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Heterologous expression of an α -amylase inhibitor from common bean (*Phaseolus vulgaris*) in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*

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Abstract

Background: Phaseolamin or α -amylase inhibitor 1 (α AI) is a glycoprotein from common beans (*Phaseolus vulgaris* L.) that inhibits some insect and mammalian α -amylases. Several clinical studies support the beneficial use of bean α AI for control of diabetes and obesity. Commercial extracts of *P. vulgaris* are available but their efficacy is still under question, mainly because some of these extracts contain antinutritional impurities naturally present in bean seeds and also exhibit a lower specific activity α AI. The production of recombinant α AI allows to overcome these disadvantages and provides a platform for the large-scale production of pure and functional α AI protein for biotechnological and pharmaceutical applications.

Results: A synthetic gene encoding α AI from the common bean (*Phaseolus vulgaris* cv. Pinto) was codon-optimised for expression in yeasts (*α AI-OPT*) and cloned into the protein expression vectors pKLAC2 and pYES2. The yeasts *Kluyveromyces lactis* GG799 (and protease deficient derivatives such as YCT390) and *Saccharomyces cerevisiae* YPH499 were transformed with the optimised genes and transformants were screened for expression by antibody dot blot. Recombinant colonies of *K. lactis* YCT390 that expressed and secreted functional α AI into the culture supernatants were selected for further analyses. Recombinant α AI from *K. lactis* YCT390 was purified using anion-exchange and affinity resins leading to the recovery of a functional inhibitor. The identity of the purified α AI was confirmed by mass spectrometry. Recombinant clones of *S. cerevisiae* YPH499 expressed functional α AI intracellularly, but did not secrete the protein.

Conclusions: This is the first report describing the heterologous expression of the α -amylase inhibitor 1 (α AI) from *P. vulgaris* in yeasts. We demonstrated that recombinant strains of *K. lactis* and *S. cerevisiae* expressed and processed the α AI precursor into mature and active protein and also showed that *K. lactis* secretes functional α AI.

Keywords: Phaseolamin, α -Amylase inhibitor, α AI, Common bean, *Phaseolus vulgaris*, Yeast heterologous expression, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*

Background

Phaseolamin or α -amylase inhibitor (α AI) is a seed storage glycoprotein present in most wild and cultivated common beans (*Phaseolus vulgaris* L.) [1]. It inhibits some mammalian and insect α -amylases, but not the

endogenous plant enzyme [2–5]. Bean α AI is synthesised as a preproprotein that undergoes glycosylation at Asn residues and proteolytic processing to become the active form, comprising α - and β - subunits [4, 6–9]. Different cultivars of *P. vulgaris* contain slightly different forms of α AI exhibiting variation in their primary structure, their N-glycans as well as some heterogeneity in the processing of the C-terminal region of the β - subunit. They all retain their inhibitory activity of α -amylases [9].

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The functional protein α AI is composed of non-covalently linked α - and β - subunits [10]. In *P. vulgaris*, the α AI precursor undergoes post-translational modifications while transiting the secretory pathway. In addition to N-linked glycosylations, three proteolytic cleavages occur during the maturation of α AI. Removal of the signal peptide occurs in the endoplasmic reticulum (ER). Two carboxy-terminal cleavages occur in the vacuole: the first cleavage at Asn²³⁹ removes a short carboxy terminal peptide (propeptide), and the second cleavage is catalysed by a bean vacuolar processing enzyme from the family of legumains (VPE) at Asn⁷⁷, resulting in the formation of α - and β - subunits [4, 6–9]. After the proteolytic cleavage at Asn⁷⁷, the asparagine residue is removed from the C-terminus of the α -subunit by a presumed carboxypeptidase in the bean [9].

The inhibition of mammalian α -amylases by α AI reduces starch metabolism and the production of simple sugars, thus its use may be beneficial in helping control diabetes mellitus and obesity in humans [11–13]. The clinical use of bean α AI has been discredited because of the ineffective commercial products released in the early 1980s promoted as starch-blockers [12, 14, 15]. Most of these dietary supplements contained protein concentrates of bean seeds with a low concentration of α AI but with significant levels of antinutritional and potentially toxic compounds, such as phytohemagglutinin (PHA) and trypsin inhibitors, in addition to an active endogenous α -amylase [13].

In contrast to the impure and inefficient commercial formulations, partially purified α AI from beans with increased specific activity reduces starch digestion in human duodenum and remains stable in gastrointestinal secretions [16]. Further studies reported that purified α AI also delayed the digestion and absorption of intestinal glucose, reduced postprandial hyperglycaemia and insulin levels in diabetic and non-diabetic subjects, supporting the notion of the beneficial effect of α AI for the control of diabetes [17–20]. More recent studies using commercial extracts of *P. vulgaris* standardising the content of α -amylase inhibitor determined that many still contain antinutritional impurities, and those with a reduced level of lectins and trypsin inhibitors also exhibit a lower specific activity α AI [21–25]. Proteins naturally present in bean seeds such as PHA-L and PHA-E and the lectin-like proteins called arcelins [4, 9, 26] are difficult to remove during purification of α AI from bean seeds [9, 27]. In an attempt to decrease the amount of lectins in bean seeds, a null PHA bean line obtained by backcrossing has been used as raw material for commercial α AI formulations, but seeds from this line still contain trypsin inhibitors [24, 26]. Given that ingestion of trypsin inhibitors increases the secretion of trypsin and α -amylase by

the pancreas [28, 29], their presence in commercial bean extracts are likely to affect the activity and efficacy of α AI.

To date there is no efficient method to recover highly pure and fully functional α AI from bean seeds nor an appropriate expression system for the production of purified recombinant α AI. Here we report the heterologous expression of α AI in yeast, using *Kluyveromyces lactis* and *Saccharomyces cerevisiae* as hosts.

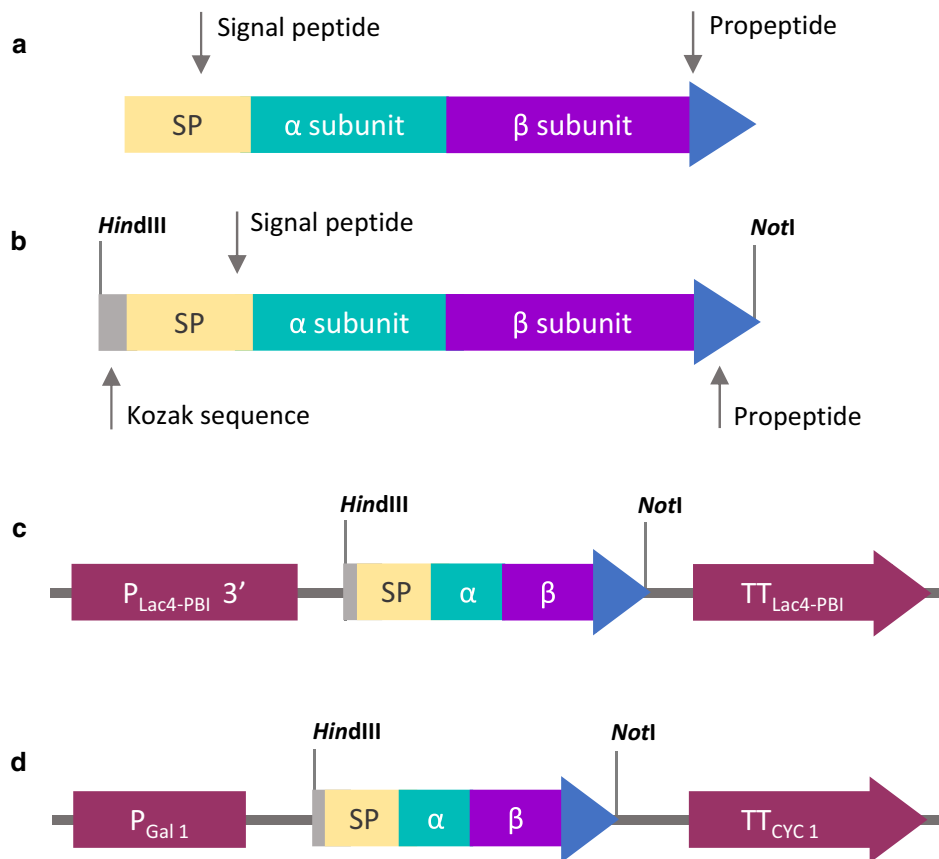
Methods

Strains and culture media

Escherichia coli DH5- α was used as host for plasmid propagation and was grown in LB medium. Yeast strains used in this work were *K. lactis* wild type GG799 (*MAT α*) and protease deficient strains YCT389 (*MAT α Δ yps1p*), YCT390 (*MAT α Δ yps7p*), YCT569 (*MAT α Δ pfam00026*), and YCT598 (*MAT α Δ Bar1p*) (purchased from NEB, Ipswich, MA, USA) [30] and *S. cerevisiae* YPH499 (*MAT α ura3-52 lys2-801_amber ade2-101_ochre trp1- Δ 63 his3- Δ 200 leu2- Δ 1*) [31]. Yeast strains were routinely grown in standard YPD medium. Cultures were induced using YPCGal medium (1% yeast extract, 2% peptone, 2% casaminoacids, 2% galactose and 2 mM ammonium sulphate, supplemented with B complex vitamins [32]) at 28 °C for 72–96 h at 250 rpm. Selection of recombinant strains of *K. lactis* was based on growth on YCB agar plates containing 5 mM acetamide [33]. *S. cerevisiae* transformants were selected by their growth in yeast synthetic drop-out medium without uracil agar (SC- \emptyset U).

Construction of expression vectors and yeast transformation

The synthetic gene *α AI-OPT* was synthesised by GenScript (Piscataway, NJ, USA) using the coding sequence (CDS) of α AI from *P. vulgaris* cv. Pinto (Fig. 1a, as Accession AY603476) and optimised for yeast codon usage bias, including a Kozak consensus sequence AAAAAG upstream of the start codon and flanking restriction sites *Hind*III and *Not*I (Fig. 1b). *α AI-OPT* gene was cloned into plasmids pKLAC2 (NEB) and pYES2 (Invitrogen, Carlsbad, CA, USA) at restriction sites *Hind*III and *Not*I, and the resulting vectors were verified by sequence analysis. In construct pKLAC2- α AI-OPT, the *α AI-OPT* CDS replaced the α MF secretion domain from vector pKLAC2. Restriction endonucleases and T4 DNA ligase were purchased from NEB and standard DNA techniques were performed as described by Sambrook [34]. Chemically competent yeast cells were prepared using the LiAc/SS carrier DNA/PEG method [35]. *K. lactis* wild type and protease deficient strains were transformed with 1 μ g of the integrative cassette from vector pKLAC2- α AI-OPT (Fig. 1c) previously linearised with *Sac*II [33]; and *S.*



Colour designations:

Promoters (P) and terminators (TT) in dark red. Kozak consensus sequence in grey.

Signal peptide in yellow; α -subunit in green; β -subunit in purple; and propeptide in blue.

Fig. 1 Schematic representation of the gene constructs used for α AI production. Diagrams of **a** the native α AI gene from cv. Pinto comprising signal peptide (SP), α - and β - subunits and the propeptide sequences; **b** the synthetic α AI-OPT gene, optimised for expression in two yeasts, including a Kozak consensus sequence and flanked by *HindIII* and *NotI* restriction sites. Diagrams of constructs **c** pKLAC2- α AI-OPT and **d** pYES2- α AI-OPT. $P_{Lac4-PBI}$ and P_{Gal1} correspond to promoters Lac4-PBI and Gal1 in pKLAC2 and pYES2, respectively. $TT_{Lac4-PBI}$ and TT_{CYC} correspond to 3' terminators Lac4-PBI and CYC in pKLAC2 and pYES2, respectively

cerevisiae YPH499 was transformed with plasmid pYES2- α AI-OPT (Fig. 1d).

Isolation and manipulation of nucleic acids

Plasmid DNA was extracted from *E. coli* and yeasts using QIAprep[®] Spin Miniprep Kit (Qiagen, Hilden, Germany) and genomic DNA from yeasts was isolated using DNeasy[®] Blood and Tissue Kit (Qiagen). Spheroplasts were obtained by incubating yeast cells with 1 U of lyticase (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. α AI-OPT gene was detected by PCR amplification of a 160 bp amplicon using primers *OPT Fwd* (TATGACGGTCAAAACGCTGA) and *OPT Rev* (GCGGAGAAACCAACTCTGAC). Multicopy

integration of cassettes was determined by PCR using primers 2, 3 and 5 [33]. Oligonucleotides were purchased from Sigma-Aldrich (Castle-Hill, NSW, Australia). Samples for nucleotide sequencing were purified using QIAquick[®] PCR Purification Kit (Qiagen). DNA sequencing reactions were amplified using primers *KLSQ Fwd* (GCGGATAACAAGCTCAAC) and *KLSQ Rev* (TTATCGCACAAGACAATC) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA, USA), and further analysed in an automatic DNA sequencer ABI 3130xl Genetic Analyser (Applied BioSystems) at the John Curtin Medical Research School, Australian National University (Canberra, ACT, Australia).

Cassette copy-number analysis by q-PCR

Q-PCR analysis was performed using FastStart SYBR Green Master without Rox (Roche Life Science, Rotkreuz, Switzerland) in the CFX96™ real-time PCR detection system (BioRad). The α AI-*OPT* gene was detected using primers *OPT Fwd/OPT Rev*, and the single-copy gene, *KIACT1*, was amplified using primers *KIACT1 Fwd/KIACT1 Rev* [36]. The conversion of mass concentration of the vector to copy concentration was as described by Whelan et al. [37]. Experiments were performed in triplicate using DNA from three independent biological samples.

Screening of recombinant α AI

Transformed colonies were transferred onto a replica plate and each colony was grown in 600 μ L of YPC-Gal media in a 96-well microplate at 28 °C for 72–96 h at 250 rpm. Cleared supernatants (200 μ L) were loaded into a dot blot apparatus connected to a vacuum pump. Pure Pinto- α AI [2] in sterile YPCGal (1 ng/ μ L) was used as positive control. Cleared YPCGal inoculated with untransformed cells (200 μ L) and sterile YPCGal (200 μ L) were used as negative controls. The nitrocellulose membrane was probed with a rabbit anti- α AI polyclonal antibody (1:10,000 dilution), and then probed with HRP-conjugated anti-rabbit secondary antibody (1:1000 dilution, Promega Corp. Madison, WI, USA). Protein antibody complexes were made visible using Pierce ECL Western Blotting Substrate detection reagent (Thermo-Fisher Scientific, Inc., Waltham, MA, USA) and autoradiography film (Kodak Biomax X-ray film). The relative amount of protein in dots was estimated by densitometry using ImageJ Software [38].

Protein analysis by SDS PAGE and western blot

Proteins from culture supernatants were precipitated using TCA/acetone and resuspended in ammonium bicarbonate buffer (100 mM ammonium bicarbonate, pH 8.2, 10% DMSO). Proteins from yeast cells were extracted using YeastBuster Protein Extraction Reagent (Novagen, Darmstadt, Germany), 50 μ L of 100 \times THP, protease inhibitors (2 μ M PMSF, 30 μ g leupeptin, 5 μ g pepstatin A) and 1 U of DNase. Pure Pinto- α AI was used as positive control for SDS-PAGE (3 μ g) and western blots (200 ng plus 3 μ g of BSA).

Samples were analysed on NuPAGE® Novex® 10% Bis-Tris Pre-Cast Protein Gels (Invitrogen), loaded in MES Running Buffer and stained with Coomassie G-250. For western blotting, separated proteins were electro-transferred onto a nitrocellulose membrane (0.2 μ m Hybond-C, Amersham Biosciences) and probed as described for antibody dot blot. Membranes were stained with AmidoBlack prior to probing with the α AI antibody [39].

Protein concentration was determined by Bradford assay [40], using Coomassie Protein Assay (Thermo-Fisher Scientific) and BSA (0–7 μ g/ μ L) as standard.

α -Amylase activity assay

α -Amylase inhibitory activity was determined using Megazyme Ceralpha reagent as substrate (nonreducing-end blocked *p*-nitrophenyl- α -D-maltoheptaoside, BPNPG7; plus glucoamylase and α -glucosidase; Megazyme International, Ireland) and porcine pancreatic α -amylase (#A6255 Type I-A, Sigma Aldrich), as described by Samah et al. [41]. Inhibitory activity was calculated as follows: 1 U of α -amylase activity is the amount of enzyme that generates 1 μ mol/min of *p*-nitrophenol under assay conditions; inhibition units or units of α -amylase inhibition (IU) is the amount of inhibitor that reduces the activity of α -amylase by 50% [2]. Experiments were performed in triplicate and results were expressed as the average \pm standard deviation. Prior to inhibitor activity assays of culture supernatants, samples were partially purified by DEAE-Sepharose to remove interfering compounds from culture media.

Statistical analysis

The significance of differences between measurements was analysed using Student's unpaired *t* test. Values are reported as the average \pm standard deviation. Differences in means were considered significant if $P < 0.05$.

Purification of recombinant α AI

Induced culture supernatants were dialysed against 0.02% sodium azide solution for 24 h at 4 °C and freeze-dried. The lyophilised powder was resuspended in 5 mL of 10 mM citrate/phosphate buffer (10 mM disodium phosphate; 50 mM citric acid; pH 8.0) and incubated with 5 g of DEAE-Sepharose® CL-6B (Pharmacia Biotech, Sweden) for 1 h at 4 °C. Unbound protein was eluted with 10 mL of 10 mM citrate/phosphate buffer. Fractions containing protein bound to the resin were eluted with 5 mL of 250 mM NaCl solution in 10 mM citrate/phosphate buffer, using a glass sintered-funnel (porosity #2) connected to a vacuum pump by a Büchner flask. Samples were dialysed against 0.02% sodium azide solution for 24 h at 4 °C and freeze-dried. Pre-purified samples were incubated with 5 mL of α -amylase-Sepharose in 20 mL of stabilization buffer (15 mM succinic acid, 20 mM CaCl₂, 0.5 M NaCl, pH 5.6) for at least 2 h at 37 °C. Unbound protein was eluted with two washes of 15 mL of stabilization buffer and protein bound to the resin was eluted with two washes of 15 mL of releasing buffer (0.15 M citric acid, pH 3.2) and pH of samples was immediately increased to 5.6 using 0.1 M NaOH. Fractions were dialysed against 0.02% sodium azide solution for 24 h at

4 °C, freeze-dried and stored at 4 °C for further analyses. All eluted fractions were analysed by Bradford assay and Ceralpha assay. Resin α -amylase-Sepharose was prepared as previously described [4].

Identification of recombinant α AI by MS

Protein (25 μ g) was loaded on SDS-PAGE and bands corresponding to the non-processed (approximately 35 kDa) and processed forms (a combination of α - and β - subunits of α AI, with a molecular mass between 15 and 20 kDa) were excised from the gel. Samples were submitted to Australian Proteome Analysis Facility (Macquarie University, Sydney, NSW, Australia), digested with trypsin and loaded onto a capillary LC system coupled to an MS/MS instrument (1D Nano LC ESI MS/MS) as described by Attack et al. [42]. Tryptic fragments were identified using Mascot Server software (Matrix Science Inc., MA, USA) using the *Phaseolus_vulgaris* database.

Results

Construction of yeast expression vectors and screening for secretion of recombinant α AI

In preliminary experiments we assessed the expression of α AI in *K. lactis* and *S. cerevisiae* using the native gene isolated from beans (shown schematically in Fig. 1a), but no protein expression could be detected (data not shown). We used the amino acid sequence of α AI from cv. Pinto to design and synthesise a gene optimised for yeast codon usage (Fig. 1b). The DNA sequence of the yeast-optimised synthetic gene called *α AI-OPT* is shown in Additional file 1: Figure S1. *α AI-OPT* was cloned downstream of the galactose-inducible promoters LAC4_{PBI} and Gal1 in plasmids pKLAC2 and pYES2, respectively (Fig. 1c, d). *K. lactis* wild type and protease

deficient strains were transformed with the integrative cassettes derived from construct pKLAC2- α AI-OPT and cells were grown in selective YCB agar plates containing acetamide. In addition, *S. cerevisiae* YPH499 was transformed with episomal plasmid pYES2- α AI-OPT and cells were grown in yeast SC- \emptyset U agar plates. Since a variable number of plasmid cassettes can be integrated in the *K. lactis* genome with consequent differences in the *α AI-OPT* gene dosage, some variability in the expression of recombinant α AI was to be expected. Therefore, we performed a preliminary screening for the expression of recombinant α AI over a large number of colonies instead of first sorting colonies by genetic analysis. Colonies (186) from each transformed strain of *K. lactis* and *S. cerevisiae* were grown and induced in micro-scale cultures, and the cleared culture supernatants were screened by protein dot blot using an antibody to α AI. In some cultures of *K. lactis* YCT390 (circled dots in Fig. 2), the signal intensity was equivalent to 200 ng of pure Pinto- α AI. Cultures from recombinant *S. cerevisiae* YPH499 and other strains of *K. lactis* (GG799, YCT389, YCT569 and YCT589) showed a signal intensity equivalent to that of the negative controls (see Additional file 2: Figure S2).

Saccharomyces cerevisiae YPH499 pYES2- α AI-OPT clones expressed α AI intracellularly

Although dot blot screening of *S. cerevisiae* YPH499/pYES2- α AI-OPT cultures showed no indications of secreted α AI, plasmid DNA from 10 randomly selected clones was analysed by PCR to screen for the gene. All colonies were positive for the gene (see Additional file 3: Figure S3). To determine whether these clones expressed recombinant α AI, we analysed the proteins in both the culture supernatants and in the lysed cells of three

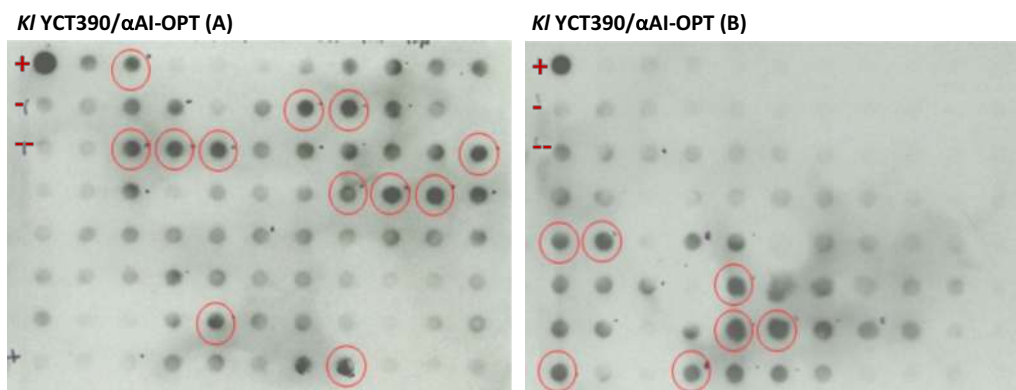


Fig. 2 Antibody dot blot screening of culture supernatants of *K. lactis* YCT390 with *α AI-OPT*. Cleared supernatants (200 μ L) of transformed colonies of *K. lactis* YCT390/ *α AI-OPT*. Pure Pinto- α AI (200 ng) used as positive control (+). As negative controls, cleared culture supernatant (200 μ L) of untransformed strain YCT390 (–) and culture media YPCGal (200 μ L) without cells (– –). *Circled dots* correspond to *dots* with a signal intensity equivalent to that of positive control

randomly selected clones (8, 27 and 92) by western blot. Immunoreactive polypeptides of the expected size of processed α AI were detected in the intracellular contents of the three recombinant colonies of *S. cerevisiae* YPH499 but were not detected in the culture supernatants (Fig. 3a). Furthermore, no bands corresponding to α AI precursor were detected in either the lysed cells or in the secreted fraction (Fig. 3a).

The intracellular fractions of recombinant clones 8, 27 and 92 of *S. cerevisiae* YPH499 were analysed by Ceralpha assay, using the parent strain with the empty plasmid (YPH499/pYES2) as negative control (Fig. 3b). The inhibition of α -amylase activity by these extracts was very low, but statistically significant ($P < 0.05$) when compared to the negative control.

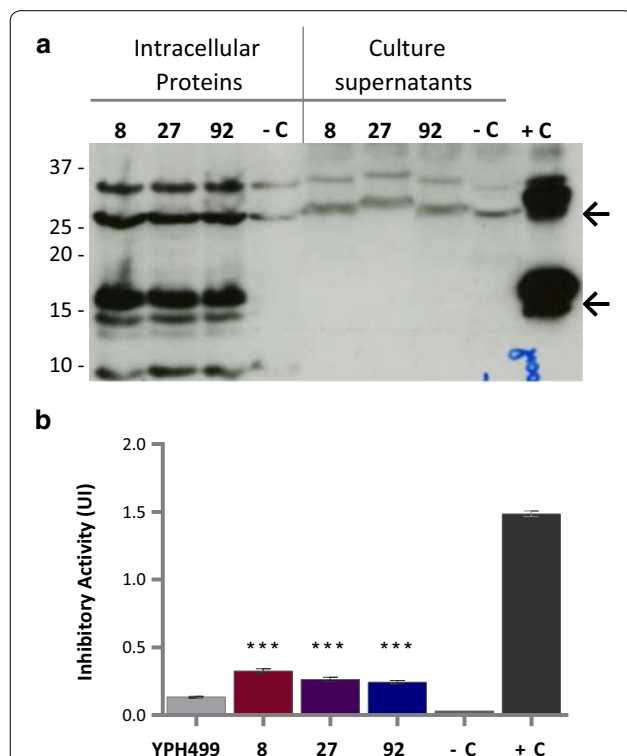


Fig. 3 Western blot and α -amylase inhibitory activity of proteins from selected colonies of *S. cerevisiae* YPH499 with α AI-OPT. **a** Western blot of proteins (25 μ g) from culture supernatants and lysed-cells of colonies 8, 27 and 92 transformed with α AI-OPT; the parent strain YPH499 transformed with empty plasmid pYES2 was used as negative control (-C) and pure Pinto- α AI (200 ng) was used as positive control (+C). Molecular markers are shown in kilodaltons at left. **b** Inhibitory activity against porcine pancreatic α -amylase of lysed-cell extracts (100 μ g of protein) of *S. cerevisiae* YPH499/pYES2- α AI-OPT colonies 8, 27 and 92. Parent strain YPH499 transformed with empty plasmid pYES2 was used as a negative control (-C) and pure Pinto- α AI (200 ng) was used as a positive control (+C). Results are expressed as inhibition units (IU). Data from triplicate experiments were expressed as the average \pm standard error. *** $P < 0.05$ as compared to *S. cerevisiae* YPH499/pYES2

Although attempts were made to purify the recombinant inhibitor from the lysed cellular contents of *S. cerevisiae* YPH499/pYES2- α AI-OPT, no active inhibitor could be recovered (data not shown).

α AI was synthesised and secreted by *K. lactis* YCT390/ α AI-OPT

We analysed the proteins from both the lysed-cells and the culture supernatants of *K. lactis* YCT390 transformants by SDS-PAGE and western blot. We chose 10 colonies from among those showing the highest signal intensities in the antibody dot blot as well as four colonies that showed intensities similar to those of negative controls. Eleven of the 14 colonies secreted polypeptides between 15 and 37 kDa (Fig. 4a, b). The top arrows on the right represent the expected molecular mass of α AI precursor; and bottom arrows on the right represent the expected sizes of processed α AI. Three of the 11 colonies (68A, 17A and 47B) secreted two immune-reactive polypeptides within the range of 15 and 20 kDa (Fig. 4b, bottom arrows on the right represent the expected sizes of processed α AI) which exhibited an electrophoretic mobility similar to the Pinto- α AI α - and β - subunits.

The intracellular extracts contained a polypeptide of approximately 32 kDa which co-migrated with the bean α AI precursor but did not contain detectable processed α AI (Fig. 4c, d, top and bottom arrows, respectively).

The genetic analysis of the 14 colonies of *K. lactis* YCT390/ α AI-OPT confirmed that the 11 colonies expressing the polypeptides of interest carried the α AI-OPT gene and also harboured more than one copy of the cassette inserted into the chromosome, whereas non-expressing colonies showed no amplification of the gene (see Additional file 4: Figure S4).

Precursor or processed α AI were not detected in cell lysates and culture supernatants of colonies from the other transformed strains of *K. lactis* (GG799, YCT389, YCT569 and YCT589), although they were positive for α AI-OPT gene (data not shown).

We measured copy number of α AI-OPT gene by q-PCR to determine the number of cassettes inserted into the *K. lactis* chromosome. All strains harboured 5–6 copies of α AI-OPT gene (Table 1).

Culture supernatants of *K. lactis* inhibited α -amylase

To determine whether the polypeptides secreted by selected recombinant strains of *K. lactis* YCT390 were active as α -amylase inhibitors, we assayed partially purified culture supernatants from five strains for activity by Ceralpha assay (Fig. 5a). The culture supernatants were partially purified with DEAE-Sepharose in order to remove compounds present in the culture media interfering with the activity assay. Strains 17A and 68A strongly

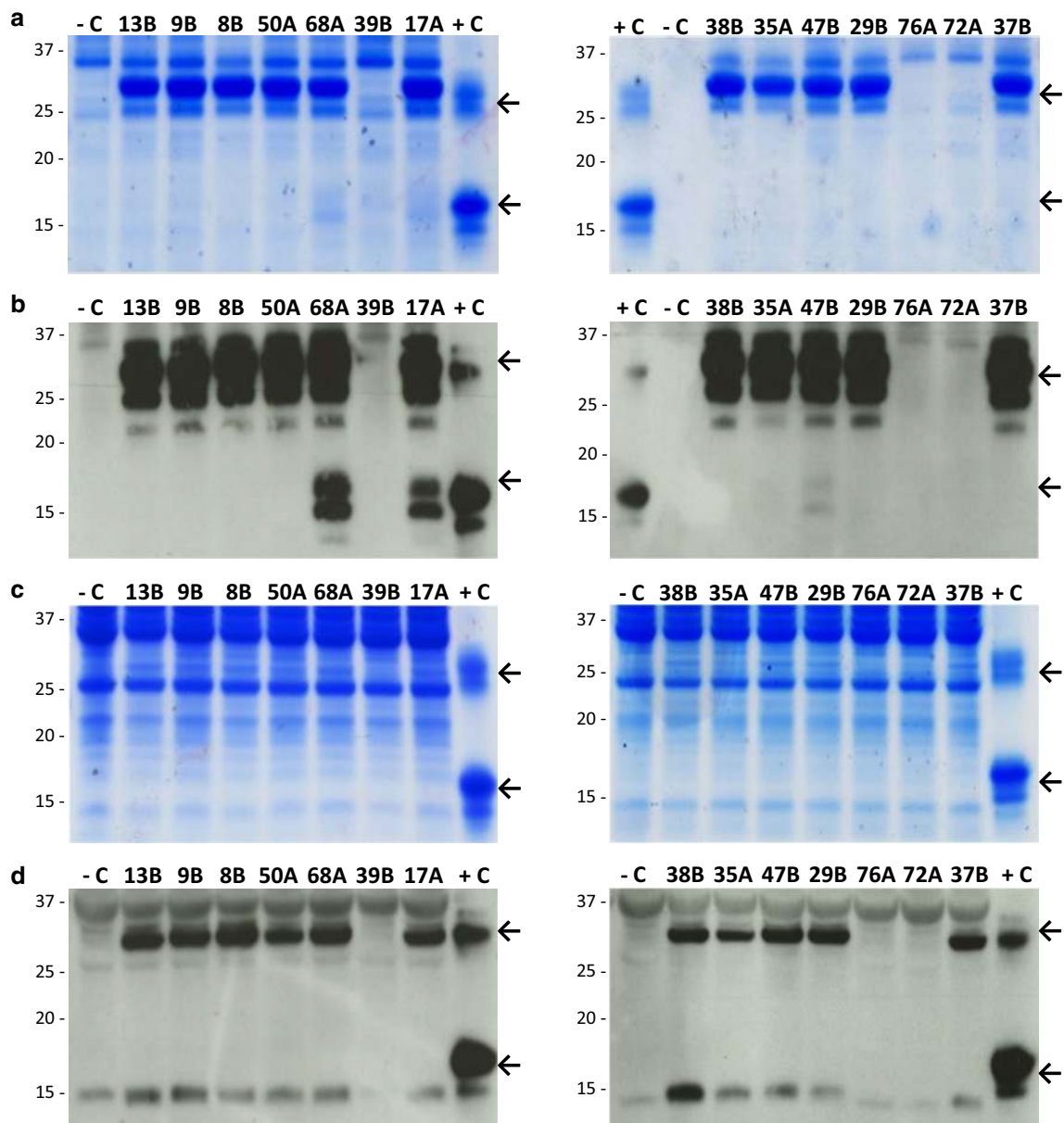


Fig. 4 SDS-PAGE and western blots of proteins from selected lines of *K. lactis* YCT390 with α AI-OPT. **a** SDS-PAGE and **b** Western blot of culture supernatants (TCA-precipitated proteins from 5 mL) of strains 13B, 9B, 8B, 50A, 68A, 39B, 17A, 38B, 35A, 47B, 29B, 76A, 72A and 37B. **c** SDS-PAGE and **d** western blot of lysed-cell contents (25 μ g of protein) of strains 13B, 9B, 8B, 50A, 68A, 39B, 17A, 38B, 35A, 47B, 29B, 76A, 72A and 37B. Untransformed strain YCT390 was used as a negative control (–C) and pure Pinto- α AI (200 ng) was used as a positive control (+C). Molecular markers are shown in kilodaltons at left

inhibited α -amylase activity while extracts from 47B and 29B showed an intermediate level of inhibition of the enzyme, namely 57 and 44%, respectively. Strain 13B culture supernatant was much less active, i.e., only slightly more active than the negative control (untransformed YCT390) (Fig. 5a). Activities from lysed-cell extracts of *K. lactis* were not significantly different ($P < 0.05$) to the negative control (Fig. 5b).

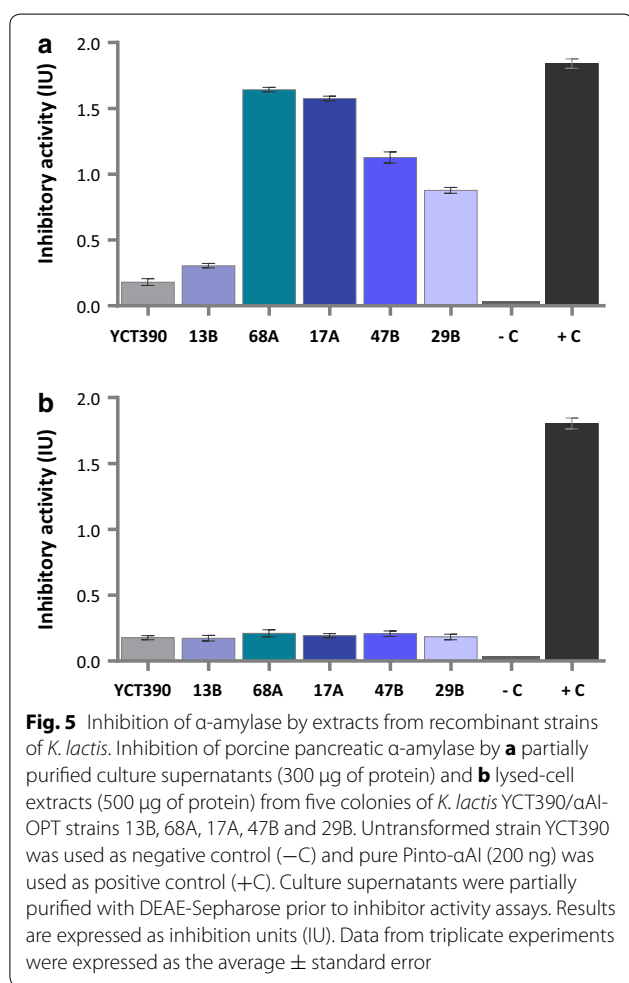
Recombinant α AI was purified from culture supernatants of *K. lactis*

The methods to purify α AI from seeds [2–4, 10, 16] were not suitable for the recovery of α AI from culture supernatants possibly because of the composition of YPCGal medium (which has a mixture of hydrolysed peptides, galactose and vitamin B complex). Soluble and functional α AI was recovered from *K. lactis* culture

Table 1 Gene copy number and relative processing of recombinant α AI among strains of *K. lactis* YCT390/ α AI-OPT

<i>K. lactis</i> strain/colony	Gene copy number		Relative processed α AI	Inhibitory activity (%)
	α AI-OPT	<i>KIACT1</i>		
Untransformed YCT390 (negative control)	0	1	–	10.3
YCT390/ α AI-OPT 68A	6	1	+++++	89.8
YCT390/ α AI-OPT 17A	5	1	+++	84.6
YCT390/ α AI-OPT 47B	6	1	++	59.2
YCT390/ α AI-OPT 29B	6	1	+	49.5
YCT390/ α AI-OPT 13B	6	1	–	16.6

Estimated copy number of genes α AI-OPT and *KIACT1* (control) in genomic DNA of selected strains of *K. lactis* YCT390/ α AI-OPT measured by q-PCR. Relative amount of processed recombinant α AI and percentage of inhibitory activity against porcine pancreatic α -amylase among pre-purified extracts of *K. lactis* YCT390/ α AI-OPT strains. Untransformed strain YCT390 was used as negative control



supernatants using a combination of ion exchange and affinity chromatography. The fractions were analysed by SDS-PAGE, western blot and Ceralpha assay (Fig. 6). Electrophoretic analysis of the purified fractions showed that anionic exchange and affinity purification with α -amylase-Sephacel yielded polypeptides with a size

between 15 and 20 kDa (Fig. 6a, lane 2, and Fig. 6b, lane 4, bottom arrow points to α - and β - subunits). Activity assay of the fractions obtained by anion-exchange and α -amylase-Sephacel showed that specific inhibitory activity increased following purification (Fig. 6c).

Prior to purification, 300 mL of culture supernatant from *K. lactis* YCT390/ α AI-OPT 68A yielded around 0.9 mg protein. After anion-exchange, we recovered about 25% of the protein containing partially purified inhibitor; and after incubation with α -amylase-Sephacel, we recovered approximately 1% of the protein in the culture supernatant as functional α AI (Table 2).

The identity of the peptides from *K. lactis* YCT390/ α AI-OPT 68A was confirmed by 1D Nano LC ESI MS/MS. After electrophoresis, the major bands between 15 and 20 kDa (expected size of α - and β - subunits) were excised from gel together in a single sample (Sample A), and the band of approximately 32 kDa (expected size of α AI precursor) was isolated separately (Sample B). α - and β - subunits of α AI were detected in both of these samples. Processed α AI and the precursor were identified by 5 and 6 distinct tryptic fragments, representing 26 and 31% of coverage, respectively (Fig. 7). No other protein was detected in these major bands.

Discussion

The α -amylase inhibitor α AI from *P. vulgaris* has been successfully expressed in different plants resulting in functional inhibitor [8, 9]. Nonetheless, the heterologous expression of α AI in a non-plant system has not been reported to date and was not expected to be straightforward due to the post-translational processing that this protein undergoes during maturation. Nevertheless, using yeasts to express α AI was attractive because of their ability to perform post-translational modifications, as well as their ability to secrete proteins into the culture media, which could facilitate purification.

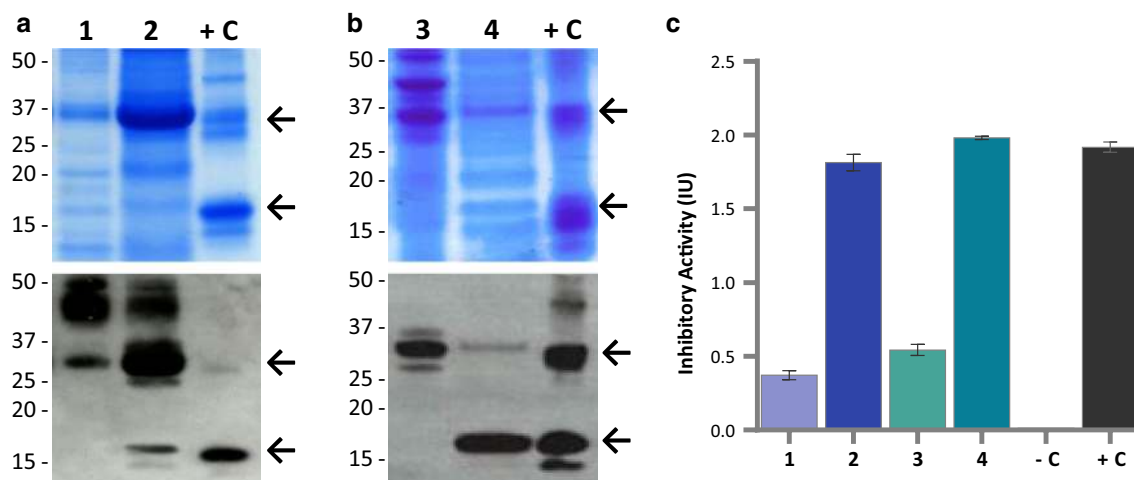


Fig. 6 Purification of recombinant α AI from culture supernatants of *K. lactis* YCT390/ α AI-OPT. SDS-PAGE and western blot of **a** fractions purified by anion exchange chromatography from *K. lactis* YCT390/ α AI-OPT strain 68A: protein unbound to DEAE-Sepharose (lane 1), protein bound to DEAE-Sepharose (lane 2); and **b** fractions purified by affinity chromatography: unbound proteins to α -amylase-Sepharose (lane 3), and proteins bound to α -amylase-Sepharose (lane 4). Pure Pinto- α AI (200 ng) was used as a positive control (+C). Molecular markers are shown in kilodaltons at left. **c** Inhibition of porcine pancreatic α -amylase by purified fractions containing unbound proteins to DEAE-Sepharose (lane 1), bound proteins to DEAE-Sepharose (lane 2), unbound proteins to α -amylase-Sepharose (lane 3), and bound proteins to α -amylase-Sepharose (lane 4). Untransformed strain YCT390 was used as a negative control (-C) and pure Pinto- α AI (200 ng) was used as a positive control (+C). Data from triplicate experiments were expressed as the average \pm standard error

Table 2 Purification of recombinant α AI from culture supernatants of *K. lactis* YCT390/ α AI-OPT strain 68A

Purification step	Total protein (μ g)	Specific inhibitory activity (IU/mg)
Dialysed and freeze-dried culture supernatant	899	ND
Anion-exchange batch-adsorption (DEAE-Sepharose CL-6B)	227	8
Affinity batch-adsorption (α -amylase-Sepharose)	8.45	234

In preliminary experiments using the plant form of the α AI gene to transform strains of *K. lactis* GG799 derivatives and *S. cerevisiae* YPH499, the yeasts expressed the gene transcript but did not express the protein (Brain-Isasi and Higgins, unpublished data). Because recombinant strains of *K. lactis* YCT390 and *S. cerevisiae* YPH499 harbouring the reconstructed gene α AI-OPT were able to synthesise functional α AI, we conclude that codon usage bias is a limiting step in the heterologous expression of α AI in yeasts.

Here we demonstrated that the native signal peptide of α AI encoded in the synthetic gene α AI-OPT was recognised by *K. lactis* YCT390 to target the nascent peptide into the secretory pathway. However, we found an unexpected differential trafficking of α AI between recombinant *K. lactis* YCT390 and *S. cerevisiae* YPH499, since recombinant colonies of *K. lactis* YCT390 secreted α AI (precursor and processed polypeptides) into the culture medium and recombinant *S. cerevisiae* YPH499 expressed functional α AI intracellularly that was not

secreted. This difference in α AI trafficking between two yeast species resembles the results obtained when PHA-L is expressed in *Pichia pastoris* and *S. cerevisiae* [43, 44]. PHA-L is also a vacuolar glycoprotein from *P. vulgaris* and shows considerable amino acid similarity to α AI, but PHA-L is devoid of the proteolytic processing site at Asn⁷⁷ required for the maturation of α AI [8]. When PHA-L is expressed in *P. pastoris* using its native signal peptide, the signal peptide is proteolytically cleaved and the protein is secreted into the culture medium [44]. In contrast, when PHA-L is expressed in *S. cerevisiae* using its native signal peptide, only about 1% is secreted and most of the protein accumulates in the vacuole because of a vacuolar targeting-determinant in the PHA-L sequence which is recognized by this yeast [43, 45]. Therefore, it is possible that α AI shows a similar behaviour to PHA-L when expressed in *S. cerevisiae*. Like α AI, several other seed proteins require proteolytic cleavage on the carboxyl side of Asn by specific VPEs to make their respective mature forms [46, 47]. To date,

Mascot Search Results

Database: Phaseolus_vulgaris **Nominal mass (Mr):** 27053 **Calculated pI:** 4.92
Protein hits: Q6J2U4|Q6J2U4_PHAVU Alpha amylase inhibitor-1 OS=Phaseolus vulgaris PE=4 SV=1

Sample A: 20-15 kDa (α- and β- subunits)

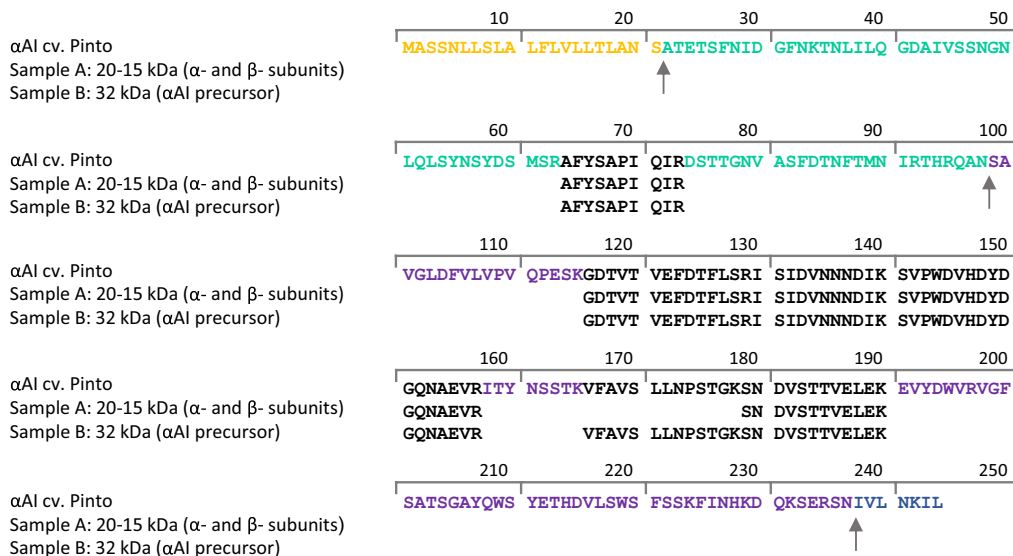
Score: 354

Sequence coverage: 26 %

Sample B: 32 kDa (αAI precursor)

Score: 546

Sequence coverage: 31 %

**Colour designations:**

Signal peptide in **yellow bold**; α-subunit in **green bold**; β-subunit in **purple bold**; and propeptide in **blue bold**.

Matched peptides shown in **bold black**. Grey arrows indicate peptidic cleavages.

Fig. 7 Tryptic peptide matches with Pinto-αAI amino acid sequence (Q6J2U4_PHAVU). Mascot search results and sequence similarity of tryptic fragments obtained from recombinant αAI from purified supernatants of *K. lactis* YCT390/αAI-OPT 68A, compared to amino acid sequence of α- and β- subunits from Pinto-αAI (Q6J2U4_PHAVU). Expected peptide cleavage sites are denoted by arrows. Identity threshold score for $P < 0.05$: 13

no specific Asn-proteases similar to VPEs are known in yeasts. Although yeast Gpi8p shows significant structural similarity to plant VPEs, it is part of the GPI-anchor complex and acts as a transamidase [48].

In other work on yeast overexpression of plant proteins requiring a proteolytic cleavage at an Asn residue, it has been shown that recombinant precursors are not proteolytically processed at the Asn site and if cleaved, this occurs at a different amino acid [49]. For example, when the 2S albumins AL1 and AL3 from soybean (*Glycine max*) were expressed in *P. pastoris*, the subunits were post-translationally processed within the same loop region as in the plant but with some differences in the precise sites [50]. Since recombinant αAI expressed in *S. cerevisiae* YPH499 and *K. lactis* YCT390 was proteolytically processed into α- and β-like subunits, it appears likely that the proteolytic cleavage was not performed at

the Asn⁷⁷ residue but this did not affect the inhibitory activity of the protein. Aberrant processing of αAI was observed in transgenic tobacco harbouring a mutation at Asn⁷⁷–Asp⁷⁷, but the specific activity of the processed protein appears to be similar to that of correctly processed αAI [8]. Also, transgenic chickpea-αAI has a subset of molecules in which the α-subunit devoid of Asn⁷⁷ and Ala⁷⁶ retains its inhibitory activity [9].

The integration of cassettes into the chromosome of *K. lactis* regularly produces strains having multiple copies of the vector [33], and it has been reported that gene dosage can affect the yield of some heterologous proteins expressed in yeasts [33, 51, 52]. Here q-PCR data showed that all the *K. lactis* YCT390/αAI-OPT colonies contained an average of six copies of the gene, indicating that there was no correlation between the gene dosage and the differences in processing of αAI. None of

the recombinant colonies of *K. lactis* YCT390 contained mutations in the α AI-*OPT* gene sequence, and there were no differences in cell growth among the recombinant *K. lactis* YCT390/ α AI-*OPT* colonies when compared to the control strain (data not shown). As previously described for the expression of recombinant phytase in *P. pastoris* [52], it seems that the level of processed α AI produced by *K. lactis* YCT390 was not related to gene dosage, mutations or toxic effects on the host.

When we characterized the heterologous expression of recombinant α AI in *K. lactis* GG799 and in four protease deletion mutants created in GG799 background [30], we found that only recombinant colonies of *K. lactis* YCT390 secreted α AI into the culture media, while in the other strains of *K. lactis* the protein was not detected. The only difference among the *K. lactis* strains assessed in this work lies in the deletion of a single protease from the secretory pathway, since *K. lactis* YCT390 is a mutant deficient in KLYps7p, an aspartyl proteinase localised in the plasma membrane and in the yeast cell-wall [30]. Our results are consistent with the notion that in many cases heterologous proteins are degraded in *K. lactis* due to aspartyl protease, and successful expression is achieved only after using a protease deficient strain [30, 53].

The screening of several hundred transformants is an important bottleneck in yeast expression systems. In our case, the selection of colonies by means of activity or immunodetection was a more efficient method of finding clones that produced a higher level of functional protein than doing an initial genetic screen. Given that the expression profile of α AI was improved in a single *K. lactis* strain (YCT390), and the other *K. lactis* strains that did not express the protein were positive for α AI-*OPT* gene, the selection of colonies by antibody dot blot allowed us to reduce the analysis from several hundreds to <14 colonies per strain.

Here we successfully expressed functional α AI in yeasts. However, it has to be noted that while we optimised the codon usage of native bean α AI for yeast expression we did not further modify the coding sequence nor the cultivation conditions. Therefore, it is possible that the amounts of processed protein may be increased further by modifying the cell culturing and induction conditions, using different strategies to enhance proteolytic cleavage, as well as other bioengineering approaches that have been demonstrated to enhance the levels of other heterologous proteins produced by yeasts [54].

Conclusion

We demonstrated that α AI from *P. vulgaris* can be expressed in two different yeast heterologous systems. We showed that functional α AI can be secreted and

purified from culture supernatants by using a two-step chromatographic method. Therefore, our work allows for the production of recombinant α AI and provides a platform for the large-scale production of pure and functional α AI protein for biotechnological and pharmaceutical applications.

Additional files

Additional file 1: Figure S1. Schematic representations of Pinto- α AI gene, synthetic gene and its nucleotide sequence. (A) Native α AI gene from cv. Pinto comprising signal peptide (SP), α - and β - subunits and the propeptide sequences; (B) the synthetic α AI-*OPT* gene, optimised for expression in yeast, including a Kozak consensus sequence and flanked by *Hind*III and *Not*I restriction sites. (C) Nucleotide sequence of α AI-*OPT* gene.

Additional file 2: Figure S2. Antibody dot blot screening of culture supernatants of *K. lactis* and *S. cerevisiae* with α AI-*OPT*. Cleared supernatants of transformed colonies of *K. lactis* strains YCT389, YCT390, YCT569, YCT589 and GG799, and *S. cerevisiae* YPH499 transformed with α AI-*OPT* gene. (+) Pure Pinto- α AI (200ng) used as positive control (+). As negative controls, cleared culture supernatant (200 μ L) of untransformed strain YCT390 (-) and culture media YPCGal (200 μ L) without cells (-). Circled dots correspond to dots with a signal intensity equivalent to that of positive control.

Additional file 3: Figure S3. Genetic analysis of *S. cerevisiae* YPH499 clones with α AI-*OPT*. (A) Colony PCR of random colonies of *S. cerevisiae* YPH499 transformed with α AI-*OPT* (primers *OPT Fwd/OPT Rev*). Purified water used as PCR negative control (-C) and plasmid DNA from pYES2- α AI-*OPT* used as positive control (+C). DNA Ladder 100bp NEB[®](M). (B) PCR determination of α AI-*OPT* gene on plasmid DNA isolated from transformants of *S. cerevisiae* YPH499 with α AI-*OPT* (primers *OPT Fwd/OPT Rev*). (C) Schematic representation of amplification product with primers *OPT Fwd/OPT Rev*. Plasmid DNA from parent strain transformed with empty plasmid (pYES2) used as negative control. DNA Ladder 1Kb Promega[™] (M).

Additional file 4: Figure S4. Genetic analysis of *K. lactis* YCT390 transformants with α AI-*OPT*. (A) Indirect determination by PCR of α AI-*OPT* gene inserted into pKLAC2 using sequencing primers *KLSQ Fwd/KLSQ Rev* in transformants of *K. lactis* YCT3908B, 9B, 13B, 29B, 37B, 38B, 39B, 42B, 47B, 9A, 12A, 17A, 35A, 50A, 68A, 72A and 76A. (B) Schematic representation of amplification product (805bp) with primers *KLSQ Fwd/KLSQ Rev*. PCR determination of (C) α AI-*OPT* gene fragment with primers *OPT Fwd/OPT Rev*, and (D) *KLACT1* gene fragment (single copy gene in *K. lactis* genome) with primers *KLACT1 Fwd/KLACT1 Rev*, in transformants of *K. lactis* YCT39013B, 29B, 37B, 47B, 17A, 35A and 68A. Genomic DNA from untransformed strain YCT390 used as negative control. Purified water used as PCR negative control (-C) and plasmid DNA from pKLAC2- α AI-*OPT* used as PCR positive control (+C). DNA Ladder 100bp NEB[®](M).

Abbreviations

α AI: α -amylase inhibitor 1; Pinto- α AI: α AI from bean cv. Pinto; PHA-L: phytohemagglutinin-L; α MF: α mating factor; CDS: coding DNA sequence; YPCGal: yeast extract-peptone-casaminoacids-galactose medium; SC- \emptyset U: synthetic drop-out medium without uracil; THP: tris(hydroxypropyl)phosphine; DEAE: diethylaminoethyl/diethylaminoethanol; kDa: kilodalton; M_r: relative molecular mass; VPE: vacuolar plant enzyme; GPI: glycosylphosphatidylinositol.

Authors' contributions

SB conducted plasmid construction, protein expression analyses and activity assays. SB and TJH planned experiments. SB, AAL and TJH contributed to the design of the study and data analysis. SB wrote the manuscript. TJH and AAL commented and reviewed the draft. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

The authors have read and agreed to the manuscript content and approved the version submitted to this journal.

Ethics approval and consent to participate

Not applicable. The manuscript does not report data from humans or animals.

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