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Production of a Recombinant Fab in *Pichia pastoris* from a Monocistronic Expression Vector

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Recombinant Fab is usually expressed using dicistronic vectors producing the heavy and light chains separately. We developed an improved vector for Fab fragment expression in *Pichia pastoris*, which allows a stoichiometric expression of both chains based on a monocistronic arrangement. The protein is produced as a unique polypeptide harbouring a KEX2 processing site between both chains. After KEX cleavage, a correctly folded mature Fab is formed. The produced recombinant protein is characterized as a heterodimeric functional Fab. The vector described is a new tool for the proper expression of antibody fragments or any heterodimeric polypeptides.

Key words: antibody engineering, fab expression, kex, Pichia pastoris.

Abbreviations: BMGY, buffered glycerol-complex medium; CH1, first heavy chain constant domain; Ck, kappa light chain constant domain; Fab, antibody's antigen binding site; Fd, heavy chain of Fab fragment; Kex, kexin-like; ORF, open reading frame; PMSF, phenylmethanesulphonyl fluoride; rFab, recombinant Fab; scFab, single chain Fab; scFv, single chain variable fragment; L, Light chain of Fab; VL, light chain variable domain.

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Recombinant antibody technology is an expanding biotech venture due to an increasing demand on antibodies for emerging therapeutic protocols (*1*). Recombinant antibodies fragments also have its importance, since they can easily penetrate tumours and it can be used for drug targeting or imaging as well (*2*). Moreover, as they are rapidly cleared from the blood they can be used for drug detoxification (*3*) or for imaging diagnosis as immunoconjugates (*4*). The Fab molecule is a monovalent antibody fragment produced enzymatically from intact immunoglobulins. It contains a fragmented heavy chain, composed of the variable and the first constant domains (Fd), and the light chain (L), composed of the light variable and constant domains. The recombinant production of Fab has been reported based on dicistronic expression vectors for production in *Escherichia coli* (*5–7*), *Pichia pastoris* (*8–10*), *Saccharomyces cerevisiae* (*11*) and animal cells (*12*).

An alternative to dicistronic coding rFab was shown by Hust and colleagues (*13*). They expressed a scFab adding a flexible peptide linker, connecting Fd and L chain and the linker remains in the recombinant protein. We had developed a new expression vector for the production of recombinant Fab in *P. pastoris*. This vector contains both the Fd and L chain-coding sequences cloned in a monocistronic reading frame. A small connector sequence coding for a KEX2 processing site between the Fd and L chain genes provides the correct processing and assembly of a scFab molecule into a mature rFab, and no additional connector peptide sequence. Thus, the two chains are expected to be joined by disulfide bonds. The construct was cloned under the control of the alcohol oxidase (AOX1) promoter (*14*) and *S. cereviseae* α-factor secretion signals for expression and secretion in culture supernatant.

The scFab ORF was initially assembled in the pIg16 vector (*15*). The Z22 L chain was fused to a human Cκ domain sequence (codons 109–214) creating a *Xho* I site in the Jk segment (codons 104 and 105) by overlap PCR. The light chain variable domain (VL) was amplified using a pair of primers (Table 1, primer 1 and 2), and a previously isolated human Cκ cDNA clone was amplified using primers 3 and 4 (Table 1). Both PCR fragments were mixed and the fusion sequence (VLCκ, the L chain) was obtained after a second round amplification using primers 4 and 2. The final PCR product was cloned between the *Bgl* II and *Eco*R I sites, originating a scFv–Cκ fusion. To generate the scFab, the scFv linker was replaced by a human CH1 domain (codons 115–222), cloned after the *Xba* I site in the JH segment (codon 113). A sequence coding for an *Aspergillus nidulans* KEX2 cleavage site (*16*) was introduced in the 3′ terminus of the CH1 domain including a *Bgl* II site in the verge of VL (including codons 1 and 2). The CH1–KEX fusion was obtained by two consecutive PCR amplifications: first, a human CH1 cDNA insert was amplified (Table 1, primers 5 and 6) from a human spleen cDNA library, followed by a second round of PCR using primers 7 and 8 (Table 1). Primer 8 includes a KEX processing site between CH1 codon 222 and the VL codon 1. The resulting scFab coding sequence was transferred to the *P. pastoris* expression vector pPIg16 (*17*) between the *Xma* I and *Eco*R I sites. Codon numbering follows Kabat

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Table 1. **Oligonucleotides used for scFab cloning.**

Oligonucleotides	Sequence	
1	3′ VL/XhoI	CGTTTAATCTCGAGTTTT GTCCCCGAGCCGAA
2	5′VL/BglII	AACGTGCTCCAGATCTCC AGATGACGGCA
3	5′JCk	ACTCGAGATTAAACGAA CTGTG
4	3′Ck/EcoRI	GGAATTCGCACTTCTCC CTCTAACACT
5	5′CH1 γ 1	CAAGGGCCCATCGGTCTT
6	3′ CH1 γ 1	CCCCCAGGAGTTCAGGTG
7	5′CH1/XbaI	TCTAGATCCACCAAGGGC CCATCGGT
8	3′ CH1/Kex	GAGATCTGGAGCACGTTT ATCCATTCTTTTGTCAC AAGATTT

numbering scheme (*18*). The scFab expression vector, pPIgFab, contains a sequence of a 1,611 nucleotide ORF coding for the chimeric Fab of the anti-Z-DNA antibody, Z22 (Fig. 1). The immature polypeptide is predicted for processing at its central KEX cleavage signal (*19*), thus yielding two polypeptides of 231 and 214 amino acid residues (predicted molecular mass of 25,146 and 23,518 Da, respectively). Due to the preservation of both Cys residues in the carboxy-terminus of L chain and in hinge residue 230 after the CH1 domain, the resulting Fab can be produced as a cystine-linked polypeptide. It is noteworthy that a putative secondary cleavage site could be found three residues upstream of the predicted site (Fig. 1). Processing at this position would imply minor changes in protein size or heterodimer assembly. This is especially important since the specificity of yeast Kex2 is based on the dipeptide KR, rather than the more stringent specificity of filamentous fungi Kex2, which is more dependent on P3 and P4 positions (*20*). The fusion open reading frame (ORF) was cloned to be expressed under the control of the methanol inducible promoter, AOX1.

Transformation of *P. pastoris* strain SMD 1168 [Δ*pep4*::*URA*3 *his4 ura3*] (Invitrogen) was carried out as described (*21*) using *Sal*I digested plasmid. Fab expressing clones were selected (*22*) using a goat anti-human κ-chain (PIERCE) as the first antibody and the secondary was an alkaline phosphatase conjugated rabbit anti-goat IgG (PIERCE). Chosen clones were recovered from the MD master plate and inoculated in liquid media. Positive clones were grown in flasks as earlier described (*22*).

The rFab was analysed on SDS-PAGE and western blot. After induction with methanol, a single immune-reactive band around 48 kDa accumulates in the culture under non-reducing conditions (Fig. 2). Moreover, pre-treatment of samples with β-mercaptoethanol yields a double band with an apparent molecular weight of ~25 kDa, in good agreement with the predicted cystine-linked heterodimer containing both the L and heavy-chain (23.5 and 25 kDa, respectively, Fig. 2). The observed size reduction supports the presence of a disulfide bridge between the carboxy-terminal Cys 214 residue on the κ light chain and the Cys 230 residue on Fd. This bridge is naturally found in chemically produced Fab, and it stabilizes the heterodimer allowing for a robust reagent with increased resistance against chaotropic denaturation (*5*).

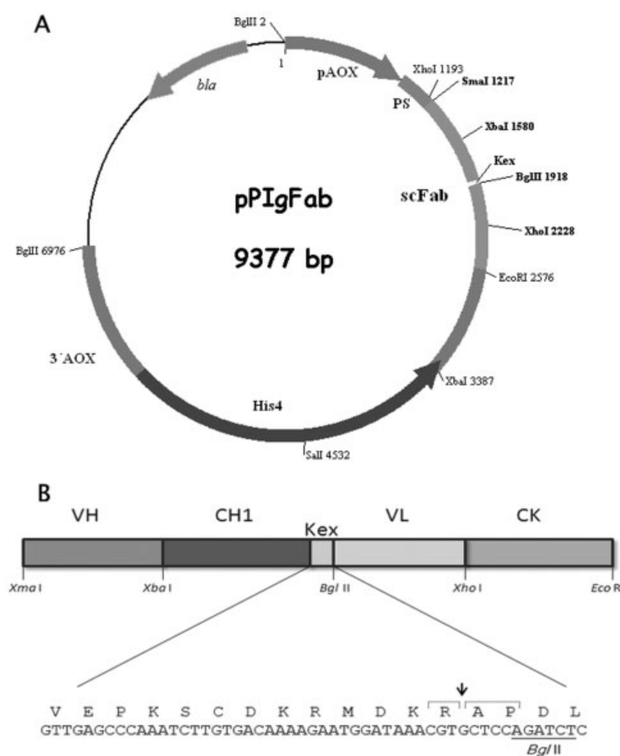

Fig. 1. **Expression vector for rFab.** (A) The plasmid pPIgFab is a second generation pPIC 9 derivative. The scFab fragment, expressed under the *AOX1* promoter, was cloned between the *Xma*I and *Eco*RI sites. The plasmid was linearized prior to transformation using the *HIS4 Sal*I site. (B) The scFab construction is shown with the restriction endonuclease sites. Fd fragment harbours a *Xma*I in its 5′terminus and a *Xba*I site between the Z22 VH and human IgG1CH1 domain. The Z22 VL is flanked by a *Bgl*II and a *Xho*I site placed between the VL and the human CK domain. The Fd and VL conecting sequenced is highlighted above and the *Bgl*II site is underlined. The predicted Kex site, including cleavage site (arrow), and carboxy and amino terminus trimming (marked with square bracket) are labelled.

The rFab was also produced in bioreactor. Fed-batch fermentations were carried out in a 2.0 l bench-top bioreactor. Cultures were grown in 100 ml of buffered glycerol-complex medium (BMGY) in a baffled flask until they reached an optical density of 2–5, and then added directly to the fermenter bottle filled with 900 ml of BMGY supplemented with 1 ml of trace salt solution, PMT4, as described (*23*). The growing conditions were: pH at 5.0–5.5, dissolved oxygen (DO) above 30% saturation and 1.2 l/min air flow rate, the cultivation temperature was kept at 30°C. Anti-foam was added when required. The 50% glycerol/0.1% PMT4 feed was kept at a rate of 15 ml/h until the culture reached an OD_{600} of 30 when the starvation started. After 1 h, the methanol feed (methanol containing 4 ml/l PMT4) was started. The initial methanol rate was 1.8 ml/h and it was gradually increased to 2.5 ml/h. The total induction time was 41 h. At every 12 h, a 5 ml sample was collected to determine the rFab production and cell density (OD_{600}). The samples' supernatants were centrifuged (2,000 *g*/10 min/4°C), and PMSF (100 μg/ml) and EDTA (0.6 mg/ml) were added.

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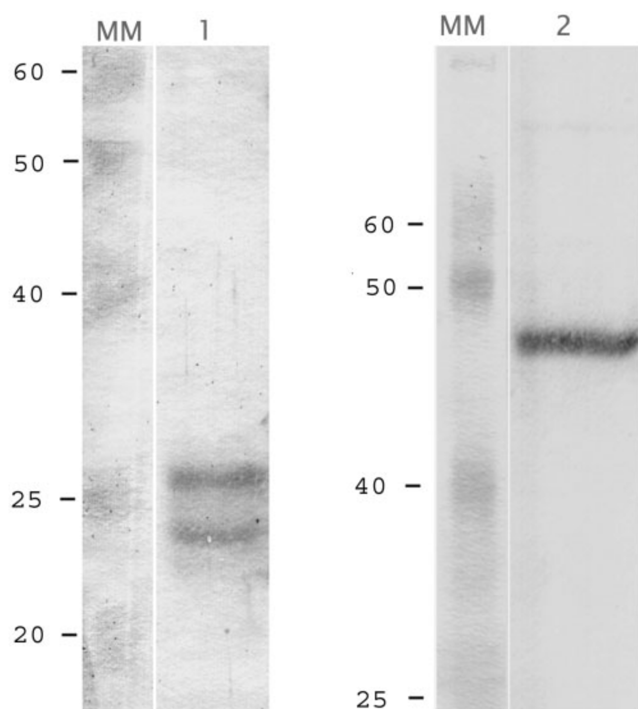

Fig. 2. **rFab is a disulfide linked heterodimer.** Concentrated rFab were analysed by SDS-PAGE followed by western blotting under reducing and non-reducing conditions (lanes 1 and 2, respectively) using a goat anti-human (H+L) IgG as a primary antibody, followed by alkaline phosphatase conjugated rabbit anti-goat IgG. These reagents allow the visualization of a double band under reducing conditions and a single one under non-reducing conditions. The molecular mass (kDa) is shown on the left.

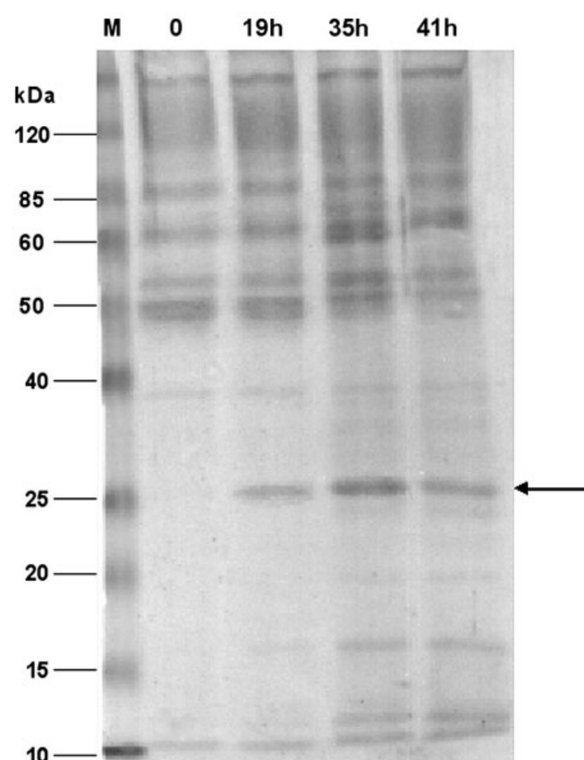

Fig. 3. **Kinetics of rFab production in biofermenter.** A producing clone was grown in a flask for 24 h and then inoculated in the bench-top biofermenter. Comassie-Blue stained SDS-PAGE containing 10 μl supernatant samples from a single culture at different induction times (0, 19, 35 and 41 h) is shown. Fd and L chain bands did not resolve and appear as a single 25 kDa overlapping band indicated by an arrow. The molecular mass standards (kDa) are shown at the right.

Table 2. **Oxygen and cell density during fermenter growth.**

Time (h)	PO_2 (%)	OD600	Methanol (ml/h)	Stirring (r.p.m)
0	115	70	0.8	1060
9	75–85	85	2.08	960
12	45–55	118	0.8	900
19	35–45	108	0.8	900
31	25–35	105	0.8	900
35	30–40	102	0.8	900
39	35–45	100	0.8	900
41	35–45	98	0.8	900

To follow the fermentation process, we periodically collected culture supernatant samples during a period of 0–41 h after methanol induction. The OD_{600} was measured for each collected sample, and it peaked at 118 after a 12 h induction. After that, the OD_{600} continuously fell to below 100 at the end of the experiment (Table 2). Each sample was analysed by SDS-PAGE under reducing and denaturing conditions and commassie blue stained gels revealed the presence of rFab in the culture supernatant (Fig. 3). The induced band was confirmed to be the rFab by western blot analysis (data not shown) revealing that both bands (Fd and L chain) co-migrates as a single unresolved band. We observed an increasing accumulation of the rFab until 35 h. At the end of process, the whole supernatant was recovered and concentrated. By comparing SDS-PAGE gels including bovine serum albumin (BSA) as protein mass standard, the estimated accumulation of rFab in the culture supernatant was 250 mg/l. The rFab production obtained in this study is comparable to others (*9*, *13*, *24*) using the conventional dicistronic expression constructs in larger scale fermenter.

The Z22 mAb antibody is a model anti-DNA antibody that recognizes polydeoxynucleotides in the Z-conformation. It had been used to detect Z-DNA *in vitro* and *in vivo* and its immunochemical proprieties is well characterized (*25*). Recombinant forms of Z22, such as the aforementioned FvFc molecule, were previously described to possess immunochemical properties comparable to the original Z22 mAb (*15*). This premise indicates that rFab should also preserve the Z22 immunochemical properties. After verifying that rFab binds to Z-DNA coated plates (data not shown), we assayed the recombinat protein specificity by inhibition assays as described (*8*). Briefly, sample/competitor were pre-mixed for 1 h and then transferred to a previously Z-DNA adsorbed microtitre plate. Z-DNA is a biotinylated derivative of poly (dG·dC) (GE Healthcare) that adopts the Z conformation

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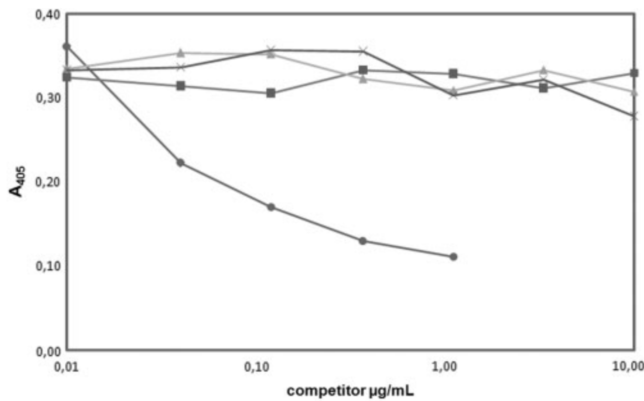

Fig. 4. **rFab binding affinity and specificity.** Concentrated rFab were assayed for its capacity to bind antigen, DNA in the Z-conformation. rFab samples were pre-mixed with the indicated concentration of competitor for 2 h at room temperature and then distributed in Z-DNA coated plates. Free rFab were detected as above. Competitors were: Z-DNA (filled circle), B-DNA (filled triangle), ssDNA (filled square) and poly-(dG-dC) (cross symbol). Only Z-DNA competes significantly for binding.

at physiological conditions. The dsDNA is calf thymus DNA (Sigma) and ssDNA is heat denatured calf thymus DNA. Each sample was tested in duplicate and experiments were repeated at least twice. Bound antibodies were detected as described for *Pichia pastoris* producing clones. As expected, only the antigen that forms Z-DNA is able to inhibit the binding of rFab to the adsorbed Z-DNA (Fig. 4). Therefore, the processed and secreted rFab preserved the binding specificity of the original mAb, suggesting the correct formation of the Fd–L chain interface and assembly of a functional Z22 paratope.

In conclusion, we proposed a single promoter, single polypeptide expression cassette harbouring a KEX processing site between the heavy- and L-chain coding sequences for production of recombinant Fab. The rational of introducing a KEX2 processing sequence was derived from the work of Contreras and co-workers (*16*) that used this same sequence to successfully produce equimolar amounts of glucoamylase and human Interleukin-6 in the fungus *A. nidulans*. Thus, we applied a similar approach in the yeast *P. pastoris*. Our point is that the continuous entering of scFab into the endoplasmic reticulum and its processing into mature Fd and L chain avoids the accumulation of unpaired immunoglobulin chains that would trigger a detrimental response reducing their ability to hyper-express heterologous genes (*26*). Using this vector, we were able to produce a mature disulfide-linked Fab molecule of 48 kDa. Additionally, the purified polypeptide preserves its immunoreactive properties as assayed by ELISA. The vector reported here represents a new and flexible strategy for producing high yields of secretable recombinant Fab in *P. pastoris* using a monocistronic expression cassette. This system is a novel alternative for the production of antibody fragments in yeast that can also be used for expressing other heterodimeric proteins.

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