.4% of the total kallikrein.

Expression of kallikrein in yeast

Kallikrein cDNA was inserted in frame with the MF α 1 prepro sequence (Bitter *et al.*, 1984), and the recombinant plasmid, PYE/k, was transformed into *S. cerevisiae* strain 20B-12. The recombinant kallikrein was synthesized as a fusion protein to the prepro sequence of the α -factor structural gene (85 amino acids) and the (Glu-Ala) dipeptides. The recombinant kallikrein was synthesized as a soluble protein in yeast. Fig. 4 shows the expression of kallikrein in yeast as analysed by protein staining and Western blot. Lanes 1 are purified rat submandibular kallikrein as a positive control. The control clone, 20B-12 harbouring vector PYE, was also included, as in lanes 2 (cellular), lanes 3 (periplasmic) and lanes 4 (medium). Lanes 5–7 are cellular, periplasmic and medium fractions of the positive clone, PYE/k, respectively. Protein staining revealed a major band of 39 kDa in the medium of the PYE/k clone (Fig. 4a, lane 7), whereas the control clone harbouring the PYE vector did not contain this band at the corresponding position (lane 4). The 39 kDa protein was recognized, in all three fractions of PYE/k clone, by both monoclonal (results not shown) and polyclonal (Fig. 4b, lanes 5–7) antibodies against kallikrein. The higher molecular mass of the yeast recombinant protein is probably due to glycosylation from the yeast system, although further study is needed to characterize the glycosylation process. In addition, the antibodies also recognized another band of 49 kDa in the cellular fraction of PYE/k (lane 5). The difference between the molecular masses of these two components may represent the molecular mass of the α -factor leader sequence, which is 85 residues in length. It therefore appears from Fig. 4 that all the secreted kallikreins were processed and appeared as a band of 39 kDa. The kallikreins within the cytosol were partially processed, and the unprocessed molecules migrated at a position corresponding to 49 kDa. The yield of immunoreactive kallikrein synthesized/litre of culture medium was 0.6–1.1 mg, as measured by direct radioimmunoassay. Most of the synthesized kallikrein (90%) was secreted into the medium.

Purification of recombinant tissue kallikreins from *E. coli* and yeast

Kallikrein was purified from *E. coli* lysate (HB101/pGP1-2 harbouring pETskbn) to homogeneity with DEAE-Sepharose CL-6B and aprotinin-affinity column chromatography. Since the carboxy side of arginine is a preferred cleavage site for kallikrein-like serine proteinases, the radiochemical Tos-Arg-OMe esterase assay has been routinely used as a measure of kallikrein-like enzyme activity. The fractions containing kallikrein were therefore monitored by Tos-Arg-OMe esterase assay. Two peaks of esterolytic activity were eluted from the column with an NaCl gradient. The first peak was esterolytic activity derived from *E. coli*, as the proteins contained in this peak did not bind to aprotinin-affinity column nor did they have corresponding kinin-

Fig. 5. Purification of kallikrein from *E. coli* HB101/pGP1-2 harbouring pETskbn

Conditions for cell growth and enzyme purification were described in the text. Electrophoresis was performed on a 7.5–15% linear gradient slab gel and stained with Coomassie Blue. Lane 1, molecular-mass standards; lane 2, purified rat submandibular kallikrein; lane 3, Triton X-100-extracted cell homogenate; lane 4, DEAE-Sepharose column fraction of purified kallikrein; lane 5, aprotinin-affinity column fraction of purified kallikrein.

releasing activities (results not shown). The second peak, eluted at approx. 0.37 M-NaCl, was pooled and passed through an aprotinin-affinity column (1.5 cm $\times$ 15 cm) equilibrated in 20 mM-Tris/HCl (pH 8.0)/0.2 M-NaCl/1 mM-EDTA. The proteins absorbed were eluted with 0.1 M-glycine/HCl, pH 2.8. The purity of each fraction was monitored by SDS/PAGE. In Fig. 5, the lanes include purified rat submandibular kallikrein (lane 2), cell homogenate extracted with Triton X-100 (lane 3), fraction from DEAE-Sepharose CL-6B (lane 4), and eluate from the aprotinin-affinity column (lane 5). The purified recombinant kallikrein migrated as a single band of 36 kDa on the gel (lane 5). Isoelectric focusing of the purified protein showed the pI of the recombinant protein to be 3.8–4.0 (results not shown). The same two-step chromatography was also used to purify kallikrein from yeast cells (20B-12 harbouring PYE/k). The medium was directly applied to a DEAE-Sepharose CL-6B column equilibrated with 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA. The fractions containing esterolytic activity were pooled and subjected to an aprotinin-affinity column and eluted with 0.1 M-glycine/HCl, pH 2.8. Radioimmunoassay indicated, however, that only 1% of the kallikrein eluted from DEAE-Sepharose CL-6B absorbed to the aprotinin affinity column and therefore could be purified, whereas the remaining portion was contained in the flow-through. The flow-through was analysed by protein staining and Western blot (Fig. 6). Purified rat submandibular kallikrein (lanes 2) and recombinant kallikrein from *E. coli* (lanes 3) were used as positive controls. One major band of 39 kDa was observed on protein staining of the flow-through against a relatively clean preparation (Fig. 6a, lane 4) and was confirmed to be recombinant kallikrein by Western blot, using sheep anti-kallikrein antiserum (Fig. 6b, lane 4). The absence of activity could be partly explained by the possibility that a portion of the recombinant proteins in the yeast was improperly folded. Submandibular kallikrein used as the control (Figs. 5 and 6, lane 2) contains a band of approx. 30 kDa on SDS/PAGE under reducing conditions, and *N*-terminal-amino-acid sequencing of this band revealed that it is a

Table 1. Comparison of recombinant kallikreins from *E. coli* and yeast with rat submandibular kallikrein

Parameter	Kallikrein source ...	Rat submandibular gland	<i>E. coli</i>	Yeast
Molecular mass (kDa)		38	36	39
N-Terminal sequence		1 2 3 4 5 6 7 8 9 VVG ¹ GYNCE ⁹ M	1 2 3 4 5 6 7 8 9 MVVG ¹ GYNCE ⁹ M	
pI range		3.7–4.0	3.8–4.0	
Tos-Arg-OMe esterolytic activity				
Activity* (E.U./mg of kallikrein)		150 ± 20	452 ± 51	329 ± 80
pH optimum		9.0	9.0	9.0
Kininogenase				
Activity (μg of kinin/min per mg kallikrein)				
Rat kininogen		1.26	3.43	3.99
Bovine kininogen		2.35	6.40	5.97
pH optimum		9.0	9.0	9.0
Inhibition [IC ₅₀ (M)]				
Aprotinin		4.3 × 10 ⁻⁸	8.5 × 10 ⁻⁸	8.4 × 10 ⁻⁸
Leupeptin		1.6 × 10 ⁻⁶	3.1 × 10 ⁻⁶	3.2 × 10 ⁻⁶
Antipain		2.1 × 10 ⁻⁵	3.5 × 10 ⁻⁵	3.3 × 10 ⁻⁵

* Mean ± S.E.M., *n* = 3.

Fig. 6. Comparison of kallikreins from rat tissue, *E. coli*, and yeast by protein staining and Western blot

(a) SDS/PAGE was performed on a 7.5–15% linear gradient slab gel and stained with Coomassie Blue. (b) Western blot with ¹²⁵I-labelled polyclonal sheep anti-(rat tissue kallikrein) antiserum. Lane 1, molecular standards; lanes 2, purified rat submandibular kallikrein; lanes 3, purified recombinant kallikrein from *E. coli*; lanes 4, flow-through from aprotinin-affinity column during purification of recombinant yeast kallikrein.

Fig. 7. Log-logit transformations of a typical radioimmunoassay curve of rat tissue kallikrein (O) and serial dilutions of recombinant kallikreins purified from *E. coli* (◇) and from yeast (●)

B/B_0 is the percentage of bound radioactivity in the presence and absence of unlabelled kallikrein using sheep anti-(rat urinary kallikrein) antiserum.

cleavage product of the 38 kDa submandibular kallikrein (J. Chao and L. X. Wang, unpublished work).

N-Terminal sequence analysis of recombinant *E. coli* kallikrein

Table 1 shows that ten amino acid residues of the N-terminus of purified recombinant kallikrein from *E. coli* match the amino acid sequence deduced from cDNA, which demonstrates the accuracy of transcription initiation and further verifies the correct construction of a full-length kallikrein cDNA molecule.

Characteristics of recombinant tissue kallikreins

Appropriate dilutions of the eluate from aprotinin-affinity column were used to obtain a range of antigen concentrations

comparable with those of the rat tissue kallikrein standard (0.08–10 ng/tube). The log-logit transformations of the radioimmunoassay standard curve for rat tissue kallikrein and serial dilutions of the purified recombinant kallikreins from both *E. coli* and yeast are shown in Fig. 7. The linear displacement curves for the purified recombinant enzymes were parallel with the kallikrein standard curve, indicating the immunological identities of the two recombinant enzymes with rat tissue kallikrein. Both recombinant kallikreins have Tos-Arg-OMe esterolytic activities and kininogenase activities comparable with, or higher than, that of tissue kallikrein (Table 1). Correspondingly, their Tos-Arg-OMe esterolytic activities and the kininogenase activities share an optimum pH at 9.0. In addition, the recombinant kallikreins were both inhibited by three proteinase inhibitors (aprotinin, leupeptin and antipain) in a dose-dependent manner. IC₅₀ (inhibitor concentration giving 50% inhibition of the enzymes)

esterolytic activity) values of the recombinant enzymes were comparable with those of rat tissue kallikrein (Table 1).

DISCUSSION

We have described the construction of a full-length rat tissue-kallikrein cDNA by oligonucleotide engineering through the extension of RSK1105, a partial cDNA clone isolated previously in our laboratory. Swift *et al.* (1982) previously isolated, through primer extension, four overlapping cDNA clones that gave a continuous mRNA sequence of the rat tissue kallikrein. Sequence analysis demonstrated that the sequence of our cDNA matches the reported sequence of the rat tissue-kallikrein cDNA (Swift *et al.*, 1982). The advantages of using oligonucleotide engineering over traditional library-screening methods can be recognized from the time-saving procedure of the construction process and the convenience of creating appropriate restriction sites at the end of the molecules such that when the cDNA clone is completed, it already contains adequate enzyme sites for cloning.

We have shown that kallikrein was overproduced in *E. coli* under the control of the T7 promoter. Since the products could not be completely solubilized, the level of production cannot be accurately assessed. It was estimated by video densitometry of SDS/PAGE that 30–50 mg of kallikrein was produced from each litre of culture. Our yield is 100-fold higher than that achieved by Angermann *et al.* (1989), in whose study, by the same method of estimation, 300–500 µg of human recombinant kallikrein was produced from each litre of *E. coli* culture. When eukaryotic proteins are produced in large amounts in *E. coli*, it is not unusual for them to form insoluble aggregates, despite the fact that they are normally soluble (Bikel *et al.*, 1983; Scheuermann & Echols, 1984; Lin *et al.*, 1987). There have been many speculations about the mechanism through which the insoluble inclusion bodies are formed (Marciani *et al.*, 1987; Schein, 1989). Among them, growth temperature above 30 °C and rapid production rate are by far the most important factors. Although frequently mentioned, the solubility problem still remains a serious one, partly owing to lack of thorough investigation. To release the recombinant protein from the tightly formed inclusion bodies, strong chaotropic reagents like 6 M-urea or 8 M-guanidinium chloride have to be used, and the renaturation process that follows can be tedious and the recovery very poor for large proteins with disulphide bonds. Previous reports showed that, in many cases, this can be overcome by lowering the culture temperature (Bishai *et al.*, 1987; Piatak *et al.*, 1988; Sturtevant *et al.*, 1989). Additional support for this temperature-sensitive protein folding comes from the phenomenon that heat-shock with a 5–7 °C temperature increase induces insolubilization of proteins, both foreign and native, in eukaryotic cells (Nover & Scharf, 1984; Nguyen *et al.*, 1989). However, this does not seem to be the case with kallikrein, since lowering the temperature of induction or growth had no obvious effect on the solubility of the product (results not shown). Alternatively, overproduction could lead to incorrect intramolecular or intermolecular disulphide bondings which, in turn, causes improper folding of the protein.

Although only a portion of the recombinant kallikreins was released from *E. coli*, the high level of production combined with a rapid purification procedure allowed us to isolate an average of 170 µg of fully active enzyme from 1 litre of culture, and made this system a valuable one for future studies. The purified enzyme exhibited relatively high Tos-Arg-OMe esterolytic activity (452 ± 51 E.U./mg), and kininogenase activity (6.40 µg/min per mg kallikrein using bovine low-molecular-mass kininogen as the substrate). The kininogenase activity is 1.5 times that of tissue kallikrein purified from rat heart (Xiong *et al.*, 1990b) and 2.7

times that from rat submandibular gland. The facts that kallikrein synthesized in *E. coli* is not glycosylated and that it is fully active indicate that glycosylation has no significant effect on its enzymic activity.

Kallikrein was also produced at a very high level in yeast under the control of the α -factor promoter. Moreover, it was found that most of the proteins synthesized were being secreted into the cell-free medium. In fact, 0.5–1 mg of kallikrein was being secreted from each litre of culture, surpassing most of the reported values for large proteins (Zsebo *et al.*, 1986). We have observed that the fusion protein containing the leader sequence was effectively processed by the KR endoprotease (Zsebo *et al.*, 1986) within the yeast cells, resulting in the secretion of mature kallikrein into the medium. Whereas approximately half of the cellular products were processed, all the proteins in the periplasmic space and in the medium appeared to be processed. Our results support the notion that the leader region of the α -factor precursor contains the essential information necessary for recognition by the yeast processing and secretory apparatus (Brake *et al.*, 1984).

Since the need to lyse cells and to solubilize proteins was not necessary, purification of recombinant kallikrein from yeast proved simpler than from *E. coli*. The same two-step chromatography as in *E. coli* kallikrein preparation was also employed. Unfortunately only 1 % of the total secreted protein was enzymically active and therefore able to bind to an aprotinin-affinity column. This is in keeping with the report by Angermann *et al.* (1989) that only about 1 % of their expressed kallikrein was proteolytically active. The inactive form, according to Angermann *et al.* (1989), was probably due to incorrect disulphide-bond formation. The active form expressed in yeast has kininogenase activity and Tos-Arg-OMe esterase activity comparable with those of recombinant kallikrein expressed in *E. coli*. The fraction contained in the flow-through from the aprotinin-affinity column lacked both activities, yet was identified by anti-kallikrein antibodies on Western blot. Direct radioimmunoassay of the aprotinin flow-through fraction revealed that the linear displacement curve for the serial dilutions of this fraction was parallel with the log-logit transformations of the radioimmunoassay standard curve for rat tissue kallikrein. These results indicate that the proteins in the flow-through contained recombinant kallikrein, only in inactive conformations. Having ten cysteine residues, kallikrein is among molecules which demand the most strict conditions under which to fold properly. It is therefore not surprising that the heterologous synthesis of kallikrein resulted in improper conformations of the molecules.

In addition to the high-level expression of the tissue kallikrein cDNA in both *E. coli* and yeast, the feasibility of kallikrein purification in both cases makes them attractive systems to work with in future studies of the structure–function relationships of kallikrein-like enzymes by site-directed mutagenesis. This will also provide models with which the mechanisms underscoring their solubilities and secretions can be investigated.

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