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Expression and purification of dalcochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts

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Abstract

The coding sequence of the mature dalcochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, was cloned and expressed in various systems. Expression in *Escherichia coli* resulted in an insoluble protein, which could be made soluble by co-expression with bacterial chaperonin GroESL. However, the enzyme had no activity. Recombinant expression in *Pichia pastoris* and *Saccharomyces cerevisiae* yielded an active enzyme. Dalcochinase was expressed under methanol induction in *P. pastoris*, since this was much more efficient than constitutive expression in *P. pastoris* or in *S. cerevisiae*. Addition of 0.5% casamino acids to the culture medium stabilized the pH of the culture and increased the protein yield by 3- to 5-folds. Insertion of a polyhistidine-tag either after the N-terminal α factor signal sequence or at the C-terminus failed to assist in purification by immobilized metal-ion affinity chromatography (IMAC) due to post-translational processing at both termini. A new construct of dalcochinase with an N-terminal truncation following the propeptide and eight histidine residues enabled its purification by IMAC, following hydrophobic interaction chromatography. The purified recombinant dalcochinase was apparently composed of differently post-translationally modified forms, but had kinetic properties and pH and temperature optima comparable to natural dalcochinase. The procedures reported here overcome the limitation in enzyme supply from natural sources, and allow further studies on structure–function relationships in this enzyme.

Keywords: Dalcochinase; β-Glucosidase; Thai rosewood; *Pichia pastoris*; Optimized expression

β-Glucosidases (E.C. 3.2.1.21) represent a group of ubiquitously expressed, hydrolytic enzymes, which catalyze the hydrolysis of β-*O*-glucosidic linkages between β-D-glucose and an aglycone or another sugar [1]. β-Glucosidases exhibit similar specificity for a β-glucoside substrate, but distinct specificities for the aglycone linked to the glucosyl group [1], suggesting their diverse biological functions. In plant physiology, β-glucosidases are implicated in growth regulation, stress response, cellobiose degradation, lignification, and defence [2]. Human β-glucosidase hydrolyses glucosylceramide in lysosomes, and its deficiency leads to Gaucher's disease [3]. In cellulolytic organisms, such as fungi and bacteria, β-glucosidase is part of the cellulase complex, which breaks down cellulose to glucose [4].

Previous studies on β-glucosidases have focused on the identification of catalytic residues and catalytic mechanisms [5–10]. β-Glucosidases are classified as retaining enzymes since their product (β-D-glucose) retains the same anomeric configuration as the substrate (a β-D-glucoside)

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[11]. Most plant β-glucosidases are glycosyl hydrolase family 1 (GH1)[1] hydrolases that catalyze the hydrolysis of their substrates via a double-displacement mechanism (http://afmb.cnrs-mrs.fr/CAZY/) [12]. Although the active site residues have not been precisely mapped for all β-glucosidases, the two glutamate residues present in the highly conserved TL/FNEP and I/VTENG motifs in all GH1 β-glucosidases are likely to act as the catalytic acid/base and nucleophile residues [11]. The three-dimensional structures of eight GH1 enzymes reveal a common $(\beta/\alpha)_8$ barrel structure, even though they share only 17–44% sequence identity [13,14].

While the mechanism of catalysis has been studied extensively, the molecular basis of substrate specificity is not as well understood. Naturally occurring glucosidic substrates contain a broad range of aglycone groups, including cyanogenic glucosides [13,15], cellobiose [16], phenolic glucosides [17], thioglucosides [18], and isoflavonoid glucosides [19]. Differences in the aglycone specificity-determining sites have been studied in maize and sorghum β-glucosidases, whose sequences show 70% identity [10–24]. Enzymatic studies of chimeric β-glucosidases and X-ray crystallographic structures suggested that the determinants of substrate specificity in the maize ZmGlu1 and sorghum SbDhr1 enzymes include both homologous and non-homologous residues. The identification of β-glucosidases, their substrates, and the nature of their interactions will not only shed light on the structure and function of the enzymes, but also help define their biological significance *in vivo*.

We have purified and characterized a β-glucosidase from *Dalbergia cochinchinensis* Pierre (Thai rosewood) [25], and determined its primary structure from the cloned cDNA [26]. This enzyme, named dalcochinase, has an isoflavonoid glucoside, dalcochinin-8′-*O*-β-D-glucoside, as its natural substrate [19]. Dalcochinase shows 81% amino acid sequence identity to another β-glucosidase from *D. nigrescens*, but the substrate specificities of the two enzymes differ [27]. Therefore, it is interesting to investigate the different amino acid residues that may function in determining substrate specificities in these two related β-glycosidases. Apart from hydrolysis, dalcochinase can also synthesize oligosaccharides and glycosides by reverse hydrolysis [28], and transglucosylation [29]. These capabilities of dalcochinase have potential applications for the synthesis of novel oligosaccharides or glycosides. To investigate the molecular basis of enzymatic catalysis in this useful enzyme, and to engineer it to achieve desirable catalytic properties, a reliable expression and purification system was developed in this study.

[1] *Abbreviations used:* IMAC, immobilized metal-ion affinity chromatography; GH1, glycosyl hydrolase family 1; IPTG, isopropyl-β-D-thiogalactoside; *AOX1*, alcohol oxidase 1; *GAP*, glyceraldehyde-3-phosphate dehydrogenase; *ADH1*, alcohol dehydrogenase 1; 4-MU-Glc, 4-methylumbelliferyl-β-D-glucopyranoside; *p*NP-Glc, *para*-nitrophenyl-β-D-glucopyranoside; *p*NP-Fuc, *para*-nitrophenyl-β-D-fucopyranoside.

Material and methods

Plasmids and host strains

Plasmids pPICZαB and pGAPZαB (Invitrogen, Carlsbad, CA, USA) were used for methanol-inducible and constitutive expression of a polyhistidine-tagged protein in *Pichia pastoris* strain GS115 (*his4*), respectively. Plasmids pVT 100-U and pYEX-BX (gifts from Dr. Sarawut Jitrapakdee, Mahidol University, Thailand, and from Professor Tony Weiss, Sydney University, Australia, respectively) were used for expression of dalcochinase in *Saccharomyces cerevisiae* strains DY150 and DBY746. Plasmid pBS536 (a gift from Dr. Nick Dixon, Australian National University, Australia) contains the *GroESL* operon for expression of chaperonin in *Escherichia coli*, and was maintained in *E. coli* in the presence of 25 μg/mL chloramphenicol and 50 μg/mL tetracycline. The plasmid pET15-b was used for expression of a polyhistidine-tagged protein in *E. coli* strain BL21(DE3).

Two modified pPICZαB *P. pastoris* expression vectors were produced for this study, one with a thrombin site before the C-terminal tag (pPICZαB-thrombin) and one with an N-terminal tag of eight histidine residues (pPICZαBNH8). First, two complementary oligonucleotides, thrombin_sense and thrombin_antisense (Table 1) for pPICZαB-thrombin, and PICZαBNH8F and PICZαBNH8R (Table 1) for pPICZαBNH8, were annealed by heating to 95–100 °C and slowly cooling to room temperature. The annealed thrombin oligonucleotides were ligated into the *Sac*II–*Xba*I sites of the pPICZαB vector, creating pPICZαB-thrombin. The pPICZαBNH8 hybrid, corresponding to the coding sequence of eight histidine residues, followed by the *Pst*I, *Sna*BI, and *Eco*RI sites, was cloned between the *Pst*I and *Eco*RI sites of pPICZαB.

Cloning, expression, and purification of dalcochinase in E. coli

The coding sequence of mature dalcochinase [26] was PCR amplified with the 5NDE and 3BAM primers (Table 1), cloned into pGEM-T easy vector, digested with *Nde*I and *Bam*HI, gel purified, and subsequently inserted into the *Nde*I–*Bam*HI sites in pET15b, giving pET-His_6-TRBG. After confirming the cloned sequence by restriction digestion and DNA sequencing, pET-His_6-TRBG was transformed into *E. coli* BL21(DE3) via electroporation. For co-expression of the *GroESL* operon with dalcochinase, pBS536 was transformed into *E. coli* BL21(DE3) harboring pET-His_6-TRBG.

A single colony of *E. coli* BL21(DE3) harboring either pET-His_6-TRBG or both pET-His_6-TRBG and pBS536 was grown in LB medium containing 100 μg/mL ampicillin overnight at 37 °C, 220 rpm. One-twentieth of the overnight culture was inoculated into 2TY medium containing 100 μg/mL ampicillin and grown at 37 °C, 220 rpm until the OD at 600 nm reached 0.5–0.8. Protein expression was induced by adding either 0.5 mM isopropyl-β-D-thiogalac-

Table 1
Sequences of oligonucleotides (5′ → 3′) used in Materials and methods

Primers	Sequences
3BAM	GGA TCC TCA AAA GCC TTC AAT GCC CCA CTT GG
3SAC	GGC GCC AAA GCC TTC AAT GCC CCA
PMCTERM1	AAG ATC TAG ATC AAA AGC CTT CAA TGC CTC TC
5HIN	AAG CTT CCA TGG GCA GCA GCC ATC ATC
5PST	CTG CAG GCA TTG ACT TTG CAA AAG
5NDE	CAT ATG ATT GAC TTT GCA AAA GAA GTC CG
5αFHIN	AAG CTT ATG AGA TTT CCT TCA ATT TTT ACT GC
PICZαBNH8F	CAT CAC CAT CAC CAT CAT CAC CAT GCT GCA GTA CGT AG
PICZαBNH8R	AAT TCT ACG TAC TGC AGC ATG GTG ATG ATG GTG ATG GTG ATG TGC A
Thrombin_sense	GGT TGG TTC CTA GGG GTT CTA TT
Thrombin_antisense	GGC GCC AAC CAA GGA TCC CCA AGA TAA GAT
trncTRBGF	CAT TCC TGC AGT TCC TCC ATT CAA CCG AAG C

toside (IPTG) or 5 mM lactose, and the culture was grown for 5 h at 20 °C. The cell pellet was collected by centrifugation and resuspended in 1/100 culture volume PBS (50 mM phosphate buffer, pH 7.4, 150 mM NaCl). Cells were lysed in 1 mg/mL lysozyme at 30 °C for 30 min in the presence of 1 mM phenylmethylsulfonylfluoride, followed by repeated freezing and thawing, and sonication. The suspension was incubated with 10 mM $MgCl_2$ and 10 μg/mL DNaseI at 4 °C for 30 min until the viscosity reduced, and then was centrifuged at 15,000 rpm for 30 min to separate the insoluble and soluble fractions.

To extract dalcochinase from inclusion bodies, the insoluble fraction was washed three times in 1/50 culture volume wash buffer (2 M urea and 2% Triton X-100 in PBS), resuspended in 1/50 culture volume extraction buffer (6 M guanidinium hydrochloride, 50 mM phosphate buffer, pH 7.4), and heated to 50–60 °C for 10–15 min. The suspension was then centrifuged at 15,000 rpm for 30 min, and the supernatant was applied to a Ni-NTA spin column (QIAGEN, Hilden, Germany) for small-scale protein purification according to manufacturer's instructions.

Dalcochinase in the soluble fraction was isolated by small-scale protein purification on a Ni-NTA spin column under native conditions, following manufacturer's instructions.

Cloning and expression of dalcochinase in P. pastoris

The coding sequence of mature dalcochinase [26] was PCR amplified with 5PST and 3SAC primers (Table 1), and inserted between the *Pst*I and *Sac*II of pPICZαB-thrombin vector following the KEX cleavage site to produce pPICZ-TRBG-His_6 (Fig. 1a). For constitutive expression under control of the glyceraldehyde-3-phosphate dehydrogenase *(GAP)* promoter, the *Pst*I–*Sac*II fragment of mature dalcochinase from pPICZ-TRBG-His_6 was inserted into pGAPαB, to give pGAPZ-TRBG-His_6.

A construct for expression of dalcochinase with an N-terminal polyhistidine-tag was also made. The dalcochinase cDNA was PCR amplified with the 5PST and PMCTERM1 primers, cloned into pGEM-T easy vector, excised, and inserted between the *Pst*I and *Sac*II sites of the pPICZαBNH8 vector to create pPICZ-His_8-TRBG (Fig. 1b). A second N-terminally His-tagged protein expression construct was made with a truncated N-terminus. The dalcochinase cDNA clone was amplified with the trncTRBGF and PMCTERM1 primers, and cloned into pPICZαBNH8, as described for pPICZ-His_8-TRBG, to create pPICZ-His_8-trncTRBG (Fig. 1c).

All recombinant plasmids were checked by restriction digestion and DNA sequencing, linearized with *Sac*I or *Pme*I, transformed into *P. pastoris* by electroporation, and selected on YPDS plates with 100 μg/mL zeocin, following the protocols from Invitrogen. Colonies of *P. pastoris* harboring dalcochinase sequence were grown in BMGY medium, and induced in BMMY medium for a further 1–5 days, according to the Invitrogen *Pichia* manual. The β-glucosidase activity in the culture medium was assayed each day.

For constitutive expression using pGAPZαB, clones of *P. pastoris* harboring the dalcochinase expression cassette

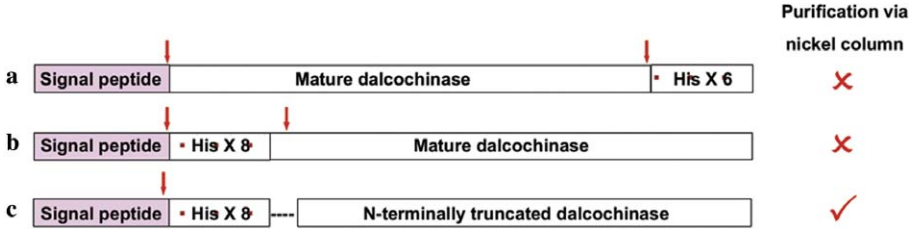

Fig. 1. Construction of recombinant plasmids harboring coding sequence of dalcochinase. (a) pPICZ-TRBG-His_6 for expression of mature enzyme with C-terminal polyhistidine-tag; (b) pPICZ-His_8-TRBG for expression of mature enzyme with N-terminal polyhistidine-tag; (c) pPICZ-His_8-trncTRBG for expression of N-terminally truncated enzyme with N-terminal polyhistidine-tag. Arrows indicate possible cleavage sites.

was grown in YPD medium, as advised by Invitrogen. β-Glucosidase activity in the production culture medium was assayed every 24 h for 5 days to check dalcochinase expression.

Optimization of P. pastoris culture medium conditions

Culture conditions of *P. pastoris* expressing dalcochinase were varied to optimize enzyme yield. First, three different media formulae were tried: (i) cell growth in BMGY and induction in BMMY; (ii) cell growth in BMGH and induction in BMMH (similar to BMGY and BMMY, respectively, but containing 4×10^{-3}% histidine and lacking yeast extract and peptone); and (iii) cell growth in MGYH and induction in MMH (similar to BMGH and BMMH, respectively, but not buffered). Second, the content of glycerol in the growth media was varied between 0.5, 1.0, and 1.5%. Third, the content of methanol in the induction media was varied between 0.5, 1.0, and 1.5%. Fourth, the concentration of casamino acids in the induction media was varied between 0, 0.5, and 1.0%.

Cloning and expression of dalcochinase in S. cerevisiae

For expression in *S. cerevisiae*, the N-terminally polyhistidine-tagged dalcochinase was cloned into the plasmid pVT-100U as: (i) a cytoplasmic protein, His_6-TRBG, and (ii) a secretory protein with the α mating factor propeptide fused to the mature N-terminus, αF-His_8-TRBG. The His_6-TRBG sequence was PCR amplified from the pET-His_6-TRBG template with the 5HIN and 3BAM primers (Table 1) and cloned between the *Hin*dIII and *Bam*HI sites in pVT-100U, giving pVT-His_6-TRBG. The αF-His_8-TRBG sequence was PCR amplified from the pPICZ-His_8-TRBG template with the 5αFHIN and 3BAM primers (Table 1) and was cloned between the *Hin*dIII and *Bam*HI sites in pVT-100U, giving pVT-αF-His_8-TRBG. Both recombinant plasmids were checked by restriction digestion and DNA sequencing, and transformed into *S. cerevisiae* strains DY150 and DBY746 via electroporation. Transformants were selected on uracil drop-out solid medium (0.8% yeast nitrogen base, 1.1% casamino acids, 2% glucose, 0.01% adenine, 0.01% tryptophan, 0.01% leucine, and 2.5% agar).

Expression of dalcochinase in pVT-100U was under the constitutive control of the alcohol dehydrogenase 1 (*ADH1*) promoter. Recombinant *S. cerevisiae* clones were grown in YPD medium at 30 °C, 220 rpm overnight. One one-hundredth volume of starter culture was inoculated in YPD and grown at 30 °C, 220 rpm for approximately 10 days. The culture medium was assayed for β-glucosidase activity every 24 h.

Purification of recombinant dalcochinase from P. pastoris culture medium

Culture medium of *P. pastoris* expressing dalcochinase was adjusted to a final concentration 1 M ammonium sulfate, and applied to a phenyl Sepharose CL-4B (Amersham Bioscience, Little Chalfont, UK) column. The column was washed with five column volumes of 1 M ammonium sulfate, 10 mM potassium phosphate, pH 7.0, and eluted with a reverse gradient of 1–0 M ammonium sulfate in the same buffer. Fractions containing β-glucosidase activity were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 8.0. The dialysate was adjusted to pH 8.0, and applied to a Ni-NTA column, which was washed and eluted with imidazole according to the manufacturer's instructions (Qiagen, Hilden, Germany). In some cases, the protein was purified with Talon resin (BD Biosciences Clontech, Mountain View, CA, USA), which gave protein similar to that purified with Ni-NTA resin. Fractions containing β-glucosidase activity were pooled, and the final enzyme preparation was concentrated and imidazole removed by ultrafiltration (30,000 M_rCO, Amicon Ultra, Millipore, Beverly, MA, USA).

Protein content was determined by the coomassie-binding method (Pierce, Rockford, IL, USA). SDS–PAGE, with 7.5% resolving gels, was performed according to the method of Laemmli [30]. Western blot analysis and chemiluminescent detection was done with mouse monoclonal antibody against natural dalcochinase, horseradish peroxidase-conjugated rabbit polyclonal antibody against mouse immunoglobulins (Dako, Denmark) and ECL Plus Western Blotting Detection reagents (Amersham Biosciences) according to the manufacturer's instructions. Non-denaturing PAGE and activity staining with 1 mM 4-methylumbelliferyl-β-D-glucoside (4-MU-Glc) was performed as reported previously [25]. To remove conjugated oligosaccharides, the glycosylated proteins were treated with endoglycosidase H (New England BioLabs, USA) for 1 h at 37 °C under denaturing conditions according to manufacturer's instructions. Purified proteins were subjected to Dubois's phenol–sulfuric acid assay for a direct estimation of total carbohydrate content, using mannose as standards [31].

Enzyme assays and kinetic measurements

Hydrolytic activities of recombinant dalcochinase in culture medium toward *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) and *p*-nitrophenyl-β-D-fucopyranoside (*p*NP-Fuc) were performed in 0.1 M sodium acetate, pH 5.0, at 30 °C for 30 min, in a 0.5 mL reaction. The reaction was stopped by adding 1 mL 2 M sodium carbonate, pH 10, and *p*-nitrophenol released was measured at 400 nm. Kinetic properties of purified recombinant dalcochinase towards various substrates were assayed in 0.1 M sodium acetate, pH 5.0, at 30 °C for 5 min in a 0.1 mL reaction. To compare the kinetic properties towards *p*NP-Glc and *p*NP-Fuc, the reaction was stopped by adding 1 mL 2 M sodium carbonate, pH 10, followed by measuring *p*-nitrophenol released at 400 nm. To compare the kinetic properties towards *p*NP-Glc and dalcochinin-8′-*O*-β-D-glucoside, the reaction was stopped by boiling for 2 min. The glucose released was

quantified by adding 1 mL glucose oxidase reagent (BM Laboratories, Thailand), and measuring absorbance at 505 nm. Kinetic parameters were calculated from the Michaelis–Menten equation using KaleidaGraph (Synergy Software).

The temperature optimum was determined by measuring *p*-nitrophenol released from 1 mM *p*NP-Glc and *p*NP-Fuc in 0.1 M sodium acetate, pH 5.0, for 10 min at temperatures ranging from 20 to 70 °C at 5 °C increments, and completing the assay as described above. Stability was tested by incubating the enzyme from 0 to 60 min at the above temperatures, then assaying the activity with 1 mM *p*NP-Glc and 1 mM *p*NP-Fuc at 30 °C for 10 min. The pH optimum was determined by hydrolysis of these substrates at 30 °C for 10 min in the following 0.1 M buffers: glycine–HCl, pH 2–3; sodium citrate, pH 3–4; sodium acetate, pH 4–5; MES, pH 5–6; sodium phosphate, pH 6–8; Tris–HCl, pH 8–9; and CAPS, pH 9–10.

Results and discussion

Expression of dalcochinase in E. coli

Previously, recombinant dalcochinase was expressed as a secreted protein in *P. pastoris* to produce the enzyme in the media [26]. However, enzyme was produced in relatively low amounts and was difficult to purify, so an attempt was made to produce an N-terminally His-tagged protein in *E. coli*. The majority of dalcochinase was found in the insoluble fraction, even when the induction was done at 20 °C (Fig. 2, lanes 1 and 2). The insoluble enzyme was solubilized with 6 M guanidinium hydrochloride, and could be purified with a nickel column under denaturing conditions to give considerable amounts of purified recombinant dalcochinase (Fig. 2, lane 3). However, extensive refolding steps did not yield active enzyme (results not shown). The small amount of recombinant dalcochinase in the soluble fraction could also be purified with the nickel column under non-denaturing conditions, but it was inactive (results not shown). Presumably the recombinant enzyme was not correctly folded or modified in the bacterial cytoplasm.

To improve production of soluble dalcochinase from bacterial cultures and to assist its folding, the *groESL* operon, encoding bacterial chaperonin, in pBS536 was transformed into *E. coli* BL21(DE3) harbouring pET-His_6-TRBG. Expression of dalcochinase was as described earlier. In this case, a protein of approximately 60 kD was found in the soluble fraction. This protein is the same size as recombinant dalcochinase, and could be purified with a nickel column under non-denaturing conditions (Fig. 2, lanes 4–6), but it did not exhibit β-glucosidase activity. MS/MS analysis of this purified protein revealed it to be bacterial chaperonin groEL, whose molecular weight is also about 60 kD (results not shown). This 60 kD protein was absent in the eluate from nickel columns when *E. coli* cultures harboured pBS536 and pET15b vector without the dalcochinase insert (Fig. 2, lanes 7–9). This suggested that the 60 kD

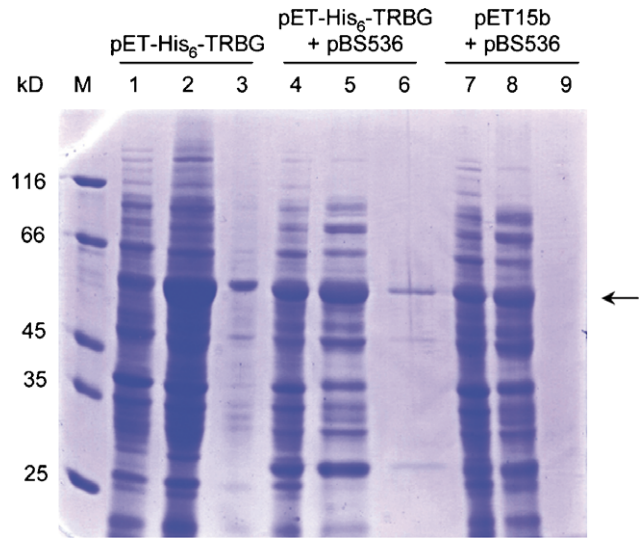

Fig. 2. Expression and purification of recombinant dalcochinase in *E. coli*. Lanes 1–3, expression of recombinant dalcochinase alone and purification under denaturing conditions; lanes 4–6, co-expression of recombinant dalcochinase with chaperonin GroESL and purification under non-denaturing conditions; lanes 7–9, co-expression of plasmid pET15b with chaperonin GroESL and purification under non-denaturing conditions. Lanes 1, 4, and 7 contained 5 h induced samples, Lanes 2, 5, and 8, extracted samples prior to IMAC columns, and lanes 3, 6, and 9, eluates from IMAC columns. Lane M is protein standards. The position of the recombinant dalcochinase is indicated by arrows.

chaperonin alone does not bind to nickel columns and could be co-purified with the polyhistidine-tagged dalcochinase to which it bound. Keresztessy et al. [32] reported a similar tight association between cassava linamarase and groEL. For linamarase, the recombinant enzyme was active and could be separated from groEL after adding excess MgATP. However, in our case, no β-glucosidase activity was obtained, suggesting that some post-translational process which does not occur in *E. coli* may be necessary for proper folding and function of dalcochinase.

Expression of dalcochinase in eukaryotic systems

Previously, the cDNA of dalcochinase was cloned into pPIC9K and expressed in *P. pastoris* as an N-terminal fusion protein with the *S. cerevisiae* α mating factor propeptide to produce 0.077 U/mL culture medium [26]. This construct of dalcochinase did not contain an affinity tag to facilitate purification, so a new construct of dalcochinase was generated with a thrombin site, followed by a polyhistidine tag at the C-terminus in pPICZ-TRBG-His_6 (Fig. 1a). β-Glucosidase activity could be detected at 0.055 U/mL culture medium following 5-day induction with 0.5% methanol (when assayed with 5 mM *p*NP-Glc).

Constitutive expression in yeast was tried as an alternative system to improve the production of dalcochinase. In the pGAPZ-TRBG-His_6 construct, recombinant dalcochinase was expressed constitutively under the control of the *GAP* promoter as a secretory protein in *P. pastoris*. For constitutive expression in *S. cerevisiae* under control of the *ADH1*

promoter, two constructs of dalcochinase with N-terminal polyhistidine tags were made: (i) a cytoplasmic protein called His_6-TRBG, and (ii) a secretory protein called αF-His_8-TRBG (with the α factor propeptide fused to the N-terminus of the mature protein). Ten colonies for each of the three constitutive expression systems were selected for trial expression experiments. Constitutive expression under the *GAP* promoter in *P. pastoris* and under the *ADH1* promoter in *S. cerevisiae* yielded dalcochinase activities at about 1/10 of the activities obtained from the pPICZ-TRBG-His_6 construct. Thus, constitutive expression was less efficient in the production of dalcochinase compared with methanol-inducible expression.

Optimization of culture conditions for expression of dalcochinase in P. pastoris

From the expression results above, it appeared that active recombinant dalcochinase was best expressed under methanol-inducible control in *P. pastoris*. To improve yields of expression in *P. pastoris* cultures under methanol induction, culture parameters were varied in order to find the optimal conditions. First, three different media formula were tried: (i) cell growth in BMGY and induction in BMMY; (ii) cell growth in BMGH and induction in BMMH; and (iii) cell growth in MGYH and induction in MMH. The combination of BMGH and BMMH gave the highest yield of activity in the culture media (results not shown), and it is also helpful in purification due to the absence of yeast extract in both media. So, from this point on, all cultures were grown in BMGH, and changed to BMMH for induction.

Then, the content of glycerol in BMGH media was varied between 0.5, 1.0, and 1.5%. While the cell densities did not differ in different glycerol concentrations, the highest dalcochinase activities were obtained when cultures were grown in BMGH containing 1% glycerol prior to induction (Fig. 3a). When the content of methanol in BMMH was varied between 0.5, 1.0, and 1.5%, the yield of dalcochinase did not differ significantly, but was highest with 0.5% methanol, followed by 1.0 and 1.5% methanol in BMMH. Thus, from this point onwards, 0.5% methanol was used for induction, with methanol added to make up 0.5% of the culture volume everyday.

Clare et al. [33] reported that the presence of casamino acids in culture media of *P. pastoris* could prevent extracellular proteolysis of heterologously expressed protein. When casamino acids were added to BMMH to a final concentration of 0, 0.5, and 1.0%, the highest dalcochinase yield was obtained in the presence of 0.5% casamino acids, resulting in 3- to 5-fold greater enzyme activity than in *P. pastoris* cultures lacking casamino acids. Furthermore, the cultures with 0.5% casamino acids could continue to grow and express dalcochinase for at least 10–12 days after induction, while dalcochinase activity in culture media without casamino acids declined after 3–5 days of induction (Fig. 3b). In addition, the pH of culture media decreased steadily in the absence of casamino acids, but was stable in the media containing casamino acids (Fig. 3c). Thus, the presence of casamino acids in the media appeared to help overcome extracellular proteolysis and stabilize pH. While similar beneficial effects of casamino acids were obtained for expression of *Arabidopsis thaliana* α-1,3-fucosyltransferase and murine *N*-acetylgalactosaminyltransferases in *P. pastoris*, other factors, such as expression control, protein sequence and media conditions, may also affect expression levels of proteins [34]. So, the optimal expression system must be determined for each protein studied.

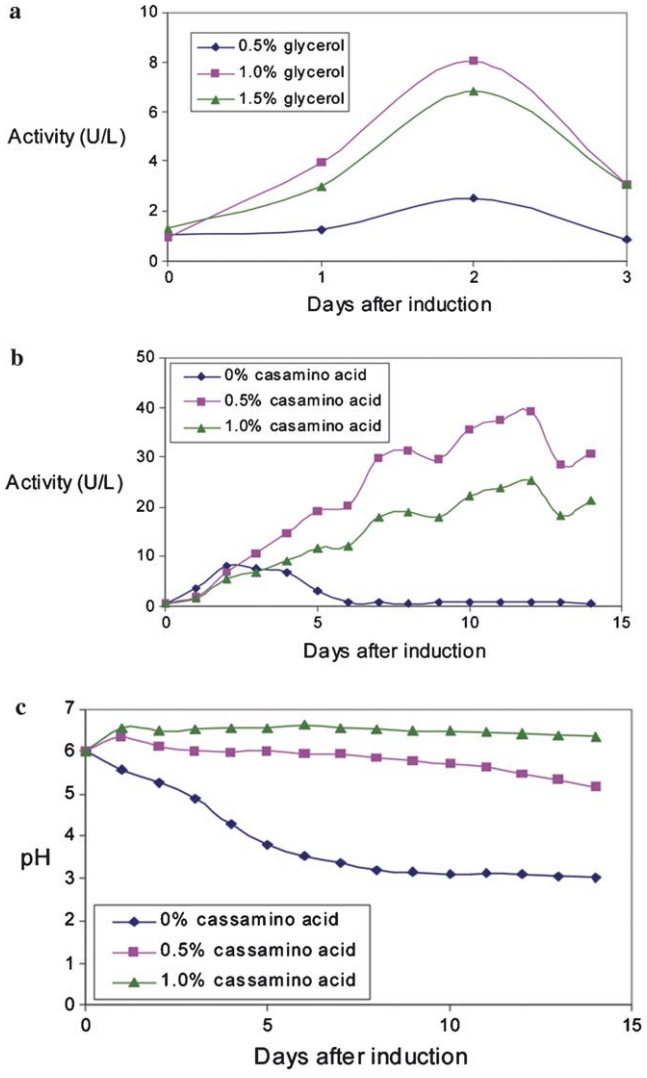

Fig. 3. Optimization of culture conditions for expression of recombinant dalcochinase in *P. pastoris* cultures. (a) β-Glucosidase activity when the *P. pastoris* cultures were grown in BMGH containing 0, 0.5, and 1% glycerol; (b) β-glucosidase activity when the cultures were induced in BMMH containing 0, 0.5, and 1% casamino acids; (c) pH of BMMH containing 0, 0.5, and 1% casamino acids. In (a) and (b) β-glucosidase activity was obtained with 1 mM *p*NP-Glc as described in Materials and methods.

Purification of dalcochinase via IMAC

In the chosen expression system, dalcochinase was made with C-terminal polyhistidine tag and secreted into the cul-

ture medium, which should facilitate simple purification via immobilized metal-ion affinity chromatography (IMAC). However, the recombinant enzyme did not bind to IMAC columns under the conditions tested. This could be due to post-translational processing at the C-terminus, which removed the polyhistidine tag.

To overcome cleavage at the C-terminus of dalcochinase, a new construct of dalcochinase, pPICZ-His_8-TRBG, was made with the polyhistidine tag at the N-terminus of the mature dalcochinase sequence following the KEX cleavage site (Fig. 1b). Similar levels of β-glucosidase activity could be detected in recombinant *P. pastoris* cultures expressing dalcochinase with an N-terminal polyhistidine tag compared with the previous constructs. The recombinant *P. pastoris* media was passed through IMAC columns under different conditions, but most of the recombinant dalcochinase with the N-terminal polyhistidine tag did not bind. Recombinant dalcochinase was then purified from *P. pastoris* culture media following the procedures used to purify natural dalcochinase from *D. cochinchinensis* seeds [25]. The N-terminus of the purified enzyme produced in *P. pastoris* was found to begin at the sequence EVPPFN, which indicated that the first 12 residues of the mature dalcochinase were cleaved from the enzyme together with the N-terminal polyhistidine tag. This explained the lack of binding of the secreted recombinant dalcochinase to the IMAC resin.

So, a new construct of recombinant dalcochinase was made with a truncated N-terminus (starting at the VPPFN sequence after the cleavage site) following an α mating factor propeptide and eight histidine residues, resulting in pPICZ-His_8-trncTRBG (Fig. 1c). The expression level of β-glucosidase from this construct in *P. pastoris* culture medium was similar to those of the previous constructs under methanol induction. Truncation at the N-terminus of dalcochinase is not expected to affect its activity, since this part of the molecule points outward and is not near the catalytic pocket in the crystal structure of white clover β-glucosidase (which has 60% sequence identity to dalcochinase) [13].

A clone of *P. pastoris* expressing the highest level of β-glucosidase activity was cultured in the optimal BMMH, 0.5% methanol expression conditions. Typically, after 10–12 days of induction, the β-glucosidase activity was approximately 0.3 U/mL culture medium, when assayed with 5 mM *p*NP-Glc. The medium was collected and purified by hydrophobic interaction chromatography, followed by IMAC, as summarized in Table 2. The final yield of dalcochinase was 24.1 U and 1.8 mg from one litre of *P. pastoris* culture, approximately 44% of the amount present in the *P. pastoris* culture medium. Although this expression level is relatively low compared with some reports of protein expression in *P. pastoris*, it is not uncommonly low [35]. The low level of expression might be due to a number of factors, including an inappropriate codon usage, since 40 out of 612 amino acid residues in the recombinant dalcochinase are encoded by codons that are used at 10% or less in *P. pastoris*. The specific activity of the purified recombinant dalcochinase was 13.8 U/mg protein, while the value of 30.4 U/mg was obtained for the purified natural enzyme using the same methods to assay enzyme activity and protein content. The discrepancy may result from lower levels of enzyme purity or differences in processing of the recombinant dalcochinase, compared to the natural enzyme.

The purified recombinant dalcochinase with N-terminal truncation and polyhistidine tag gave a broad band with an apparent molecular weight of 66–69 kD on SDS–PAGE (Fig. 4a, lane 3), which was clearly higher than the apparent 63–64 kD band for the natural enzyme purified from seeds of *D. cochinchinensis* (Fig. 4a, lane 1) and larger than the theoretical molecular weight of 59,872. Both natural and recombinant dalcochinase could be detected by monoclonal antibody raised against natural dalcochinase (Fig. 4b, lanes 1 and 3, respectively), and both showed activity when their non-denaturing PAGE was stained with 1 mM 4-MU-Glc (Fig. 5a and b). These results indicate that the recombinant dalcochinase produced in *P. pastoris* culture medium is similar to the natural enzyme in immunoreactivity and hydrolytic activity against the fluorogenic substrate.

Hypermannosylation has been reported for some *P. pastoris*-produced proteins, with up to 50–150 mannose residues in length [35]. Heterogeneity in the preparation of purified recombinant dalcochinase, causing an appearance of a broad band rather than a distinct band on SDS–PAGE, may have been due to differently glycosylated forms of the recombinant enzyme. Variation in the amino and carboxy termini, caused by inconsistent cleavage of α factor propeptide [35] and proteolysis may also partly contribute to the heterogeneity in the sample. The difference in apparent subunit molecular weights between natural and recombinant dalcochinase is likely to result from different glycosylation machineries in plant and in yeast. Using Dubois's phenol–sulfuric acid assay for a direct total sugar estimation [31], the natural and recombinant dalcochinase were found to contain approximately 13.6 and 16.1% (by weight), respectively. The purified natural and recombinant

Table 2
Purification of recombinant dalcochinase from culture medium of *P. pastoris* harboring pPICZ-His_8-trncTRBG

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture medium	55.0	92.8	0.6	1.0	100.0
Phenyl sepharose	38.8	32.8	1.2	2.0	70.6
Ni^{2+} affinity	24.4	2.0	12.5	21.1	44.5
Ultrafiltration	24.1	1.8	13.8	23.3	43.9

One litre of culture was used for purification, and assays were performed with 1 mM *p*NP-Glc as described in Materials and methods.

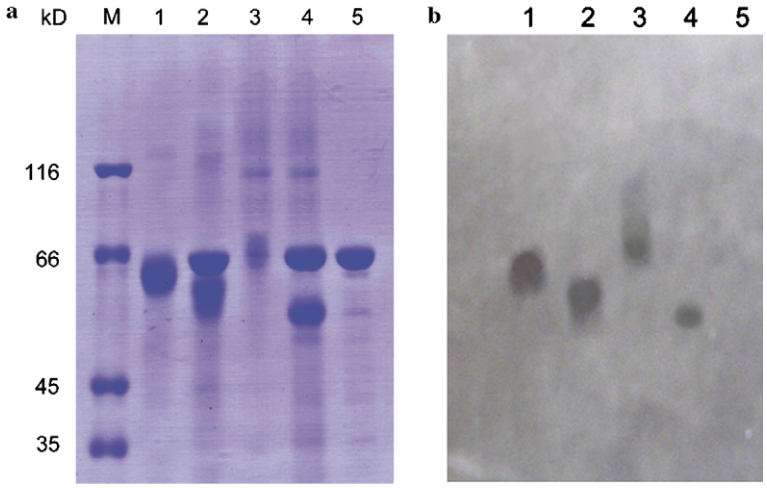

Fig. 4. SDS–PAGE of purified recombinant dalcochinase. (a) Coomassie stain; (b) Western blot: lane 1, purified natural dalcochinase; lane 2, purified natural dalcochinase treated with endoglycosidase H; lane 3, purified recombinant dalcochinase from *P. pastoris* culture medium; lane 4, purified recombinant dalcochinase treated with endoglycosidase H; lane 5, endoglycosidase H. Lane M is protein standards.

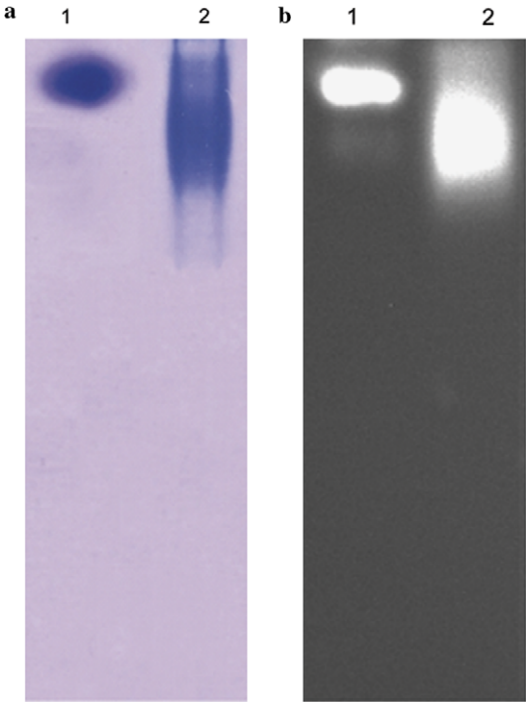

Fig. 5. Non-denaturing electrophoresis of purified recombinant dalcochinase. (a) Coomassie stain; (b) activity staining with 1 mM 4-MU-Glc. Lane 1, purified natural dalcochinase; lane 2, purified recombinant dalcochinase from *P. pastoris* culture medium.

dalcochinase were treated with endoglycosidase H, which cleaves between the innermost *N*-acetylglucosamine residues, removing high mannose and hybrid oligosaccharides from N-linked glycoproteins. The resulting products appeared as protein bands of different sizes on SDS–PAGE (Fig. 4a, lanes 2 and 4, lower bands), suggesting differences in their carbohydrate chains. The deglycosylated forms of natural and recombinant dalcochinase retained the same level of immunoreactivity as the glycosylated forms (Fig. 4b, lanes 2 and 4), but their hydrolytic activity towards 4-MU-Glc could not be determined since the deglycosylation procedures involved denaturation.

Kinetic properties of purified recombinant dalcochinase

The N-terminally truncated and polyhistidine-tagged recombinant dalcochinase purified from *P. pastoris* culture media exhibited kinetic properties similar to those of natural dalcochinase (Table 3). It had an optimal pH of 5–6, which agrees with the value of 5.0 reported for the enzyme purified from seeds [25]. The activity was maximal at a temperature of 50–60 °C, and the enzyme appeared to be stable at 60 °C for up to 50 min, which is similar to the native enzyme from seed. This suggests that the differences in the N-terminus and post-translational modification between the dalcochinase produced in *P. pastoris* and that produced in the plant have little affect on its catalytic properties and stability.

Table 3
Kinetic parameters of purified recombinant dalcochinase with N-terminal truncation and polyhistidine-tag

	K_m (mM)		V_{max} relative to *p*NP-Glc	
	Recombinant	Seed[a]	Recombinant	Seed[a]
*p*NP-Glc	5.78 ± 0.41	5.37 ± 0.09	1	1
*p*NP-Fuc	0.57 ± 0.04	0.54 ± 0.04	0.47	0.49
Dalcochinin-8′-*O*-β-D-glucoside	1.48 ± 0.08	1.68	0.76	1.07

Enzymatic assays were performed as described in Materials and methods.
[a] Kinetic parameters for the natural dalcochinase were taken from [19] and [25].

Expression of recombinant dalcochinase in *P. pastoris* has eliminated the need to rely on seasonal availability of natural sources, and offers an advantage of large-scale production via fermentation. Oxygen-limited fed-batch cultivation and expanded bed adsorption have been applied for production and recovery of recombinant dalcochinase from *P. pastoris* high-cell-density cultures [36,37]. The expression and purification procedures reported here allow simple, lab-scale preparation of recombinant dalcochinase for further enzymatic characterizations. This will enable investigations into the structure–function relationships in this enzyme using tools in molecular biology and protein engineering.

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