

High-level secretory expression, purification, and characterization of an anti-human Her II monoclonal antibody, trastuzumab, in the methylophilic yeast *Pichia pastoris*

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ABSTRACT

DNA fragments encoding the light chain and heavy chain genes of an anti-human HER II antibody, trastuzumab, fused with an egg-lysozyme signal peptide were synthesized based on the codon bias of the methylophilic yeast *Pichia pastoris*. These fragments were inserted into a site between the AOX 1-promoter and -terminator in pPICZ A to be expressed by *P. pastoris*. The expression vector was linearized, and introduced into *P. pastoris* GS115 by electroporation. After the checking of several transformants with PCR to ensure a precise insertion, one was selected and cultured to examine antibody production. The level of production reached 10 mg/L in a flask with medium containing 1% methanol. The heavy chain and light chain of the product were assembled to form a hetero tetramer, as detected by dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). N-terminal amino acid sequencing revealed that the signal peptides of both chains were well processed. The mobility of the product in SDS-PAGE after treatment with Peptide N-Glycosidase F indicated the heavy chain to be N-glycosylated. Further analysis of the N-glycans with a mass spectrometer revealed a mixture of Man₉-GlcNAc₂, Man₁₀-GlcNAc₂, Man₁₁-GlcNAc₂ and Man₁₂-GlcNAc₂, but no hyper-mannosylated glycans. ELISA, surface plasmon resonance, and flow cytometric studies showed the affinity curve and K_d value for the antigen, HER II, and reactivity to a HER2-overexpressing breast cancer cell-line, SK-BR-3, to be almost the same as for the clinically used trastuzumab produced by CHO.

Keywords: *Pichia Pastoris*; Antibody; Secretory Production; Glycosylation; Her II

1. INTRODUCTION

Recombinant monoclonal antibodies (mAbs) are useful for biological research, diagnostics and therapeutic purposes. In most cases, the host cells for production are mammalian cell lines, such as Chinese ovary cells [1] and NS0 [2]. All approved monoclonal antibodies at present are produced by mammalian cell lines [3]. Since mammalian cell cultures are far more complex and expensive than microbial cell cultures, expression systems with microorganisms have gained importance. However, bacterial systems, such as *Escherichia coli*, are often not able to modify the product as mammalian cells do.

With their capacity for post-translational modifications and potential to produce large quantities of rather complicated heterologous proteins, yeast production systems are seen as promising substitutes for mammalian cell systems [4,5]. Among several yeast systems, one using *Pichia pastoris* is increasingly being applied to both research and the production of proteins for diagnostic and therapeutic purposes. Use of its AOX 1-promoter [6], which controls the expression of the enzyme alcohol oxidase, led to the development of methanol-inducible expression systems. The hyperglycosylation that tends to occur with *Saccharomyces cerevisiae* is much less a problem with *P. pastoris* [7], and the expressed proteins can either be kept inside the cells or be secreted into the culture medium. For secreted proteins, the purification process is expected to be simpler than that inside cells,

since a reasonably high purity could be achieved in the supernatants. The amount of protein produced is usually in the range of 30% - 80% of all the secreted proteins [8,9]. Since the ability to attain very high cell densities (150 - 400 g wet cell/L can be reached in defined media [9,10]) by fermentative growth using bioreactors allows for high volumetric productivity, *P. pastoris* has become a robust system for the production of recombinant proteins.

Trastuzumab, an antibody directed against the human Her II protein produced by CHO, has been clinically used for the treatment and diagnosis of breast cancer [11]. However, the production costs are high, and alternative systems have been desired.

In this paper, we describe the construction of a *P. pastoris* strain capable of the extra-cellular expression of trastuzumab, its production in a flask culture, and results on the comparative characterization of the secretory product with a commercially available trastuzumab produced by CHO.

2. MATERIALS AND METHODS

2.1. Antibodies, Bacterial and Yeast Strains, and a Plasmid

Herceptin (trastuzumab produced by CHO) and Retuxin (rituximab produced by CHO) were purchased from Roche (USA).

Other antibodies used in immunoassays were from R&D Sciences (USA) and Life Span Biosciences (USA).

JM 109 competent cells used for plasmid construction were from Toyobo Biochemicals (Japan).

Pichia pastoris GS115 and pPICZ A for expression of the target genes were from Life Technologies (USA).

2.2. Genes for Trastuzumab Heavy (H) and Light (L) Chains

Genes for trastuzumab heavy (H) chain and light (L) chain amino acid sequences [11] as listed below, where codons were optimized for expression in *P. pastoris*, were designed, and synthesized by GenScript (USA).

Trastuzumab H chain 451 amino acids;

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYI
HWVRQAPGKGLEWVARIYPTNGYTRYADSVKGR
FTISADTSKNTAYLQMNLSRAEDTAVYYCSRWGG
DGFYAMDYWGQGLTVVSSASTKGPSVFPLAPSS
KSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV
NHKPSNTKVDKKEPPKSCDKTHTCPPCPAPPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPVYTLPPSRDELTKNQVSLTCLVKGFYP
SDIAVEWESNGQPENNYKTTTPVLDSGSSFFLYSK

LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK

Trastuzumab L chain 214 amino acids;

DIQMTQSPSSLSASVGRVTITCRASQDVNTAV
AWYQQKPGKAPKLLIYSASFLYSGVPSRFSGRSG
TDFLTISLQPEDFATYYCQQHYTTPPTFGQGTKV
EIKRTVAAPSVEFIPPSDEQLKSGTASVCLLNIFY
PREAKVQWKVDNALQSGNSQESVTEQDSKSTYS
LSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSF-
NRGEC Both sequences were N-terminally fused to a secretion signal peptide sequence of egg white lysozyme for secretion by *P. pastoris*.

2.3. Construction of Plasmids

The H and L chain genes synthesized were inserted between the AOX 1 promoter and AOX 1 transcriptional terminator in pPICZ A to construct an H chain and an L chain expression vector, respectively. The DNA fragment that contained the AOX 1 promoter, L chain gene and AOX 1 transcription terminator, the L chain expression cassette, was cut out from the L chain expression vector with *Bgl* II and *Bam*H I double digestion. The L chain expression cassette was then inserted into the *Bam*H I site in the H chain expression vector to create an expression vector for trastuzumab. In order to improve the insertion of the whole H chain and L chain expression cassette into the host genome, a DNA fragment (approximately 450 bp) covering a 5'-upstream region of the AOX 1 promoter and N-terminal region of the Polyamine oxidase gene was amplified from the host genome by PCR using

5'CCTTTCGTCTTTGGATGTTAGATC3' and

5'CTCACGTTAAGGGATTTTGGTCATGAGATCAgt
agtttgaagtactataaacaattccg3' (where the upper-case letters are the pPICZ A vector sequence and the lower-case letters are the polyamine oxidase gene sequence 349-381 bp from its start codon) as primers, and sub-cloned into the *Bgl* II site, *i.e.* at the 5'-end of the AOX 1 promoter of the H chain expression cassette, in the antibody expression plasmid (see **Figure 1** for details on the plasmids) using a PCR fragment cloning kit (In-Fusion HD EcoDry Cloning Kit, Takara, Japan).

2.4. Transformation of *P. pastoris*

The trastuzumab expression vector was linearized by *Bgl* II digestion. *P. pastoris* cells were transformed by electroporation according to the manual provided by Life Technologies. Transformants that appeared on YEPG (1% yeast extract, 2% peptone, 1 M sorbitol, 2% agar and 2% glucose) plates containing 100 mg/L Zeocin (Life Technologies) were checked for integrated DNA fragments by PCR using as primers,

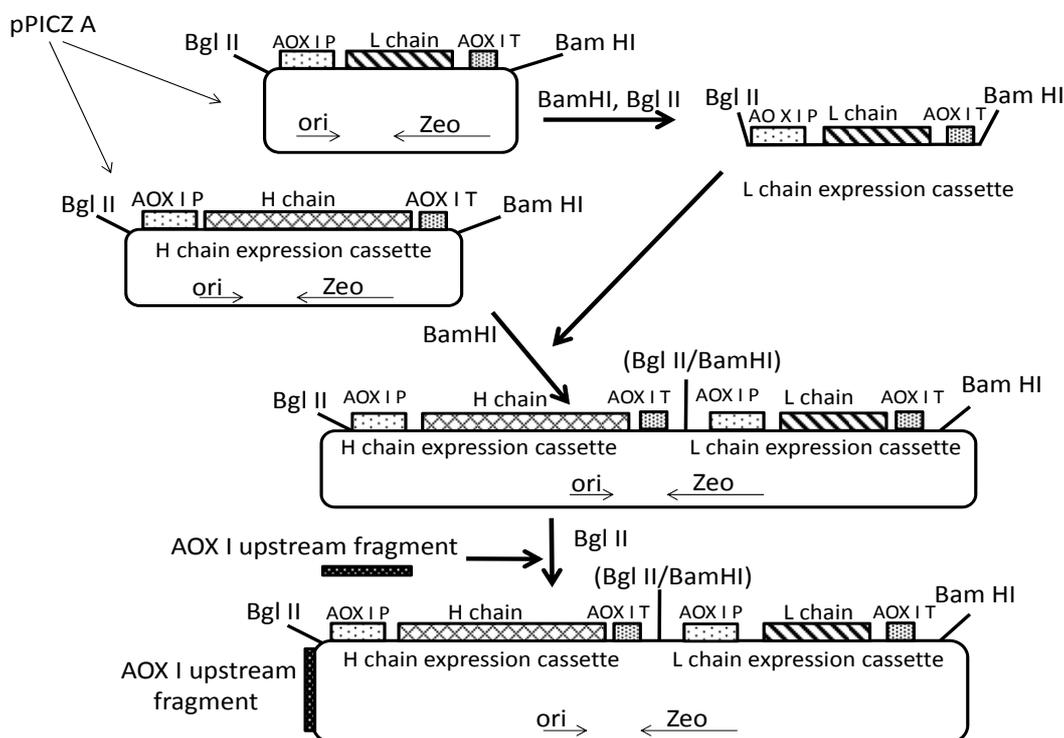


Figure 1. Construction of the expression plasmid for trastuzumab: AOX I P: the Alcohol oxidase 1 promoter region. AOX I T: the Alcohol oxidase 1 transcriptional terminator region. Zeo: Zeocin resistance gene. Ori: plasmid replication origin. AOX I upstream fragment: a DNA fragment amplified by PCR that covers a region upstream of the AOX 1 promoter and a 5' portion of the Polyamine oxidase gene.

5'GACTGGTTCCAATTGACAAGC3' and 5'GCAAATGGCATTCTGACATCC3', located in the AOX 1 promoter and AOX 1 transcriptional terminator, respectively.

2.5. Production of Trastuzumab

P. pastoris containing the trastuzumab expression vector was cultured from in 10 mL of BMGY medium [1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate (pH 6.0), 4×10^{-5} % biotin, and 1% glycerol] overnight at 30°C with shaking at 275 rpm. A 100-mL volume of BMGY medium was inoculated with 1 ml of the starting culture. The second culture was incubated for approximately 12 h at 30°C with shaking at 275 rpm. Cells were harvested at an OD_{600nm} of between 1 and 4 and pelleted by centrifugation for 10 min at 5000 rpm at 4°C. Induction of protein expression was initiated by resuspending the yeast cells in 100 mL of BMMY medium [1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate (pH 6.0), 4×10^{-5} % biotin, and 1% methanol]. Incubation was continued at 30°C with shaking at 300 rpm for 24 h. A supplemental volume of methanol equal to 1/100 of the culture volume was added at 16 h. Following induction for 24 h, the culture was chilled on ice, and the cells and culture medium were separated by centrifuga-

tion for 10 min at 5000 rpm at 4°C. The culture medium was stored at 4°C.

2.6. Purification of Trastuzumab from Culture Media

Trastuzumab was recovered from the culture medium with an AKTA explorer 100A (GE Healthcare, NJ) using Streamline rProtein A (GE Healthcare, NJ). The rProtein A resin was equilibrated, and washed with 20 mM Tris-HCl pH 7.0. The culture supernatant was loaded at a maximum capacity of 32 mg/mL resin at a residence time in excess of 6 min. Trastuzumab was eluted using a pH gradient from 4.0 to 3.0 in 100 mM sodium citrate. Eluate fractions were adjusted to pH 5.0 with 1 M Na_2HPO_4 , before being diluted five-fold with H_2O . The pH was then re-adjusted to 4.5 with 1 M acetic acid before being loaded on a Source 30S (GE Healthcare, NJ) column equilibrated with 25 mM sodium acetate, pH 4.5. Trastuzumab was eluted using the following stepwise elution protocol: 2 column volumes of Buffer A (12.5 mM sodium acetate, 12.5 mM sodium phosphate, pH 6.0), 2 column volumes of 4% Buffer B (12.5 mM sodium acetate, 12.5 mM sodium phosphate, 0.5 M sodium chloride, pH 6.0), 3 column volumes of 6% Buffer B, 15 column volumes of 8% Buffer B, 3 column volumes of 12% Buffer B, and 2 column volumes of 100% Buffer B.

2.7. N-glycosidase Treatment

Peptide- N^4 -(*N*-acetyl- β -glucosaminyl)-asparagine amidase was purchased from Takara (Japan). The samples were treated with the enzyme according to the manufacturer's directions.

2.8. Elisa

Assays were conducted essentially as described [12]. Aliquots (100 μ l) of antigen solution (3 mg/mL in PBS) were added to wells of an ELISA plate (COSTAR 9018), and incubated at 4°C overnight. The plate was washed 3 times with PBS. The wells were blocked with 1% BSA in PBS at 37°C for 2 hrs. The plate was washed 3 times with 0.05% Tween 20 in PBS. Samples (100 μ L) were put into the wells, and incubated for 1 hr at room temperature. The plate was again washed 3 times with 0.05% Tween 20 in PBS. Next, 100 μ L of 10^{-4} diluted anti-human L kappa goat antibody conjugated with HRP (Life Span Biosciences, USA) was put into the wells and incubated for 1 hr at room temperature. The plate was again washed 3 times with 0.05% Tween 20 in PBS. Detection of bound antibodies was done by measuring absorbance at 450 nm using 1,2-Phenylenediamine in Citrate buffer containing H_2O_2 [13].

2.9. Surface Plasmon Resonance

CHO-derived and Pichia-derived trastuzumabs' affinity to Her II was measured with Biacore 3000 (GE Healthcare, NJ, USA). The assay was done in HBST (20 mM HEPES, pH 7.4, 150 mM NaCl and 0.05% Tween 20). Her II-Fc chimera (R&D Systems) was immobilized on a CM5 chip (GE Healthcare, NJ). The antibody was injected at the concentration between 1.56 nM and 50 nM. The off rate was calculated by monitoring the assay for at least 2 min. The data obtained were analyzed with global fitting using BIA evaluation soft (GE Healthcare).

3. RESULTS AND DISCUSSION

3.1. Construction of Expression Plasmids for Trastuzumab

A plasmid containing the antibody heavy chain and light chain secretory expression units under the control of the AOX 1 promoter was constructed (Figure 1). To increase the efficiency of integration of the vector into the host AOX 1 promoter region by homologous recombination, digestion at a unique site in the AOX 1 promoter region to linearize the original vector is recommended by the manufacturer. However, since the antibody expression plasmid contained two AOX 1 promoters, linearization of the expression vector with a restriction enzyme that cuts inside the AOX 1 promoter would separate the H chain and the light chain expression cassettes. A DNA fragment of the upstream region of the AOX 1 gene

promoter and a part of the flanking gene, Polyamine oxidase, was amplified from the genome by PCR, and then put into the *Bgl* II site, *i.e.* at the 5'-end of the AOX 1 promoter of the H chain expression cassette. Linearization of the plasmid with *Bgl* II digestion would be expected to facilitate integration of the entire antibody expression construct through homologous recombination between the host genome and the plasmid in the region surrounding the *Bgl* II site.

3.2. Isolation of Transformants, and Checking of the Antibody Expression Construct

Using the transformation conditions recommended by the manufacturer, only three colonies appeared on plates containing Zeocin (100 mg/mL), despite that the original vector, pPICZ A, could transform hundreds of colonies under the same conditions. It is thought that the expression of the antibody might be harmful to the cells. Genomic DNA of these three colonies was isolated, and checked for the insertion of the expression construct by PCR. Two of them showed integration of the construct. One was selected for further cultivation and production.

3.3. Secretion of Trastuzumab, and Its Characterization

After cultivation and induction of the transformant selected, the culture medium was separated from the cells by centrifugation. The production of the antibody in the culture medium was checked by western blotting using anti-human IgG Fc and anti-human κ (Figure 2 (a)). The heavy chain produced by *P. pastoris* was detected as two bands. The main was at a slightly higher molecular weight position than the one produced by CHO, but the weaker band was seen at the same position. The light chain was detected as one band almost at the same position as that produced by CHO. The antibody in the culture medium was then affinity-purified with a Protein A column, and the product was analyzed by SDS-PAGE under reduced and non-reduced conditions. Under non-reduced conditions, the molecular weight of the antibody was approximately 250 kDa, and almost the same as that produced by CHO (Figure 2 (b)). The antibody is considered to be correctly assembled to form a heterotetramer. The light chain and heavy chain bands were separated by SDS-PAGE, and transferred to a membrane filter to analyze their N-terminal amino acid sequences with a sequencer. The N-terminal sequences of the heavy chain and light chain were revealed to be EVQLV- and DIQMT-, respectively. These results were consistent with the amino acid sequences predicted from the DNA. The chicken lysozyme signal peptide of both chains was precisely processed in *P. pastoris*.

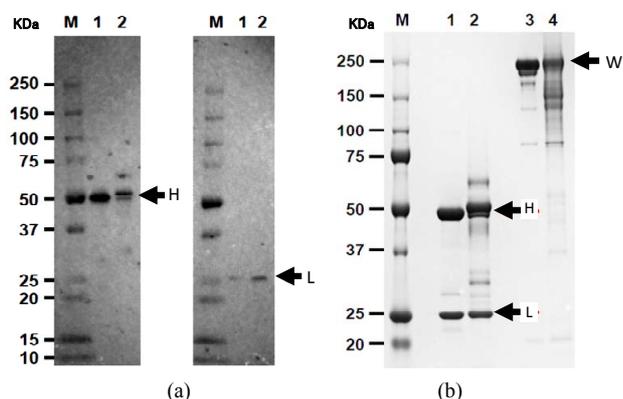


Figure 2. Western blotting and SDS-PAGE of trastuzumab secreted in the *Pichia pastoris* culture medium. (a) Left panel; western blotting with anti-human IgG Fc. H indicates the antibody's heavy chain. Right panel; western blotting with anti-human light chain kappa. L indicates the antibody's light chain. Lane M; molecular weight markers. Lane 1; trastuzumab produced by CHO. Lane 2; trastuzumab produced by *P. pastoris*; (b) Lanes 1 and 2; A Coomassie blue-stained SDS-PAGE gel under reduced conditions. Lanes 3 and 4; A Coomassie blue-stained SDS-PAGE gel under non-reduced conditions. H, L and W indicate the H chain, L chain and hetero-tetramer, respectively. Lane M; molecular weight markers. Lanes 1 and 3; trastuzumab produced by CHO. Lanes 2 and 4; rProtein A-captured trastuzumab produced by *Pichia pastoris*.

3.4. Analysis of Sugar Chains

The antibody was further purified with an ion exchange column, Resource S (GE Healthcare Japan), and treated with Peptide- N^4 -(N -acetyl- β -glucosaminyl)-asparagine amidase. The samples were analyzed by SDS-PAGE (**Figure 3**). After treatment with the enzyme, the heavy chain showed a decrease in molecular weight and the same mobility as the one produced by CHO (**Figure 3**). From this result, the heavy chain produced by *P. pastoris* has major sugar chains linked to asparagine as is the case with that produced by CHO. The sugar chains removed from the heavy chain produced by *P. pastoris* were further analyzed with MALDI-TOF mass spectrometry. **Figure 4** shows the mass spectra. From the peaks in the spectra, the sugar chain structures were estimated to be $\text{Man}_9\text{-GlcNAc}_2$ (Mw: 1962), $\text{Man}_{10}\text{-GlcNAc}_2$ (Mw: 2124), $\text{Man}_{11}\text{-GlcNAc}_2$ (Mw: 2286) and $\text{Man}_{12}\text{-GlcNAc}_2$ (Mw: 2448). They were all high-mannose type sugars, however, there were no hypermannosylated or fucosylated sugar chains detected. Hypermannosylated sugar chains are typical of yeast proteins, and thought to be immunogenic for human [14-16]. However, high-mannose type sugar chains are frequently observed in human sugar chains. Fucosylation of the sugar chains of antibodies, usually observed in the CHO product, was reported to have an adverse effect on effector functions [17,18]. The antibody produced by *P. pastoris* could be expected to have

higher ADCC (antibody-dependent cell mediated cytotoxicity) than the one produced by CHO.

3.5. Affinity for Human Her II

Binding to Her II was compared between the antibody produced by *P. pastoris* and that produced by CHO using ELISA and Surface Plasmon Resonance. The affinity curves and constants were almost the same (**Figure 5** and

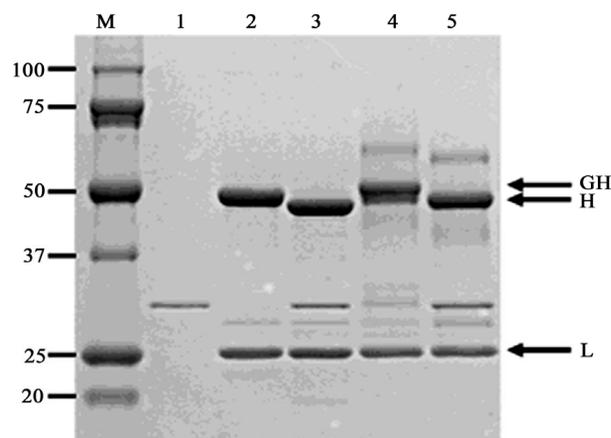


Figure 3. Trastuzumab treated with Peptide- N^4 -(N -acetyl- β -glucosaminyl)-asparagine amidase. Samples were analyzed by SDS-PAGE under reduced conditions. Lane M; molecular weight markers (kDa). Lane 1; Peptide- N^4 -(N -acetyl- β -glucosaminyl)-asparagine amidase. Lanes 2 and 3; trastuzumab produced by CHO. Lanes 4 and 5; trastuzumab produced by *P. pastoris*. Lanes 2 and 4; trastuzumab without Peptide- N^4 -(N -acetyl- β -glucosaminyl)-asparagine amidase treatment. Lanes 3 and 5; trastuzumab with Peptide- N^4 -(N -acetyl- β -glucosaminyl)-asparagine amidase treatment. L indicates the L chain. H indicates the H chain without N-linked glycans and GH indicates the H chain with N-linked glycans. The gel was stained with Coomassie blue.

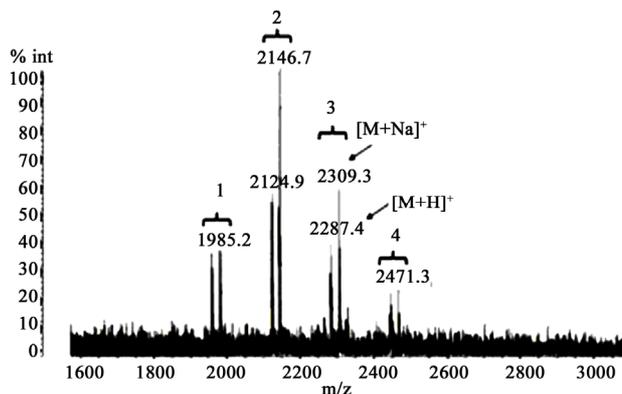


Figure 4. MALDI-TOF mass spectra of N-linked glycans attached to trastuzumab produced by *P. pastoris*. Each N-glycan has two peaks, one with an H ion and another with an Na ion. Structures deduced from peaks 1, 2, 3 and 4 are $\text{Man}_9\text{GlcNAc}_2$ (Mw: 1962), $\text{Man}_{10}\text{GlcNAc}_2$ (Mw: 2124), $\text{Man}_{11}\text{GlcNAc}_2$ (Mw: 2286) and $\text{Man}_{12}\text{GlcNAc}_2$ (Mw: 2448), respectively.

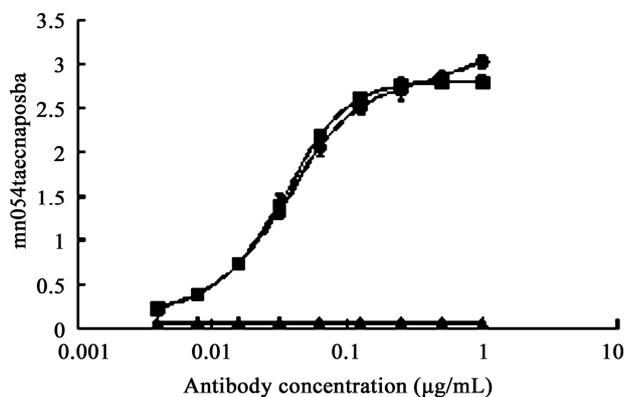


Figure 5. Comparison of reactivity to the antigen with the CHO product. Assays were carried out with ELISA as described in materials and methods. ▲: Rituximab (an antibody to CD20, a negative control). ●: trastuzumab produced by CHO. ■: trastuzumab produced by *P. pastoris*.

Table 1). Reactivity to the breast cancer cell line SK-R-3 overexpressing Her II was also compared using flow cytometry (**Figure 6**). The reaction pattern was very similar, whereas rituximab did not show any bindings to the cell line.

4. CONCLUSIONS

An N-terminal processed and folded trastuzumab hetero-

Table 1. Kinetic parameters of trastuzumab produced by CHO and *Pichia pastoris*.

Trastuzumab by	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_A (M^{-1})	K_D (nM)
CHO	4.25×10^5	5.21×10^{-5}	8.17×10^9	0.12
<i>P. pastoris</i>	4.26×10^5	8.72×10^{-5}	5.33×10^9	0.19

k_a and k_d values were calculated by SPR analyses (see the text); K_A and K_D values were based on k_a and k_d .

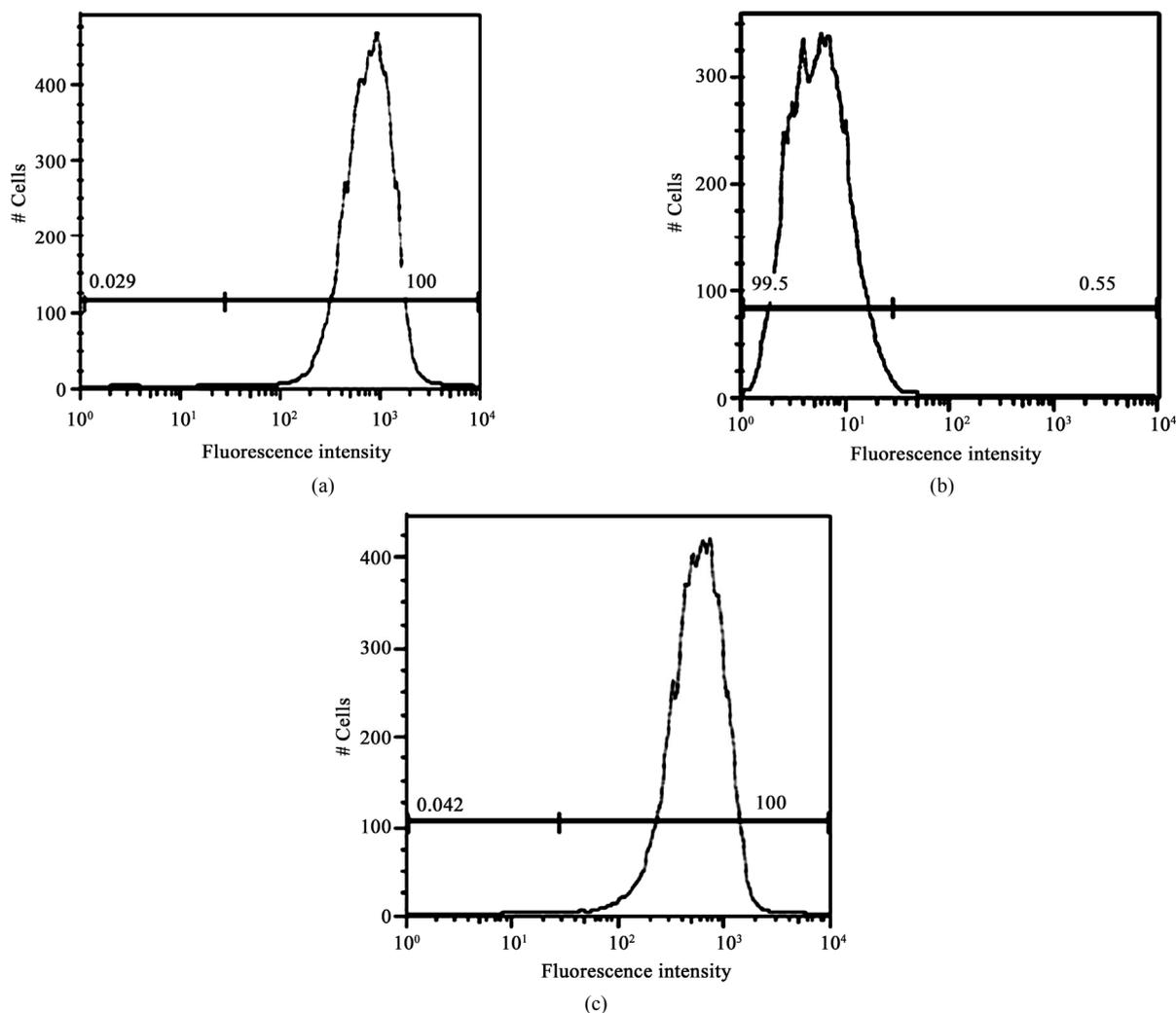


Figure 6. Reactivity to the Her II-overexpressing breast cancer cell line SK-BR-3 analyzed by flow cytometry. (a) trastuzumab produced by CHO. (b) trastuzumab produced by *P. pastoris*. (c) rituximab. Reactivity to the cells was detected with a flow cytometer using an Alexa 488-conjugated anti-human IgG Fc antibody as a secondary antibody. Numbers in the figures indicate the percentage of cells in peaks.

tetramer was highly expressed at 10 mg/L in flask cultures by *Pichia pastoris* transformed with an expression plasmid. The trastuzumab produced by *P. pastoris* showed similar HER2-binding activity to that produced by CHO, and had N-linked sugar chains. Glycan structures predicted from MALDI/TOF mass spectra were Man₉GlcNAc₂, Man₁₀GlcNAc₂, Man₁₁GlcNAc₂ and Man₁₂GlcNAc₂, but no fucosylated or hyper-mannosylated glycans were observed. Reactivity to the antigen, Her II, was almost the same for that produced by CHO. Antibody production using *Pichia pastoris* could be an effective substitute for the expensive systems using mammalian cells.

REFERENCES

- [1] Kito, M., *et al.* (2002) Construction of engineered CHO strains for high-level production of re-combinant proteins. *Applied Microbiological Biotechnology*, **60**, 442-448. [doi:10.1007/s00253-002-1134-1](https://doi.org/10.1007/s00253-002-1134-1)
- [2] Barnes, L.M., Bentley, C.M. and Dickson, A.J. (2000) Advances in animal cell recombinant protein production: GS-NS0 expression system. *Cytotechnology*, **32**, 109-123. [doi:10.1023/A:1008170710003](https://doi.org/10.1023/A:1008170710003)
- [3] Farid, S.S. (2006) Established bioprocesses for producing antibodies as a basis for future planning. *Advances in Biochemical Engineering/Biotechnology*, **101**, 1-42. [doi:10.1007/10_014](https://doi.org/10.1007/10_014)
- [4] Cregg, J.M., *et al.* (2000) Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology*, **16**, 23-52. [doi:10.1385/MB:16:1:23](https://doi.org/10.1385/MB:16:1:23)
- [5] Romanos, M.A., Scorer, C.A. and Clare, J.J. (1992) Foreign gene expression in yeast: A review. *Yeast*, **8**, 423-488. [doi:10.1002/yea.320080602](https://doi.org/10.1002/yea.320080602)
- [6] Cregg, J.M., *et al.* (1985) *Pichia pastoris* as a host system for transformations. *Molecular Cell Biology*, **5**, 3376-3385.
- [7] Grinna, L.S. and Tschopp, J.F. (1989) Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. *Yeast*, **5**, 107-115. [doi:10.1002/yea.320050206](https://doi.org/10.1002/yea.320050206)
- [8] Goodrick, J.C., *et al.* (2001) High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. *Biotechnology and Bioengineering*, **74**, 492-497. [doi:10.1002/bit.1140](https://doi.org/10.1002/bit.1140)
- [9] Schilling, B.M., Goodrick, J.C. and Wan, N.C. (2001) Scale-up of a high cell density continuous culture with *Pichia pastoris* X-33 for the constitutive expression of rh-chitinase. *Biotechnology Progress*, **17**, 629-633. [doi:10.1021/bp010041e](https://doi.org/10.1021/bp010041e)
- [10] Hellwig, S., *et al.* (2001) Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. *Biotechnology and Bioengineering*, **74**, 344-352. [doi:10.1002/bit.1125](https://doi.org/10.1002/bit.1125)
- [11] Carter, P., *et al.* (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proceedings of National Academy Science of the USA*, **89**, 4285-4289. [doi:10.1073/pnas.89.10.4285](https://doi.org/10.1073/pnas.89.10.4285)
- [12] Shibui, T. and Nagahari, K. (1992) Secretion of a functional Fab fragment in *Escherichia coli* and the influence of culture conditions. *Applied Microbiological Biotechnology*, **37**, 352-357. [doi:10.1007/BF00210991](https://doi.org/10.1007/BF00210991)
- [13] Cabilly, S. (1989) Growth at sub-optimal temperatures allows the production of functional, anti-gen-binding Fab fragments in *Escherichia coli*. *Gene*, **85**, 553-557. [doi:10.1016/0378-1119\(89\)90451-4](https://doi.org/10.1016/0378-1119(89)90451-4)
- [14] Dean, N. (1999) Asparagine-linked glycosylation in the yeast Golgi. *Biochimica et Biophysica Acta*, **1426**, 309-322. [doi:10.1016/S0304-4165\(98\)00132-9](https://doi.org/10.1016/S0304-4165(98)00132-9)
- [15] Ballou, C.E. (1990) Isolation, characterization, and properties of *Saccharomyces cerevisiae* mnn mutants with non-conditional protein glycosylation defects. *Methods Enzymology*, **185**, 440-470. [doi:10.1016/0076-6879\(90\)85038-P](https://doi.org/10.1016/0076-6879(90)85038-P)
- [16] Yip, C.L., *et al.* (1994) Cloning and analysis of the *Saccharomyces cerevisiae* MNN9 and MNN1 genes required for complex glycosylation of secreted proteins. *Proceedings of National Academy Science of the USA*, **91**, 2723-2727. [doi:10.1073/pnas.91.7.2723](https://doi.org/10.1073/pnas.91.7.2723)
- [17] Niwa, R., *et al.* (2004) Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. *Cancer Research*, **64**, 2127-2133. [doi:10.1158/0008-5472.CAN-03-2068](https://doi.org/10.1158/0008-5472.CAN-03-2068)
- [18] Yamane-Ohnuki, N., *et al.* (2004) Establishment of FUT8 knockout Chinese hamster ovary cells: An ideal host cell line for producing completely defucosylated antibodies with enhanced anti-body-dependent cellular cytotoxicity. *Biotechnology and Bioengineering*, **87**, 614-622. [doi:10.1002/bit.20151](https://doi.org/10.1002/bit.20151)