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Expression of chymotrypsin(ogen) in the thioredoxin reductase deficient mutant strain of *Escherichia coli* AD494(DE3) and purification via a fusion product with a hexahistidine-tail

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Abstract

A reliable protocol was designed for fast expression and purification of recombinant chymotrypsin(ogen). The zymogen was overexpressed in soluble form as a $(His)_6$-fusion construct in the cytoplasm of the thioredoxin reductase deficient *Escherichia coli* strain AD494(DE3). This allowed purification of chymotrypsinogen in a highly selective affinity chromatography capture step using a Ni-NTA column. After activation with enterokinase, the enzymatically active chymotrypsin was purified in a polishing step using a modified soybean trypsin inhibitor agarose column. This expression system and the use of affinity chromatography for capture and polishing, offers an easier and faster route to recombinant chymotrypsin(ogen) than the previously described use of *Saccharomyces cerevisiae*.

Keywords: *Escherichia coli*; Purification; Chymotrypsin; Chymotrypsinogen; Thioredoxin reductase

1. Introduction

In order to check previous computational calculations, characterising the propagation pathway of the conformational transition [1] which occurs during activation of chymotrypsin after proteolytic cleavage by trypsin [2,3], site-specific mutants of chymotrypsin(ogen) have to be created for stopped flow experiments. To perform these mutagenesis experiments in an efficient way, a fast and reliable expression and purification system for recombinant chymotrypsin(ogen) is necessary.

Expression of chymotrypsinogen is already achieved in *Saccharomyces cerevisiae* [4] using a yeast expression system described for carboxypeptidase A1 [5]. However, the expression in yeast is very slow and many selection steps on different media are required before the target protein can be harvested. This is very time consuming since already nine days are passed before the start of the purification procedure, which consists of different chromatographic steps. Due to these long incubation times, the autocatalytic property of the serine protease and its sensitivity to autodigestion, a significant loss of yield and activity is observed.

To overcome the problem of the long incubation times and to obtain a faster and economical expression and purification, we choose to work with a bacterial expression system.

Up to now, enzymatically active recombinant

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chymotrypsin has only been expressed in *Escherichia coli* cells using the pTrap-vector expression system [6] in modest amounts of 0.1 mg per litre culture (Szilagyi L., Hungary, personal communications), which is insufficient for stopped flow experiments.

In this work, the gene for chymotrypsinogen was overexpressed in *E. coli*. To express chymotrypsin in the proper conformation, the formation of correct disulphide bridges is necessary [7]. However, the reducing environment of the *E. coli* cytoplasm prevents the generation of protein disulphide bonds [8–10] and limits the solubility and enzymatic activity of recombinant chymotrypsin. To circumvent these limitations, the recombinant protein was either targeted to the periplasm as a fusion protein with the pelB signal peptide or was expressed in the cytoplasm of bacteria deficient in thioredoxin reductase, allowing possible disulphide exchange. These two methods already proved their value in expression of eucaryotic proteins containing disulphide bridges [11–15].

Due to the instability of chymotrypsinogen and the tendency to autodigestion [16,17] and dimerisation [18–24] of chymotrypsin, it is essential to use fast and adequate purification protocols at low temperatures for rapid removal of the most harmful contaminants and buffer exchange to optimal buffer conditions, in which stability of the enzyme is ensured.

This fast purification was achieved following the three-phase purification strategy (capture, intermediate purification and polishing). Due to the attachment of a hexahistidine tag at the recombinant protein which allowed the development of a fast and reliable assay for the zymogen and due to the use of a highly selective affinity capture step on a Ni-NTA column, an intermediate purification was not even necessary.

2. Experimental

2.1. Oligonucleotides

Oligonucleotides for insertion of the hexahistidine-tail and clone analysis were purchased from Genset (Paris, France) and were used without further purification.

T7-promoter primer: 5′-TAATACGACTCACTATAGGGG-3′, T7-terminator primer: 5′-GCTAGTTATTGCTCAGCGG-3′, GH1718: 5′-TATCAA GAA GAC GCT ATT CCC GGG TCC TG - 3′, GH138: 5′-TATCAAACCGGTTGGGGCAAGACCAAATAC-3′, GHis1: 5′-TCCGATATCCATGGCCATC-3′, GHis2: 5′-AGATTGCATATGCATCACCATCACCATCACGAAGCTTTCCCGGTGGATG-3′.

2.2. Bacterial strains

The WK6 non-suppressor strain of *E. coli* was used for preparation of plasmid DNA, cloning and mutagenesis: Δ(*lac-proAB*) *galE strA*[F′ *traD36 proAB*$^{+}$*lacI*^{q} *lacZ*Δ*M15*].

For expression of the chymotrypsinogen-gene (preceded by the pelB-leader sequence), we used the *E. coli* strain BL21(DE3): F^{-}*ompT* [*lon*] *hsdS*$_B$ ($r_B^- m_B^-$)λ (DE3).

For expression of the $(His)_6$-chymotrypsinogen fusion construct in the cytoplasm, the thioredoxin reductase mutant *E. coli* strain AD494(DE3) was used: Δ*ara*$^{-}$ *leu7967* Δ*lacX74* Δ*phoAPvuII phoR* Δ*malF3* F′[*lac*$^{+}$ (*lacI*^{q})*pro*] *trxB*::kan (DE3).

2.3. Vectors

pET-22b(+): pBR322 derived expression vector, Ap^{r}.

2.4. Construction of the pelB-leader-chymotrypsin(ogen) expression vector

The entire gene encoding chymotrypsinogen was excised from the vector pVIF (kindly provided by Professor W.J. Rutter) by restriction cleavage using the restriction enzymes *Hin*dIII and *Ecl*136II. Restriction endonucleases were used according to the specifications of the suppliers (Boehringer Mannheim, Mannheim, Germany or New England Biolabs., Beverly, MA, USA). Digestion was followed by phenol extraction to eliminate the enzyme and by gel filtration chromatography on Sephadex G-50 (Pharmacia, Uppsala, Sweden) to change the buffer conditions. The *Hin*dIII protrusion was filled

by Klenow polymerase treatment as prescribed by Boehringer Mannheim. The restriction fragment containing the chymotrypsinogen gene was isolated by semi-preparative gel electrophoresis and purified with the QiaEx gel extraction kit (Qiagen, Hilden, Germany). The vector pET-22b(+) was dephosphorylated with calf intestine alkaline phosphatase (CIP) (Boehringer Mannheim) as described by Sambrook et al. [25] after digestion with *Ecl*136II. After heat inactivation of CIP, the chymotrypsinogen-fragment was ligated into the pET-vector by T4 DNA ligase (Boehringer Mannheim) during 4 h at room temperature. Transformation of competent cells of *E. coli* strain WK6 with the ligation product was carried out by electroporation (Tung and Chow [26]) using a Gene Pulser apparatus (Bio-Rad Labs., Hercules, CA, USA) at 200 Ω, 25 μF, 2.5 kV and 0.2 cm cuvettes. Positive clones were identified by polymerase chain reaction (PCR) clone analysis using the T7-terminator primer and the internal primer GH1718. For the isolation of pure DNA from the positive colonies Qiagen ion-exchange columns were used. The DNA was sequenced by the dideoxy chain termination method of Sanger et al. [27] using the ABI PRISM Dye Terminator Cycle Sequencing Ready reaction Kit (Perkin-Elmer, Foster City, CA, USA) with AmpliTaq^7 FS DNA polymerase.

The T7-promoter, T7-terminator and internal primers GH1718 and GH138 were used for sequence analysis. The DNA sequencing samples were run on an ABI PRISM 377 DNA sequencing instrument (Perkin-Elmer). The new construct was called pETctg.

2.5. Construction of the (His)$_6$-chymotrypsinogen expression vector

Creation of the hexahistidine-tail and elimination of the pelB-leader sequence was achieved by inverse PCR on the plasmid pETctg using the LA PCR Kit (TaKaRa Shuzo, Otsu, Japan) and the mutagenic primers GHis1 and GHis2. The PCR reaction was carried out after linearisation of the vector by *Eco*RI and dephosphorylation with CIP. The PCR product was purified using the Qiaquick PCR Purification kit (Qiagen) and digested with *Nde*I. After overnight ligation at 16°C the ligation product was precipitated and used to transform competent *E. coli* WK6 cells. Positive clones were identified, treated and sequenced as mentioned before. The new construct was called pEThisctg.

2.6. Expression of the recombinant proteins and cellular localisation

For the expression of chymotrypsinogen in the periplasm, the construct pETctg was transferred to competent BL21(DE3) cells (containing plasmid providing Cm^r and *Lac*I repressor). Ap^r and Cm^r colonies were grown in 100 ml of fresh Luria–Bertani medium containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. Expression was optimised by testing different conditions of time of induction, concentration of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Promega, Southampton, UK), induction length and growth temperature. The highest expression level was obtained by growing the cultures at 37°C until an optical density at 600 nm (O.D.$_{600}$) of 0.6 was reached and subsequently adding IPTG to a final concentration of 1.6 m*M*. The optical density of the cell cultures was measured in a Novaspec II spectrophotometer (Pharmacia). After induction the culture was continuously shaken at 37°C for 4 h and samples were taken for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. An aliquot (120 μl) of the bacterial culture (O.D.$_{600}$ of 1.6) was centrifuged. The pellet was resuspended in 60 μl water and 20 μl loading buffer (4×) and boiled for 5 min. Twenty microlitres were supplied on a SDS-gel. Total cell proteins were separated by SDS–PAGE and stained with Coomassie blue.

For production of the (His)$_6$-chymotrypsinogen fusion protein, the construct pEThisctg was introduced into competent AD494(DE3) cells. Ap^r and Km^r colonies were grown in 100 ml of fresh Luria–Bertani medium containing 100 μg/ml ampicillin and 15 μg/ml kanamycin.

Expression was optimised in the same way as mentioned before and the optimal conditions appeared to be the same. For preparative purposes cultures were grown in 4 l of fresh Luria–Bertani medium with 100 μg/ml ampicillin and 15 μg/ml kanamycin and induced as mentioned before.

The cellular location and solubility of the ex-

pressed recombinant protein was determined following the procedure as described by Qiagen.

2.7. SDS–PAGE and Western blotting

To assay the effectiveness of the expression and the purification steps, qualitative and quantitative analysis was performed using SDS–PAGE according the method of Laemmli [28]. Approximately 10 μg of protein was dissolved in 4× SDS–PAGE buffer (200 m*M* Tris–HCl, pH 6.8, 8 m*M* EDTA, 0.4%, w/v, bromophenol blue, 4%, w/v, SDS and 40%, v/v, glycerol) and loaded on gel after boiling for 5 min and centrifugation for 3 min. As reference sample the molecular mass standard low range marker of Bio-Rad Labs. was used. The SDS–PAGE was run on a Mini Protean II device (Bio-Rad) with tank buffer of 25 m*M* Tris–HCl, pH 8.3, 192 m*M* glycine, 0.1% (w/v) SDS. The proteins were detected by Coomassie blue staining. Immunoblotting was used as a fast and reliable assay for the recombinant zymogen. For immunoblotting the fractionated proteins were electrophoretically transferred to poly(vinylidene fluoride) (PVDF) membranes (Millipore, Bedford, MA, USA) and blots were developed as described by Gershoni and Palade [29] using monoclonal mouse-anti-histidine TAG antibodies (Serotec, Oxford, UK). Bound antibodies were detected by alkaline phosphatase labelled goat-anti-mouse-IgG (Promega) using NBT (nitroblue tetrazoliumchloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate) as substrates. The molecular mass standard proteins were stained in amidoblack solution (0.5%, w/v, in EtOH–HOAc–water, 5:1:5, v/v/v).

2.8. Purification of the $(His)_6$-zymogen

2.8.1. Sample extraction and clarification

A sample preparation procedure (extraction and clarification) was executed prior to the first purification step.

After growth and induction of the 4-l culture of pEThisctg transformed AD494(DE3), all following steps were carried out at 4°C to reduce the degradation of the unstable zymogen and protease activity. Cells were harvested by centrifugation for 15 min at 6000 *g* using a GSA rotor in a Sorvall RC-24 centrifuge (Du Pont Instruments, Wilmington, NC, USA) and the pellet was resuspended in 50 ml of sonication buffer (50 m*M* NaH_2PO_4 pH 8.0, 300 m*M* NaCl). Lysozyme was added to a final concentration of 40 mg/l with stirring for 30 min on ice and the cells were lysed by three cycles of freezing and thawing. The viscous suspension was sonicated three times for 1 min (with 1 min intervals) on ice after adding 0.4 mg RNaseI and 0.4 mg DNaseI and 1 h incubation on 37°C. The extract was clarified by centrifugation at 10 000 *g* at 4°C for 30 min using a SS-34 rotor (Du Pont Instruments). After centrifugation the supernatant fraction was filtered using a 0.22-μm pore size filter (Millipore).

2.8.2. Capture (and intermediate purification)

The extracted and clarified sample was applied on a Ni-NTA-agarose column [6 ml of Ni-NTA resin (Qiagen) packed in an XK-16 column (Pharmacia)] that had been equilibrated with sonication buffer. The column was washed extensively with sonication buffer until a stable baseline was obtained. Then the recombinant $(His)_6$-chymotrypsinogen was eluted with a 150 ml total volume linear pH gradient using buffers pH 6 (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10% glycerol, pH 6) and pH 4 (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10% glycerol, pH 4). To confirm the desired purity of the zymogen, collected fractions were separated by SDS–PAGE and detected by Coomassie Blue staining. The protein concentration was determined by Bradford assay (Bio-Rad) using a bovine serum albumin (BSA) standard curve [30].

2.9. Activation of the zymogen and purification of the active chymotrypsin

Before activation of the zymogen with enterokinase, the eluent (approximately 25 ml) was dialysed for 4 h against 5 l activation buffer (50 m*M* Tris–HCl, 50 m*M* ϵ-amino-*n*-caproic acid, 20 m*M* $CaCl_2$, pH 8) or applied in fractions of 2.5 ml to a PD-10 column (Pharmacia) previously equilibrated with 25 ml activation buffer. Enterokinase cleavage of the fusion protein was carried out overnight at an enzyme–substrate ratio of 1:30 (w/w) (Boehringer Mannheim).

2.10. Purification of the active chymotrypsin

The digest was applied on a SBTI-column [10 ml of modified soybean trypsin inhibitor immobilized on 4% beaded agarose (Sigma–Aldrich, Bornem, Belgium) packed in an XK-16 column] which was equilibrated with 50 m*M* Tris–HCl, 0.2 *M* NaCl, 0.1 *M* $CaCl_2$. The column was washed extensively with this buffer until a stable baseline was obtained. Elution of the active chymotrypsin was performed with an acetate buffer (0.1 *M* HOAC adjusted with NaOH to pH 4, 0.1 *M* $CaCl_2$, 0.2 *M* NaCl). To ensure the purity and activity of the protein, samples were submitted to SDS–PAGE and activity assays.

2.11. Activity tests

Chymotrypsin activity was determined as described by Delmar et al. [31] with succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide (Sigma–Aldrich) as substrate. The hydrolysis rates were measured by following the absorbance spectrophotometrically at 410 nm on a Uvikon 940 double beam spectrophotometer (Kontron, Zürich, Switzerland).

3. Results

3.1. Construction of the chymotrypsinogen expression vectors pETctg and pEThisctg

Because chymotrypsinogen is a very unstable protein, we chose to work with a chymotrypsinogen chimera, in which the chymotrypsinogen propeptide is substituted with that of trypsinogen. This zymogen can be activated by enterokinase and has the same kinetic parameters as the wild-type, but is more stable [4]. The gene of this protein was kindly provided by Professor Dr. W.J. Rutter.

To obtain an overexpression of the recombinant zymogen, the T7 RNA polymerase/promoter system in the vector pET22b(+) was used [32–34].

The expression vector pETctg was obtained by insertion of the chymotrypsinogen fragment in pET22b(+) downstream the pelB-leader sequence (Figs. 1 and 2). PCR-clone analysis using the GH1718 and T7-terminator primers, and sequencing confirmed the nucleotide sequence of the region comprising the chymotrypsinogen gene, pelB-leader and T7 promoter/terminator.

By constructing the expression vector pEThisctg, the pelB-leader sequence was replaced by a hexahistidine sequence in order to obtain a highly selective affinity chromatography capture step for the expressed zymogen. This was achieved by inverse PCR, using pETctg as template and the mutagenic primers GHis1 and GHis2. Sequence analysis confirmed the correct nucleotide sequence of the entire $(His)_6$-chymotrypsinogen fusion construct.

3.2. Expression of the chymotrypsinogen in the periplasm

Expression of the recombinant chymotrypsinogen was obtained by induction with IPTG of the BL21(DE3) strain transformed with pETctg. Samples collected 4 h after induction were separated by SDS–PAGE and stained with Coomassie blue. Induction resulted in the appearance of a protein band of M_r 30 000, which is absent in the untransformed BL21(DE3) or BL21(DE3) transformed with the control plasmid pET22-b(+). Localisation and solubility tests showed that the majority of the recombinant zymogen accumulated in the cytoplasm as inactive inclusion bodies. Only a negligible fraction was secreted in the periplasm as soluble protein.

3.3. Expression of the $(His)_6$-chymotrypsinogen in the cytoplasm of the AD494(DE3) strain

Expression of the recombinant $(His)_6$-chymotrypsinogen was obtained by induction with IPTG of the AD494(DE3) strain transformed with pEThisctg (Fig. 3a). Expression tests and analysis of the solubility of the expressed protein were carried out as mentioned before and showed that more than 50% of the expressed chymotrypsinogen in the transformed AD494(DE3) cells was soluble and amounted to about 10% of the total soluble protein.

The overexpression of recombinant $(His)_6$-chymotrypsinogen was also verified by immunoblotting analysis using monoclonal mouse-anti-histidine TAG antibodies (Fig. 3b) [35].

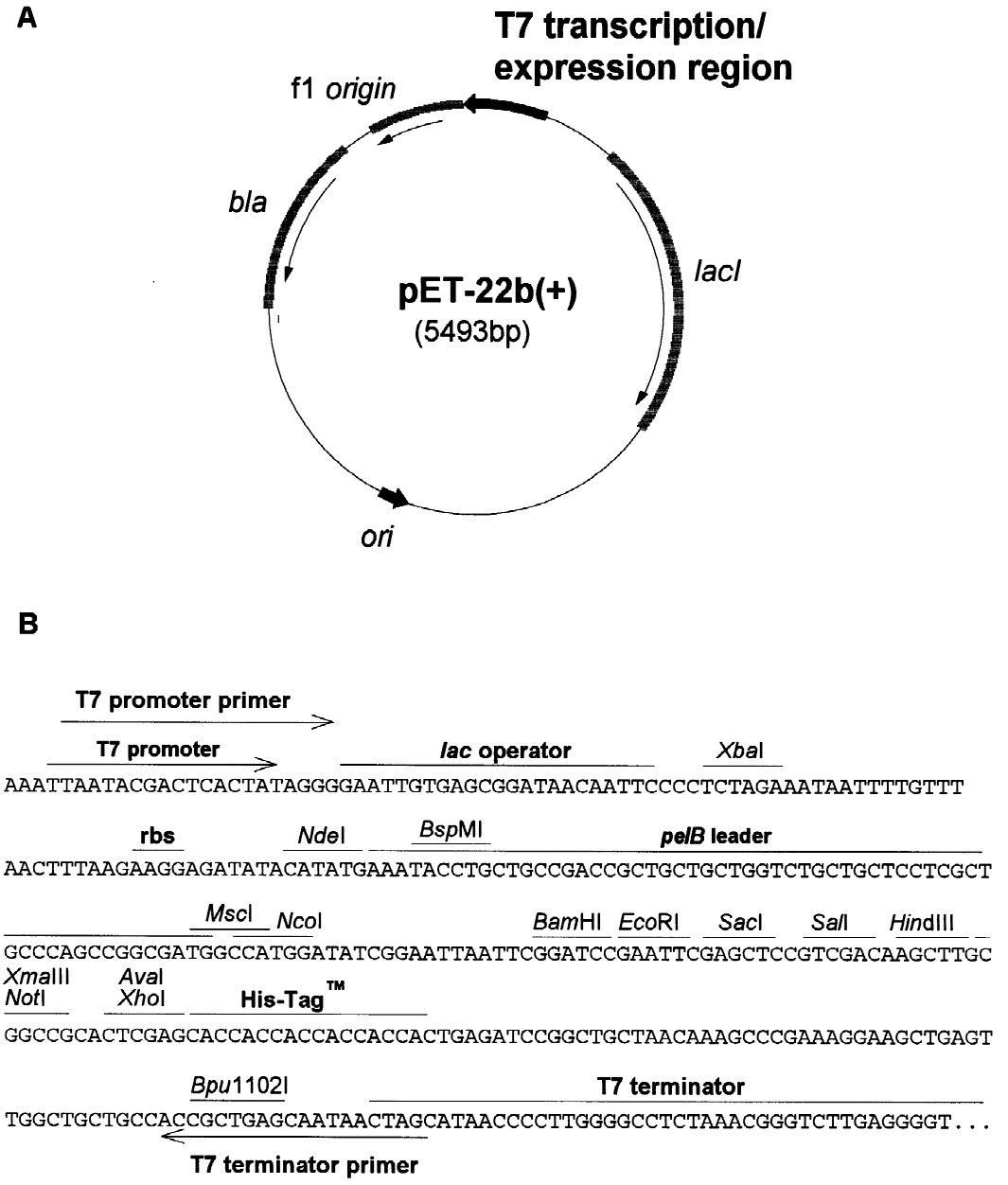

Fig. 1. Schematic presentation of (A) pET-22b(+) and (B) the T7 transcription/expression region.

3.4. Purification of the $(His)_6$-zymogen

After sample extraction and clarification, the soluble $(His)_6$-zymogen was isolated, concentrated and brought into stabilising buffer conditions in one single chromatographic step by immobilized metal chelate affinity chromatography using a Ni-NTA column [36]. This capture step focused on the rapid removal of contaminating proteins, lipids, low-molecular-mass substances and the most harmful con-

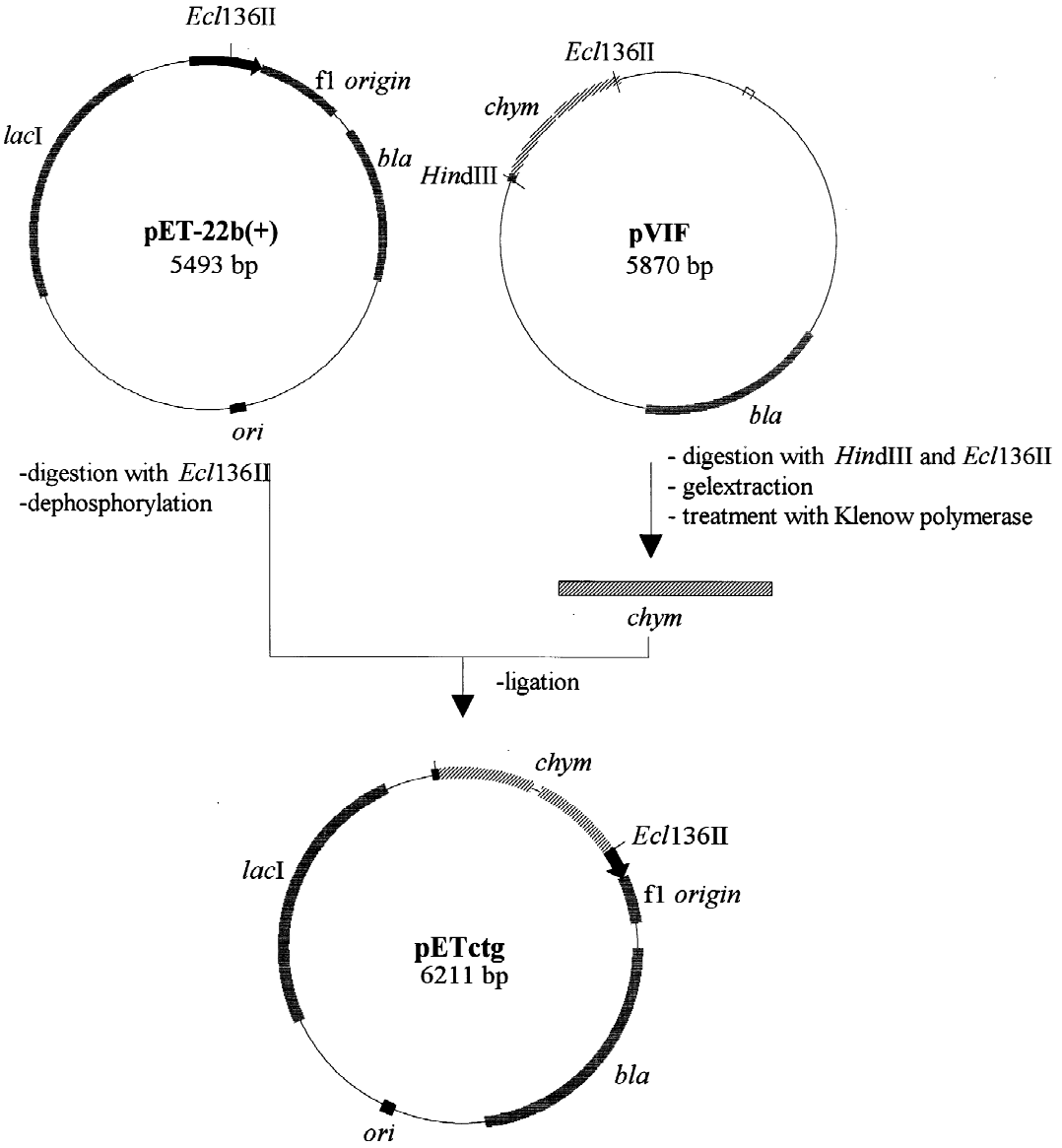

Fig. 2. Cloning of the chymotrypsinogen-gene (*chym*) in the vector pET-22b(+). The gene of rat-chymotrypsinogen was inserted into the vector pET22b(+) after the pelB-leader sequence which allows expression in the periplasm to ensure the formation of disulphide bonds. After digestion of pVIF with *Hin*dIII and *Ecl*136II, the chymotrypsinogen-fragment (*chym*) was treated with Klenow polymerase to obtain blunt ends. This fragment was ligated in the pET-vector, digested with *Ecl*136II.

taminants from the unstable chymotrypsinogen. No intermediate purification steps were needed as the high selectivity of the capture step led to a recovery of the zymogen with a purity of approximately 90% as shown by SDS–PAGE and staining with Coomassie blue (Fig. 4). The immunoblotting assay confirmed the good recovery of the zymogen from the Ni-NTA column.

Elution of the protein with a buffer of pH 4 instead of imidazole, minimised the use of additives and ensured restriction of any possible chymotrypsin activity and autodigestion in the sample.

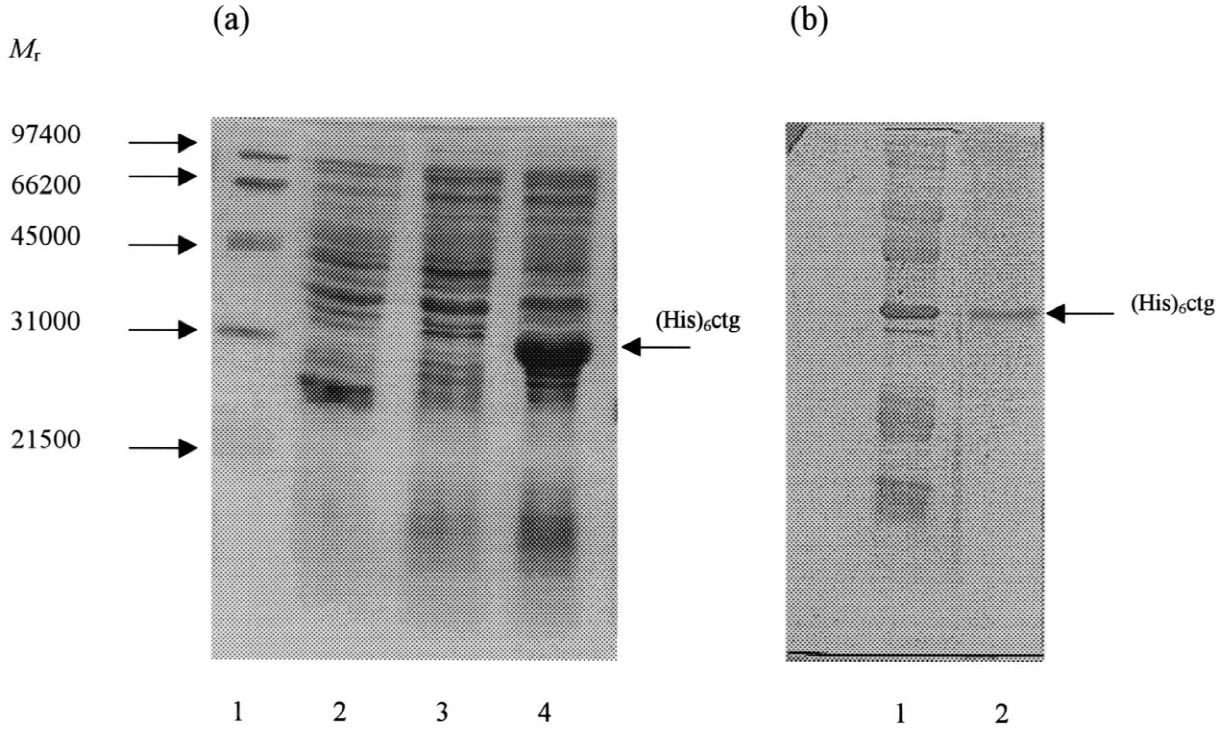

Fig. 3. Analysis of the expression of $(His)_6$-chymotrypsinogen by SDS–PAGE and (a) staining with Coomassie blue or (b) immunoblotting. (a) Lane 1: Bio-Rad low-molecular-mass protein standards; lane 2 shows the expression by a AD494(DE3)-culture [harbouring pET-22b(+)], after induction during 4 h; lanes 3 and 4 show the expression by a AD494(DE3)-culture (harbouring pEThisctg), respectively before and after 4 h induction. (b) Lane 1: Bio-Rad low-molecular-mass protein standards; lane 2 shows the expression by a AD494(DE3)-culture (harbouring pEThisctg), after 4 h induction.

3.5. Activation of chymotrypsinogen

To avoid protein inactivation by autodigestion during expression and purification, activation of the recombinant zymogen by digestion with enterokinase was postponed until right before the last purification step.

The zymogen was brought in an activation buffer of pH 8 to ensure optimal conditions for enterokinase activity. To repress autodigestion of the activated chymotrypsin, the reversible chymotrypsin inhibitor ϵ-amino-*n*-caproic acid [37,38] was added. During activation, the hexahistidine-tail provided at the N-terminus of the recombinant protein was removed. Since the pH of the activation buffer is appropriate to apply the sample directly to the soybean tryspin inhibitor (SBTI) column and the caproic acid is removed during elution of the active chymotrypsin from the SBTI column, no further sample conditioning was needed after the activation step.

3.6. Purification of the recombinant chymotrypsin

After the activation of the zymogen, the well-characterised interaction between biologically active α-chymotrypsin and the biospecific ligand (immobilized SBTI) [39] was used in the polishing step. The digest was subjected to affinity chromatography, using modified sepharose bound SBTI as affinity medium [40,41]. This resulted in a highly selective polishing step with improved resolution from trace contaminants like enterokinase and closely related substances like aggregates, digestion material and uncleaved chymotrypsinogen. After this step final purity was achieved as shown by SDS–PAGE analy-

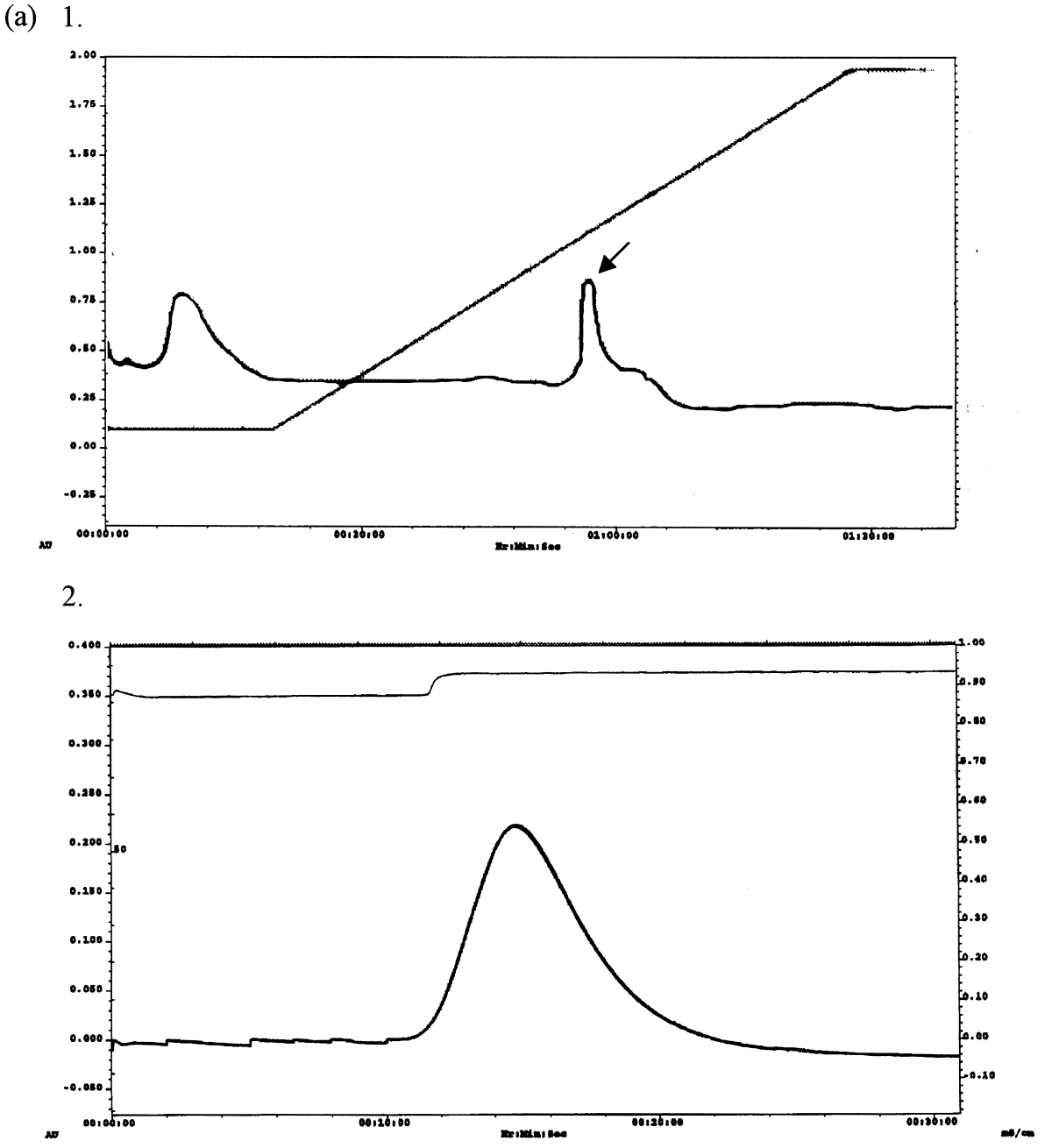

Fig. 4. (a) Chromatograms of the capture and polishing step and (b) analysis of the protein fractions by SDS–PAGE and staining with Coomassie blue. (a) 1=Elution pattern in the capture step, using a Ni-NTA column: after a washing step with sonication buffer until a stable baseline was obtained, the recombinant $(His)_6$-chymotrypsinogen (arrow) was purified using a 150 ml total volume linear pH gradient using buffers pH 6 (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10% glycerol, pH 6) and pH 4 (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10% glycerol, pH 4). 2=Chromatogram of the polishing step using a modified SBTI column: after a washing step with loading buffer (50 m*M* Tris–HCl, 0.2 *M* NaCl, 0.1 *M* $CaCl_2$), the active chymotrypsin was eluted with an acetate buffer (0.1 *M* HOAC adjusted with NaOH to pH 4, 0.1 *M* $CaCl_2$, 0.2 *M* NaCl). (b) Lane 1: Bio-Rad low-molecular-mass protein standards; lane 2: $(His)_6$-chymotrypsinogen fraction after digestion with enterokinase; lane 3: $(His)_6$-chymotrypsinogen fraction after Ni-NTA column; lane 4: chymotrypsin after purification on a SBTI column.

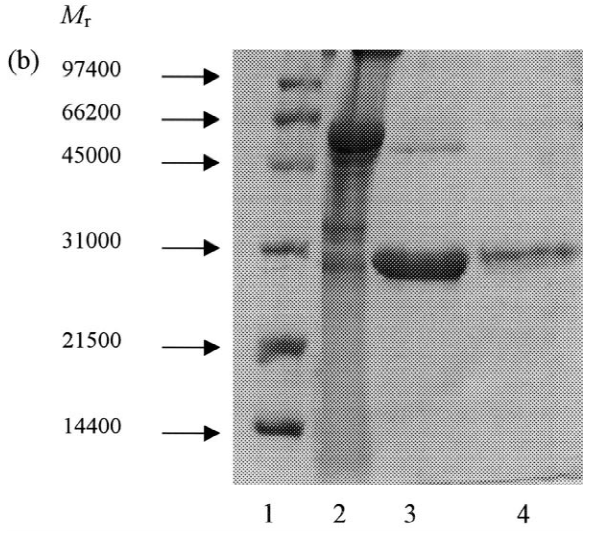

Fig. 4. (*continued*)

sis (Fig. 4). The active protein was eluted in the appropriate storing buffer of pH 4, containing $CaCl_2$.

The low pH ensured the inhibition of autodigestion since chymotrypsin is inactive at this pH due to the protonation of His57 of the catalytic triad [42]. The presence of $CaCl_2$ ensured the prevention of dimerisation [43,44], which is normally present at this low pH.

The activity of the purified chymotrypsin was assayed with the specific substrate *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide [31] and kinetic analysis showed that the purified chymotrypsin (expressed in *E. coli*) behaved in the same way as the one prepared from the yeast expression system [4].

4. Discussion

In order to elucidate the pathway of the conformational change occurring during activation of chymotrypsinogen, a fast and reliable expression and purification protocol for recombinant chymotrypsin(ogen) was needed for mutagenesis experiments. So far, expression of chymotrypsinogen in milligram amounts, required for stopped flow studies, was only achieved in *Saccharomyces cerevisiae*. Despite the high expression level, the expression in yeast is very time consuming.

In contrast with the yeast expression system, expression in *E. coli* is much faster but the production of chymotrypsinogen remained problematic as the majority of the recombinant protein was insoluble. The difference in solubility of recombinant chymotrypsinogen probably reflects the fact that proper folding of the chymotrypsinogen depends on S–S bond formation, which cannot be achieved in the reducing environment of the *E. coli* cytoplasm. However, it is possible to allow disulphide bond formation in *E. coli* in two ways: the recombinant protein can be targeted to the periplasm as a fusion protein with the pelB signal peptide or be expressed in the cytoplasm of the thioredoxin reductase deficient mutant strain of *E. coli* (AD494), which allows for the potential formation of disulphide bonds in the cell cytoplasm and gave satisfying results in expression of proteins bearing disulphide bridges [11–15]. In the present paper, these two routes have been explored.

Experiments showed that the majority of chymotrypsinogen expressed in the *E. coli* strain BL21(DE3) and directed to the periplasm was also insoluble, probably due to interference of the pelB-leader with the correct folding of chymotrypsinogen or due to the failure of the cell protein processing machinery to cleave the pelB-leader sequence.

This problem was overcome by expressing the chymotrypsinogen in the *E. coli* strain AD494(DE3). More than 50% of the chymotrypsinogen in the transformed AD494(DE3) cells was soluble and amounted to about 10% of the total soluble protein.

Hence, cytoplasmic expression using the thioredoxin reductase deficient strain AD494(DE3) appears to be much more efficient for a correct folding of this recombinant protein than the use of the pelB-leader directed transport to the periplasm.

The fusion protein $(His)_6$-chymotrypsinogen was expressed in the cytoplasm of the *E. coli* strain AD494(DE3) with a yield of 1 to 2 mg per litre culture, what is in sharp contrast with the lower expression level of 0.1 mg/l using the pTrap-vector and is much faster than the expression in the yeast system.

The developed purification strategy required even fewer steps than described in other purification procedures since capture and intermediate purification were achieved in a single step by attachment of

the histidine tag to the recombinant zymogen. This allowed the use of a Ni-NTA column giving a highly selective affinity chromatography capture step providing less sample handling and a faster isolation, stabilisation and concentration of the unstable zymogen. Thanks to the attachment of the histidine tag a fast and reliable assay for zymogen detection could be developed using monoclonal mouse-anti-histidine TAG antibodies.

After a fast activation step in stabilising conditions, using the reversible inhibitor ϵ-amino-*n*-caproic acid for suppressing autodigestion, the target protein was recovered with the desired purity using the biospecific SBTI as ligand in a highly selective polishing step. The effectiveness of the purification steps was easily confirmed by fast and reliable analytical assays and minimising the use of additives avoided the need for sample conditioning between the purification steps.

Thanks to the faster expression, faster purification and minimisation of the sample handling, the problems of instability and autodigestion of the recombinant chymotrypsin(ogen) were better suppressed than in the previously described purification protocol. This expression and purification protocol offers an easier route to recombinant chymotrypsin(ogen) with good economy and shorter time to pure product than the previously described use of *Saccharomyces cerevisiae*.

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