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Cloning, Functional Expression in *Pichia pastoris*, and Purification of Potato Cystatin and Multicystatin

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In the tubers and leaves of potato, *Solanum tuberosum*, cysteine protease inhibitors are thought to play roles in the defence against herbivores and in regulating physiological processes like senescence and cell death. The cDNAs for two such inhibitors, potato multicystatin (PMC) with 8 cystatin domains and potato cystatin (PC) with a single domain, were cloned and expressed in the yeast *Pichia pastoris*. PC yielded on average 100 mg of purified active protein from 1 *l* of culture supernatant. Purification to homogeneity was done in one step by cation exchange. The apparent equilibrium dissociation constant (K_i) for papain was 0.1 nM. Cloning of the PMC cDNA was successful despite apparent toxicity for *Escherichia coli* and a high frequency of recombination events in RecA$^-$ strains of *E. coli*. In yeast, the expression of the cloned full length PMC gene was poor compared to that of the single domain.

[**Key words:** multicystatin, cystatin, cloning, potato, *Pichia pastoris*]

The sub-phellogen layer of potato tubers contains the 85-kDa cysteine protease inhibitor potato multicystatin (PMC), a predominantly cuboidal, and occasionally prismoidal, crystalline protein (1). The protein purified from tubers is an effective inhibitor of cysteine proteases of western corn rootworm (*Diabrotica virgifera*), and suppresses larval growth (2). The PMC gene is wound-inducible (3) and its product is assumed to play a role in defence against insect pests (4), but possibly also in regulating the sprouting of potato tubers (Michaud, D., personal communication), leaf senescence and plant cell death (5). The PMC protein remains crystalline at pH 6.0 and above and is soluble at an acidic pH. PMC consists of eight perfectly repeated phytocystatin domains, and can bind to eight papain molecules simultaneously. It is stable over a pH range of 3.5 to 10, and a temperature of 60°C for 10 min (1). Upon incubation with serine proteases, PMC is cleaved into 10-, 20- and 32-kDa fragments, yet retaining full papain-binding activity (6). This suggests that expression of a single 10-kDa domain of PMC may yield an active and stable cysteine protease inhibitor similar to the cystatins found in other plant species which, except for tomato (4), usually contain only one to three domains (7–9).

Cystatins are considered to be factors which may protect agricultural crops against pests (2) or foods against decay (10). For insect bioassay studies, the use of purified recombinant protein is preferred for the higher degree of accuracy in predicting the gene activity in transgenic plants. For use as preservatives in foods, reliable production systems are required. Yeasts are suitable hosts for the production of heterologous proteins as they can perform post-translational modifications, folding and processing of eukaryotic proteins. The methylotrophic yeast *Pichia pastoris* has in the past decade increasingly been used for this purpose due to its ability to generate very high yields of recombinant proteins (11–13).

In this paper, the cloning and expression of a single- and an eight-domain potato cystatin in the yeast *P. pastoris* is described. An efficient protocol for one-step purification directly from the yeast supernatant yields a high level of pure protein.

MATERIALS AND METHODS

Oligonucleotides All oligonucleotides were obtained from Eurogentec (Seraing, Belgium), and the nucleotides underlined are the specific restriction sites included in the primers for cloning.

PMC-DN: CCCCCATGGCATACGTAAAATTATTCGCAGT GATGGCAATCGTA
PMC-UP: CCCCCCGCGGCCGCCACACCAACATAAAAG TAGTTTC
PC-DN: GTATCTCTCGAGAAAAGAGAGAGGCTGAAG CTGCAATCGTAGGGGGC
PC-UP: CCCATGCGGCCGCCTACTTTGTAGCATCACC AACAAGTTTAAATTCTTG

Cloning the PMC and potato cystatin (PC) cDNAs Total RNA was isolated from fresh potato peels (*Solanum tuberosum* cv.

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Abbreviations: BMG, buffered minimal glycerol; BMMY, buffered methanol-complex medium; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; kDa, kilo dalton; OD, optical density; PC, potato cystatin; PCR, polymerase chain reaction; PMC, potato multicystatin; RT, room temperature; TE, Tris–EDTA; YNB, yeast nitrogen base.

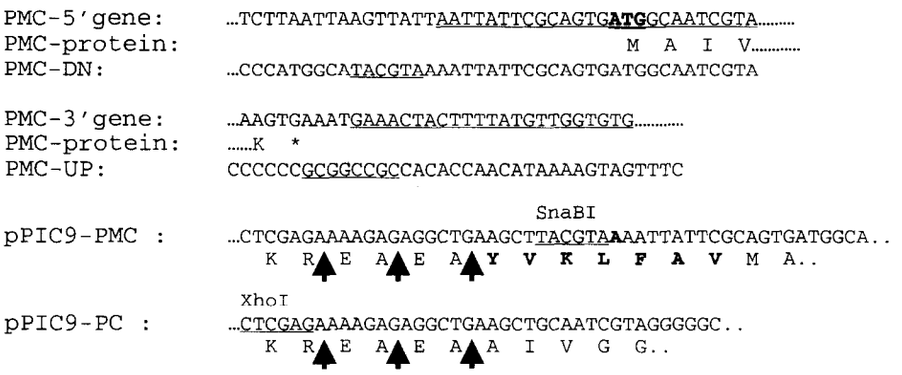

FIG. 1. Diagram depicting the original PMC gene (5′ and 3′ ends) and the way the PMC and PC coding regions were cloned into pPIC9. The primers PMC-DN and -UP were designed to avoid annealing to all 8 homologous domains by making use of non-coding 5′- and 3′- regions of the gene. This led to 7 additional amino acids (in bold) on the N-terminal after removal of the MF-alpha secretion signal and the Glu-Ala repeat by Kex2 and Ste13 peptidases, respectively (arrows). The nucleotides underlined in the 5′ and 3′ gene sequence indicate the annealing sites of the primers (PMC-DN and PMC-UP) used for PCR, while the underlined sequences in the primers are the restriction sites used for cloning.

Superior) according to the hot phenol method (14). Subsequently, mRNA was purified and first-strand cDNA was synthesised using a PolyA-Tract Series 9600 mRNA Isolation and cDNA Synthesis System Kit (Promega, Madison, WI, USA). The kit protocol was modified by extending the duration of first strand synthesis at 37°C from 30 min to 2 h to ensure the synthesis of long-length cDNA (~2.3 kb for PMC). The published genomic DNA sequence of the PMC gene (15) was used to design primers for PCR. To prevent annealing to the highly homologous repeated internal cystatin domains of the PMC gene, the primers PMC-DN and PMC-UP were designed using the non-coding 5′ and 3′ regions of the PMC mRNA to allow amplification of the full mRNA template (Fig. 1). Primers PC-DN and PC-UP were designed to clone only the last domain of the PMC gene of 303 bp to also produce a single potato cystatin domain in yeast. The prepared first strand cDNA was treated with a cocktail of RNases (Gibco BRL/Life Technologies, Breda, The Netherlands) at 37°C for 1 h and the reaction was terminated by holding the mixture at 95°C for 5 min. A fraction of 2–4 µl of this mixture was taken as a template for the PCR reaction using the Advantage Genomic PCR Kit (Clontech, Palo Alto, CA, USA). The following cycling parameters were used for the amplification of PMC: 94°C, 1 min; 60°C, 30 s; 72°C, 4 min, for 31 cycles with an additional extension time of 20 min at 72°C during the last cycle. The 303-bp PC fragment was amplified using the same conditions, except that the time for extension was reduced to 1 min instead of 4 min. The PMC cDNA fragment was cloned directly into the *Pichia* expression vector pPIC9 by digesting the vector and the 2276-bp PCR product with *Not*I and *Sna*BI, ligating them overnight at 14°C and transforming the ligated mixture into *E. coli* XL1 blue. The PC cDNA fragment was first A-tailed using standard methods (16), cloned into pGEM-T and sequenced. Subsequently, the PC cDNA and the pPIC9 vector were digested with *Xho*I and *Not*I, ligated and also transformed into *E. coli* XL1 blue. The PMC- and PC-containing pPIC9 plasmid DNA was linearised with *Sal*I and used to electroporate competent GS115 cells of *P. pastoris* according to standard protocols (Invitrogen, Breda, The Netherlands). Histidine-positive transformants were selected on MD (minimal dextrose) plates.

Expression of PMC and PC Ten single colonies per construct were inoculated into 10 ml of BMG medium contained in 50-ml pre-sterilised test tubes (buffered minimal glycerol, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 450 µg/*l* biotin and 1% glycerol). Cultures were grown overnight at 30°C on a plate agitator (Innova 4300, 2.54 cm orbital; New Brunswick Scientific, Edison, NJ, USA) at 250 rpm and the OD_{600} was checked after 16 h of culturing. The 10 clones had an OD_{600} of 1.28 to 1.50. Cells were pelleted (3500 rpm for 5 min) and re-suspended to $OD_{600}=1.0$ in 10 ml of BMMY (buffered methanol-complex medium, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol) and cultured for 6 d at 30°C on a plate agitator at 250 rpm. Every 24 h, methanol was freshly added to a final concentration of 0.5% (v/v). At the end of day 6, the PMC cultures were dialysed against buffer at pH 5 at 4°C overnight to dissolve any potential crystals formed. Expression of PC and PMC was determined by immunoblotting as described below.

Expression screening A dotblot was prepared with 50 µl of supernatant in 100 µl of loading buffer (100 mM CAPS+1% SDS) per well on BioRad TransBlot Nitro-cellulose membrane (0.2 µm). The membrane was used for an immuno dotblot with the primary antibody being rabbit IgG raised against a gluthatione-*S*-transferase–PC fusion protein produced in an earlier study in *E. coli*. The secondary antibody was sheep-anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (Jackson Immuno Research, West Grove, PA, USA). Tuber-purified potato multicystatin of known concentration was used for the reference curve in the immuno dotblot. The recombinant exhibiting the highest level of expression identified by dotblotting was analysed by Western blot procedures (Fig. 2) and was used for scaling up of the culture.

Scaling up of the expression of PC and the 8-domain PMC Fermentation of PMC and PC was performed in a BioFlo 3000 bench-top fermentor with a 2-*l* working volume (New Brunswick Scientific). The methanol concentration in the medium was monitored by a methanol concentration monitor and controller, MC168 (PTI Instruments, Woburn, MA, USA) under the software control of NB208 Windows (PTI Instruments), while the fermentation itself was monitored and controlled by AFS BioCommand based software (New Brunswick Scientific). The inoculum was grown overnight at 30°C on a plate agitator at 250 rpm in a 2-*l* shake flask containing 150 ml of complex medium with glycerol (buffered glycerol-complex medium, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 0.5% glycerol). The fermentation vessel, containing 1.5 *l* of basal salts medium (phosphoric acid, 85% 40.05 ml, calcium sulfate 1.395 g, potassium sulfate 27.3 g, magnesium sulfate·$7H_2O$ 22.4 g, potassium hydroxide 6.2 g, glycerol 60 g) was brought to the set temperature of 30°C and pH 5.0 (17). The dissolved oxygen set point was 30%. The medium in the vessel was inoculated with the entire overnight culture and the fermentation was run as described (17). The batch fermentation of PC was stopped after 70 h, at an

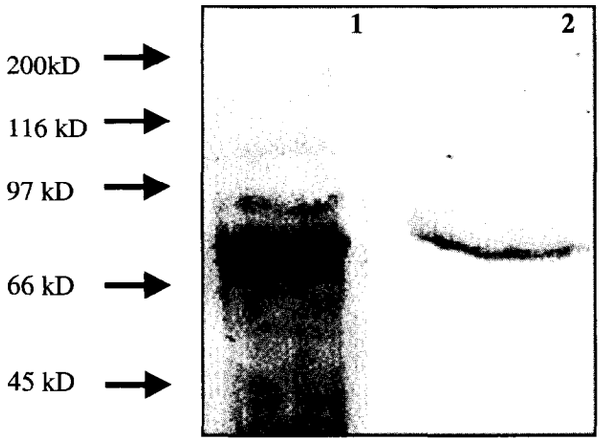

FIG. 2. Western blot of the PMC protein produced in *P. pastoris*. Lane 1, 20 ng of purified PMC from potato peel (cv. Superior); lane 2, 50 µl of supernatant of 10-ml *P. pastoris* cultures obtained after overnight acidification. Numbers in kD are molecular weight markers of protein standards run on this 10% SDS–PAGE gel.

OD_{600} of 239. Two batch fermentations of PMC were carried out at pH 5 and 3, but the monitoring of the expression revealed only very low expression levels.

FPLC purification of PC The cultures were centrifuged at 4°C, 3500×*g* and the supernatant was sterilised by passage through a 0.45-µm filter. The filtered supernatant was diluted 1:2 with equilibration buffer (20 mM Na–citrate, pH 2.8) for ion exchange chromatography and the pH was further reduced to 2.8 with concentrated HCl. Routinely, 0.5–1.0 *l* of the filtrate was loaded onto a 5-ml SP HI-trap column (AP Biotech, Uppsala, Sweden), previously equilibrated with the same buffer, at a flow rate of 1 ml/min. The column was then washed with five volumes of equilibration buffer. The adsorbed PC protein was eluted with a linear 0–1 M NaCl gradient in equilibration buffer or in one step with 400 mM NaCl. The column flow through was monitored at 280 nm. A peak was observed at approx. 400 mM NaCl, fractions were analysed by SDS–PAGE, pooled according to the desired level of purity and sterilised by passage through a 0.22-µm filter.

Determination of the apparent equilibrium dissociation constant The apparent equilibrium dissociation constant (K_i) of PC for papain was determined by titration of papain and curve fitting software. PC (0–34 µl, 150 nM stock solution) was added to 130–96 µl of assay buffer (50 mM MES pH 6.5 with 5 mM cysteine and 0.1 mg/ml BSA fraction V; Sigma, St. Louis, MO, USA) and 20 µl of 150 nM papain (2× crystallized, titrated with E-64, *trans*-epoxysuccinyl-L-leucylamido-4-guanidino butane; Sigma) to a final volume of 150 µl. After a 30-min pre-incubation at room temperature, the amount of uncomplexed papain was measured by adding 50 µl of substrate (0.9 mg/ml Z-Phe-Arg-pNA [Bachem, Bubendorf, Switzerland] in methanol diluted 20x in assay buffer) and spectrophotometrically recording the change in OD_{405} (18) on a benchtop microtiterplate reader (BioRad). The residual protease activity was plotted vs. the volume of the inhibitor. The data sets were fitted to the equation $[I^\circ]=(1-a)/a^*K_i+[E]^*(1-a)$, where I° represents the initial concentration of the inhibitor, E° the initial concentration of the enzyme, and a the fraction of the free enzyme (19). A nonlinear regression analysis program was used for determination of K_i and the initial concentration of the inhibitor (Sigmaplot 5.0; SPSS Science, Chicago, IL, USA).

RESULTS AND DISCUSSION

Cloning of PMC The cDNA of the cysteine protease inhibitor PMC from potato, *S. tuberosum* cv. Superior, was cloned by PCR into the pPIC9 vector and transformed into the methylotrophic yeast *P. pastoris* for large-scale production of PMC protein. The 2276-bp cDNA fragment of PMC proved to be difficult both to amplify and clone, although the primers were stringently designed to allow only amplification of the entire coding region. Figure 1 shows that as the first two amino acids are unique to the first domain of PMC only the last 6 nucleotides at the 3′ end of primer PMC-DN correspond to sequences recurring in several internal domains of the PMC gene. None of the PMC-UP nucleotides were overlapping with those in the other domains. Nevertheless, despite the use of hot starts, the PCR reaction routinely generated a ladder of products which were multiples of 300 nucleotides (300, 600, 900, 1200, etc.). This may be explained by the priming of incomplete amplification products on internal domains which could not be avoided by the prolonged extension times. The full-length product was cut out of a gel and ligated into several different standard cloning vectors. The cloning of the 2.3-kb PMC cDNA fragment, however, routinely resulted in a small 303-bp product in all the vectors tested independent of the cloned orientation of the 2.3-kb fragment (pGEM-T, pSK^+ and pPCR-SCRIPT). When sequenced, the 303-bp product was identified as a recombination product of gene fragments encoding domains 1 and 8. This problem in cloning the full-length gene is probably due to a combination of the high toxicity and high homology of the 8 domains, because the same gene with introns could be cloned without a problem in these vectors. Apparently, during the growth of a colony recombined bacteria had a strong growth advantage despite the use of $RecA^-$ *E. coli* strains such as XL1-blue. It is unclear why the non-directional cloning procedures using the pGEM-T vector still resulted in the same phenomenon, considering that the 50% chance of an inverse orientation in this vector would not be expected to result in a toxic product. Also, the addition of 2% glucose to the selective medium (as a repressor of Lac promoter activity [20]), did not result in the cloning of the entire fragment. However, the direct cloning into the yeast expression vector pPIC9 in the same *E. coli* background reduced the recombination in this vector to a great extent. Plasmid isolations from single colonies would typically yield a mix of plasmids containing both 8-domain and 4-domain genes. Apparently, the recombination occurred during the growth of the colony and subsequent culturing, but in a less frequent and marked manner in this vector background.

Cloning of PC A 309-bp PC cDNA fragment obtained by PCR was cloned into pGEM-T. The sequence of this cDNA fragment is presented in Fig. 3. The sequence of PC did not show complete identity to any of the eight domains of PMC (Fig. 4). The PC cDNA fragment that was cloned is, therefore, expected to be a fragment of one of the other 4 to 6 cystatin genes in potato (14). PC was expressed in *P. pastoris* by cloning the 309-bp PCR product into the secretion vector pPIC9 as shown in Fig. 1.

```
      K  R  E  A  E  A  A  I  V  G  G  I  I  N  V  P  F  P  N  N  P  E
GAGCTCAAAAGAGAGGCTGAAGCTGCAATCGTAGGGGGCATTATCAATGTTCCATTCCCAAACAACCCCGAG
 F  Q  D  L  A  C  F  A  V  Q  D  Y  N  K  K  E  N  A  H  L  E  F  V  E
TTCCAAGATCTTGCTTGTTTTGCTGTTCAAGATTATAATAAGAAAGAGAATGCTCATTTGGAGTTTGTAGAA
 N  L  N  V  K  E  Q  V  V  A  G  M  M  Y  Y  I  T  L  V  A  T  D  A
AATTTGAATGTGAAAGAACAAGTTGTTGCTGGAATGATGTACTATATAACACTTGTGGCAACTGATGCT
 R  K  K  E  I  Y  E  T  K  I  L  M  K  E  W  E  N  F  K  E  V  Q  E
AGAAAGAAGGAAATATATGAGACCAAGATTTTGATGAAGGAATGGGAGAATTTCAAGGAAGTTCAAGAA
 F  K  L  V  G  D  A  T  K  *
TTCAAGCTTGTTGGTGATGCTACAAAGTAGGCGGCCGC
```

FIG. 3. The 303-bp sequence of the PC cDNA cloned by PCR from potato cv. Superior. The primers PC-DN and PC-UP are underlined. In bold, at the N-terminus, are 6 amino acids of the MF-alpha secretion signal removed during secretion into the medium, and therefore, not present in the purified PC.

```
AIVGGIINVPFPYNPEFQDLVCFAVQDYNKKENAHLEFVENLNV   (PC 1 to 44)
-----LVD---ENKV--D--AR-------Q-NDSS---KKV---    (DOMAIN 1A)
TMP---V---N-N-E-AR-I------Q----------------    (DOMAIN 2A)
-KL---TD----N-------AR-I-V------V-----------    (DOMAIN 3A)
-KT-------N-NS------AR--------NTQ-----------    (DOMAIN 4A)
KKL--FTE----NS------TR---HQ---DQ------------    (DOMAIN 5A)
-KL---------N-------AR----------------------    (DOMAIN 6A)
--I--FTD----N-------AR----------------Y------    (DOMAIN 7A)
-KP----I----NS------AR-----F-----G----------    (DOMAIN 8A)

KEQVVAGMMYYITLVATDARKKEIYETIKLVKEWENFKEVQEFKL (PC 45 to 89)
-Q-I---I-----FE--EGGN-KE--A---LRK--DL-KVVG---    (DOMAIN 1B)
-------I------A---DAG-KKIYKAKI-----D--K-V----    (DOMAIN 2B)
-Q-----------A-I--G--K------W-----D--K-V----    (DOMAIN 3B)
---L-S--------A----GN-KE--A--W-----D--K-ID--L    (DOMAIN 4B)
-K------L----FA---GG--K------W--V-----K-V----    (DOMAIN 5B)
---L----L-------I--G--K---A--N--------K-I----    (DOMAIN 6B)
---L----I----------G--K---A--N-----D--K-V----    (DOMAIN 7B)
-------------A-------------------------------    (DOAMIN 8B)
```

FIG. 4. Alignment of the 8 domains of PMC at the amino acid level indicating a minor variation between the 8 domains. None of the 8 domains are identical to the cloned domain of PC but greatest homology is with domain 8 of PMC. The first 44 amino acids here are designated with A and the last 45 amino acids with B.

Expression of recombinant PC and PMC in *P. pastoris* Ten to twelve different colonies of recombinant yeast expressing PMC and PC were grown overnight and induced with methanol for expression. This resulted in stable and biologically active protein in the case of PC. There was no variation in the expression level of PC between clones. Out of the 12 clones tested, 7 expressed PC at a similar level and clone-3 was chosen for scaling up. For PMC, variable expression levels were observed among ten positive clones identified. It was determined using dot blots that 2 were high, 5 were medium and 3 were low expressers, while 2 clones exhibited no detectable expression. A Western blot revealed that PMC produced in all yeast cultures was similar in size (85 kD) to PMC extracted from potato tuber peel (Fig. 2), indicating that in *P. pastoris*, unlike in *E. coli*, the gene was stably expressed and maintained in the selected yeast cultures.

Scaling up of the expression and purification of PC in *P. pastoris* Several media and pH conditions in a bench-top fermentor were tested for scaling up of the fermentation of the selected PMC clone. However, production of significant (>1 mg/*l*) levels of secreted PMC sufficient for purification could not be achieved. The amount of PMC present in the pellet was several fold higher than in the supernatant in the scale-up cultures indicating problems in secretion of the large PMC protein (data not shown). Although cellular expression in the cytoplasm could be an alternative, it was not tried in this investigation. The expression may also have been affected by the presence of the *Sna*BI site used for cloning the gene. Recently, it was found that the *Sna*BI site is identical to an mRNA efficiency element and may cause 10-fold reduction of gene expression in *P. pastoris* using the pPIC9 vector (13).

Fermentation of PC was performed in 1.5 *l* of fermentation medium using an aerated bench top fermentor. Fermentation was stopped at an optical density of 239 and the PC protein was the major protein in the culture supernatant (Fig. 5, lane I). A rapid one-step purification protocol was developed which removed all media components and separated the PC protein from the other minor proteins (Fig. 5C). A 3-fold dilution of the culture supernatant in equilibration buffer sufficiently lowered the salt concentration to achieve efficient binding of PC to the SP-sepharose. Figure 5A and B shows the results of a purification in which the salt gradient was continued up to 1 M NaCl. This shows that PC is specifically bound to the column (less protein in the flow through F). During the elution at the higher salt concentrations (>400 mM) other minor protein bands also elute. For this reason, the protocol was adapted to elute the protein batch-wise at 400 mM NaCl resulting in highly pure protein (Fig. 5C, lane B). From 1.5 *l* of culture supernatant ca. 150 mg of PC was finally recovered, which was estimated to be >95% pure (Fig. 6). Titration of recombinant PC against the cysteine protease papain resulted in a K_i

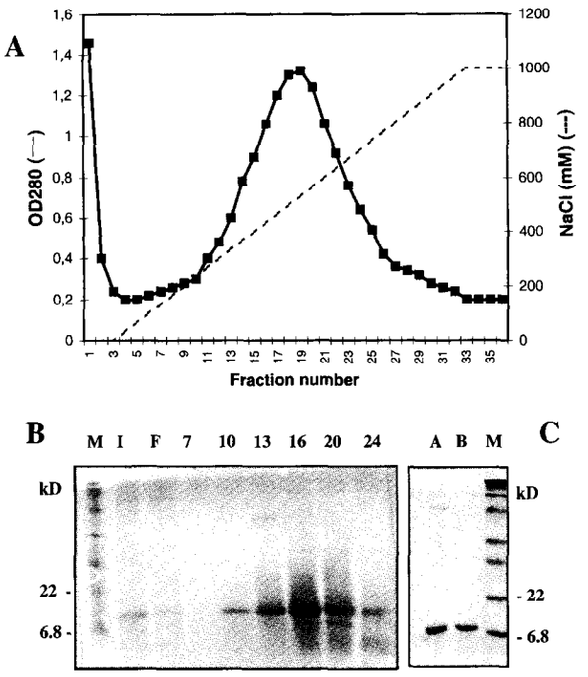

FIG. 5. One step SP HI-trap sepharose cation exchange purification of 0.5 *l* culture supernatant diluted 1:2 in equilibration buffer of pH 2.8. (A) Eluted protein profile using a 1 M NaCl gradient. (B) SDS–PAGE of input (I), flow through (F) and consecutive fractions (7–24) from the purification shown in A. (C) SDS–PAGE of batch-purified potato cystatin at 400 mM NaCl. Purification A yielded 30 mg of protein in 60 ml and purification B yielded 120 mg of PC in 300 ml. The protein size markers are loaded in lane and relevant sizes are indicated.

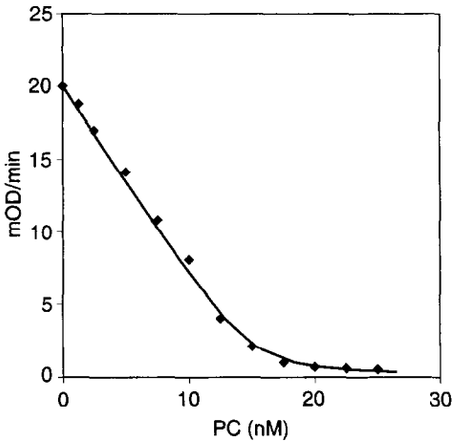

FIG. 6. Active site titration of 15 nM papain with purified PC. The y-axis shows mOD/min, which is the change in absorption at 405 nm expressed in milli-OD/minute, using Z-Phe-Arg-pNa·HCL (Bachem) as a substrate.

(apparent equilibrium dissociation constant) of 0.1 nM (Fig. 6). This value is in the range of the constants reported for potato wild-type cystatins (6).

This is the first report on the production of a plant cystatin in *P. pastoris*. Phytocystatins from rice, chestnut, papaya and corn have so far been exclusively produced in *E. coli* (21–23). It was shown that yeast provides a good alternative expression system for these inhibitors with the advantage of a rapid one-step purification step from the supernatant. Expression levels were rather low with the PMC gene, but the gene could at least be maintained in the yeast background unlike the situation in *E. coli*. The efficient production of PC has enabled further study of this protein for its potential effects against insects (24).

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