Co-expression of alpha-1 antitrypsin with cytoplasmic domain of v-SNARE in *Pichia pastoris*: preserving biological activity of alpha-1 antitrypsin

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Running title: Co-expression of A1AT with v-SNARE in *P. pastoris*

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Abstract:

Alpha-1-antitrypsin (A1AT) is a major serum protein in human with protease inhibitory activity. Because of its extensive application in medicine, recombinant DNA technology has been considered for its production. The current study is going to examine co-expression of

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A1AT, and soluble domain of v-SNARE in *Pichia pastoris*, which can prevent the secretion of A1AT after passing the secretory pathway thoroughly. This was done mainly to preserve the biological activity of A1AT which in the secretory mode might be impaired in the fermentation and early clarification conditions. SNARE proteins are the driving force for vesicle docking and membrane fusion in the term defined as exocytosis. Intracellular expression of the cytoplasmic domain of v-SNARE and its subsequent interaction to form SNARE complex can intensify the competition for A1AT secretory vesicles to be fused and released to the media. Our investigation shows successful co-expression of A1AT in the form of post-Golgi vesicles (PGVs), and the cytoplasmic domain of v-SNARE. Our findings confirmed the reduction of A1AT secretion by 45% which trapped in post-Golgi A1AT secretory vesicles inside the cell were biologically more active than secreted A1AT protein. These results indicate that the inhibition of A1AT secretion can protect its biological activity in fermentation and clarification processes.

Keywords: A1AT, Intracellular, Secretion, v-SNARE, Co-expression

Introduction:

Human alpha-1-antitrypsin (A1AT) is a major serum protein which is produced and secreted from liver cells. A1AT is a protease inhibitor whose primary physiological role is to inhibit neutrophil elastase in the lungs. The genetic deficiency of A1AT, including Z-allele, which is considered the most common cause of the deficiency, leads to lung diseases such as emphysema in adults and liver diseases in children [1,2]. So far, the A1AT for augmentation therapy has been obtained from human blood, which is source-limited and may contain infectious agents. Thus, to meet the large clinical demand for this pharmaceutical protein, recombinant production of A1AT in hosts like *E. coli*, various yeasts, CHO cells, and transgenic plants has been considered as a potential alternative method [1-3].

A1AT is a glycoprotein of 394 amino acids with molecular weight of 54 kDa and has three asparagine-linked carbohydrate side chains at positions 46, 83, and 247 [4,5]. Carbohydrates represent 12% of A1AT by molecular weight, and even though they are not essential for biological activity, they enhance *in vivo* lifetime, as well as *in vitro* stability of the protein [3,5,6]. Thus, for glycosylation and other post-translational modifications (PTM) of A1AT, eukaryotic systems are preferred to prokaryotic systems like *E. coli* [1,4,6]. Although in higher eukaryotes like mammalian cells A1AT is glycosylated with the complex pattern of carbohydrate similar to plasma-derived one, their expression systems are highly costly and have low productivity [7,8]. Alternatively, lower eukaryotes like yeast can be cost-effective host cells for large-scale production of heterologous proteins such as A1AT mainly due to the ease of molecular genetic technology as well as their fast growth [6].

A1AT expression *via Saccharomyces cerevisiae,* the most well-characterized and established yeast system, has been studied in various research papers to not only understand the mechanism of A1AT expression but also to find out the most efficient secretion method [4,6,9]. Although successful expression and secretion of A1AT protein were reported with relatively high yields, some drawbacks made commercial production of A1AT *via* the baker's yeast cumbersome [3]. Instability of expression plasmids and more acute antigenicity due to higher hypermannosylation in the Golgi compared to other yeasts like *P. pastoris* are considered the major disadvantages of this host cell [8,10].

Currently, methylotrophic yeasts such as *Pichia Pastoris (P. pastoris)* have attracted major attention for the large-scale production of heterologous proteins with relatively cheap broth media [10-12]. The efficient and tightly-regulated methanol oxidase gene (AOXl) promoter is used to express large quantities of heterologous proteins in *P. pastoris* along with post-translational modifications, such as protein glycosylation, disulfide bond formation, and proteolytic processing [11,12]**.** Yet, the number of studies which have examined the expression of A1AT in methylotrophic yeasts are quite limited. Some studies have reported secretory expression of A1AT in *P. pastoris*; however, they are not comprehensive and further investigation is required to examine the credibility of results [13-15]. In contrast to the most research papers which have examined extracellular expression in order to simplify the consequent downstream processes, in this paper, the intracellular expression was considered as a more appropriate choice. This is due to the fact that high-level production of heterologous proteins higher than 20 kDa usually encountered low secretion level [4,12]. Most importantly, the secreted A1AT may lose their biological activity during fermentation and initial clarification processes due to lower stability of this recombinant protein compare to plasma-derived ones [3,8]. On the other hand, intracellular A1AT vesicles can better preserve their biological activity and are less susceptible to degradation by enzymes such as protease [16].

In yeasts like *P. pastoris*, Snc1 and Snc2, members of the synaptobrevin/VAMP family of v-SNAREs, are driving forces for vesicle docking and membrane fusion at the cell surface in the term defined as exocytosis [17,18]. Exocytosis takes place when a SNARE complex is formed by pairing the t-SNARE in the target membrane with a cytoplasmic domain of v-SNARE in the transport vesicle [19,20]. In the current study, we investigated the co-expression of A1AT, which had v-SNARE domain, and the cytoplasmic domain of v-SNARE in *P. pastoris* under the control of inducible alcohol oxidase 1 (AOX1) promoter. This co-expression may lead to conjugation and saturation of t-SNARE proteins of the plasma membrane with recombinant v-SNARE protein which results in inhibition of exocytosis and therefore, accumulation of A1AT vesicles inside the yeast cell after PTM. In the downstream stage, these A1AT vesicles can be purified *via* multiple centrifugation process, after being released into the appropriate disruption buffer [16,21].Here, we investigated the possibility of improving the purification of A1AT by co-expression with v-SNARE in *P. pastoris.*

1. MATERIALS and METHODS:

Materials

All buffers were prepared with analytical grade chemicals purchased from Merck (Germany) company and water for injection (WFI) from the Pasteur Institute (Iran).

P. pastoris strains and vectors

Wild-type *P. pastoris* X-33 (Invitrogen, USA) was used as a host cell to express recombinant protein. The plasmid pPICZα A was used to construct the methanol-inducible A1AT expression vectors. DNA fragments both for A1AT and cytoplasmic domain of v-SNARE was ordered to be synthesized by Gene synthesis service of BIONEER company, Daejeon , Korea, Republic of(south korea).

Two types of expression cassettes, including inducible promoters were used for protein expression. Cassette Ⅰ contained the codon-optimized gene of the A1AT with 5′*EcoRI* and 3′ *NotI* restriction ends with a His-tag c-terminal was designed to express A1AT in the secretory mode. The constructed cassette Ⅱ contained a v-SNARE cytoplasmic domain with His-tag epitope at the carboxyl-terminal and restriction end *BamHI*. In our experiments, *P. pastoris* X-33 in 3 different modes (strains) were investigated. As a negative control, wild-type *P. pastoris* X-33 was selected. The *P. pastoris* with cassette Ⅰ in pPICZα A plasmid expresses A1AT in the secretory mode was named CONI(Fig. 1 (A)). The *P. pastoris* which was named CONⅡ, both cassette I and cassette Ⅱ were inserted in the plasmid pPICZα A in order to co-express the A1AT and cytoplasmic v-SNARE domain in the secretory, and intracellular mode, respectively (Fig. 1 (B)).

P. pastoris transformation and cultivation

The *E. coli* strain DH5α was used for bacterial transformation and recombinant plasmids propagation. Low salt broth (LB) was used in recombinant bacteria culture (0.5% w/v yeast extract, 1% w/v tryptone, 0.5% w/v NaCl with Zeocin 25 µg/ml) [14]. P. pastoris cells were grown overnight in YPD broth (2% peptone, 1% yeast extract, 2% dextrose) at 30 Cº/ 250 rpm. Afterward, the transformation was performed based on manufacturer's (Invitrogen) suggestion. The linearized pPICZα A with *sacI* was transformed into *P. pastoris via* electroporation. Transform-competent cells were cultivated again on the YPD agar plates in the presence of 1 M sorbitol, zeocin 100µg/ml in 30°C incubator and then, colonies were selected after 3-5 days.

Fermentation

Three fermentation processes were carried out for each strain to compare the secretion rate differences among them. The fermentation medium contained ammonium sulfate, glycerol, magnesium sulfate, EDTA, dipotassium hydrogen phosphate, calcium chloride and trace elements. Many of the selected colonies of wild-type X33, CONI and CONII strains were inoculated into three 2L shake flasks with 450 ml of media in 29°C/250 rpm inside the IKA shaker incubator KS4000 for 36 h [22]. Afterward, 450 ml shake flask was transferred to stirred-tank fermenter Winpact FS01-V-B Equipped with pH, temperature and dissolved oxygen controllers. The fermentation was carried out in the fed-batch mode in pre-sterilized medium (3L) at 29℃/500-600 rpm and the pH around 4.5 via adding 20%w/v ammonia solution. [11] [22] The aeration was set at 2 vvm and feeding was controlled in response to pH and dissolved oxygen (DO) concentration of broth [11,22]. The methanol induction started after 48 hours of fermentation when glycerol was depleted and OD did not increase further. The methanol induction continued in each fermentation process until the biomass concentration remained constant

Harvesting and cell disruption

After fermentation of each of three strains, secreted proteins in the broth were harvested after separation of biomasses (cell pellets) *via* centrifugation at 3000g for 20 min. For acquiring intracellular proteins, cell disruption of *P. pastoris* was required. For this purpose, 500 mg of separated cell pellets were washed with phosphate buffer saline (PBS) and centrifuged twice at 3000 g for 10 minutes each time. After discarding the supernatants, the cell pellets were suspended to 0.5 ml of disruption buffer (pH 8, Tris 20 mM, NaCl 250 mM, EDTA 5 mM, DTT 2.5 mM and 1 mM PMSF dissolved in isopropanol). Subsequently, 500 mg of glass beads was added to the disruption buffer in a tube and the cells were disrupted by vortex mixing on ice for 20 min. In order to assure that nearly all of yeast cells were disrupted, the cells were dyed and observed by the microscope. After this validation, the released soluble cytosolic proteins were separated by ultracentrifuge at 13000 g for 30 min as supernatant, and cell debris were discarded.

Biomass weight determination

The biomass in the fermentation broth was measured by wet-cell weight as well as OD600.

Total protein analysis

The Coomassie blue method was used for determining the total protein [23,24]. 0.1 ml of the fraction of the unknown sample was added to 5.0 ml of Coomassie protein assay reagent and was thoroughly mixed and thereafter absorbance of the mixture was read at 595 nm. *Via* using the standard curve, made by measurement of the known concentration of bovine serum albumin solutions, the absorbance of our samples were converted to protein concentration [9].

SDS-PAGE and western blot analysis

The purity and molecular weight of proteins was assessed by SDS-PAGE on a 12% polyacrylamide gel [25]. In order to detect recombinant A1AT and soluble domain of v-SNARE western blot was applied [14]. First, the proteins were transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane. Then, the membrane was blocked with PBS-T-BSA (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 2% BSA and 1% Tween 20), incubated with Anti-His-HRP ,Anti-A1AT and Anti-v-SNARE antibodies developed by using ECL technique (Amersham Bioscience). Finally, the obtained results were analyzed by Bandscan 5.0 software.

ELISA and trypsin inhibitory analysis

To quantify the intracellular and secretory recombinant A1AT, the soluble cytosolic proteins and proteins in the supernatant of cell culture were analyzed *via* ELISA assay by Abcam's A1AT ELISA Kit (ab108799) (SERPINA1). Frist, the samples of cell culture supernatant and cell-free lysed supernatant were coated on 96-well plates (50 µl/well). Afterward, the biotinylated A1AT antibody was used as the first detection antibody and horseradish peroxidase (HRP) labeled streptavidin antibody was used as the second antibody. To determine the percentage of biologically active A1AT, trypsin inhibitory capacity (TIC) of samples were measured using enzymatic assay as described by Dietz, Rubinstein and Hodges (1974) [26].

Ni-NTA chromatography

Since the cytoplasmic domain of v-SNARE and A1AT are recombinant fusion proteins that are tagged with six histidine residues, Ni-NTA was used for purification and detection of these proteins.

Result and Discussion:

Biomass weight:

The biomass concentration for X33, CONI and CONII strains were obtained after methanol induction and reaching constant OD. The two types of recombinant and wild type of *P. pastoris* showed different fermentation profiles, which are demonstrated in table 1. After commencing methanol induction, cell growth for X33, CONI and CONII was halted after about 73, 36 and 14 hours, respectively. According to the short time of cell growth in CONII, it was concluded that possibly, the expression of the cytoplasmic domain of v-SNARE involved in mechanisms which inhibited yeast growth such as the accumulation of different extracellular substances inside the yeast. As shown in table 1, the highest and lowest biomass weights belonged to X33 and CONII, respectively, which was predictable due to differences in methanol induction and yeast growth time.

Total protein

In the taken samples from the supernatant of cell culture and free cell lysate, total intracellular and extracellular proteins were measured which are shown in Table 2. Based on the concentration of extracellular total protein, protein secretion of CONI was three folds more than CONII and X33. On the contrary, the concentration of total intracellular protein in CONII was much higher than the X33 and CONI due to protein accumulation inside the cell. It seems that the expression of v-SNARE in CONII, *via* SNARE complex forming, partially inhibited fusion and secretion of secretory proteins. As fusion of secretory A1AT, like other secretory proteins, happens by the formation of SNARE complex, the A1AT accumulation was also expected.

Protein characterization

For detecting the recombinant A1AT, and soluble domain of v-SNARE expressed in *Pichia pastoris*, western blotting under reducing condition was performed. Western blotting was done on both supernatant of cell media and cell-free lysate with both Anti-His and Anti-A1AT antibodies (Fig. 2,3).Cell-free lysate was detected for Snc1 expression by western analysis using Anti-Snc1 antibody(Fig. 4). As shown in Fig. 2, the molecular weight of detected A1AT is higher than the molecular weight of plasma derived A1AT because of hyper-glycosylation as well as addition of 6X-His tag. According to the obtained data from western blotting in the supernatant of cell culture, CONII demonstrated less amount of A1AT than CONI (Fig. 2). This result strengthens our hypothesis that A1AT secretion would be prevented *via* v-SNARE protein expression.

The cell-free lysates western blotting with Anti-His antibody detected His tagged A1AT in CONI in three different molecular weights. On the other hand, western blotting against A1AT by anti-A1AT antibody demonstrated only one band. We hypothesized that bands 1 and 3 in the anti-His-tag western blotting might be the aggregated and degraded types of A1AT which might have formed during cell disruption of CONI.These forms of A1AT were not detected by Anti-A1AT antibody (Fig.3). For cell free lysate of CONII , four bands for anti-His antibody western blot were observed .As both A1AT and cytoplasmic domain of Snc1, the yeast homolog of Synaptobrevin (v-SNARE) co-expressed in CONII are fused with 6X-His tag we expected to observe more than one band (Fig. 3).Western blot was carried out on cell free lysate of CONII to A1AT and 55 kDa band and a little lower molecular weight band distributed to glycosylated A1AT were observed (Fig. 3).Western blot detection of Snc1expression was performed by using Anti-His antibody and Anti-Snc1 antibody. The existence of two bands in Anti-His western blot as well as in Anti-Snc1 of CONII distributed to cytoplasmic domain of v-SNARE, confirmed the expression of Snc1 soluble domain (Fig.

3, 4). The 52 kDa band implied that the expressed recombinant cytoplasmic domain of v-SNARE could form SNARE complex which was released after cell disruption. As during the exocytosis, vesicle fusion process, the v-SNARE and t-SNARE proteins bind together and form an SDS-resistant trans-complex with a 1:1:1 stoichiometry [20], It was concluded that recombinant v-SNARE cytoplasmic domain with a c-terminal 6X-His tag had a major role in forming the SNARE complex. The other band would appear to distribute the intraction of cytoplasmic domain of Snc1and soluble domain of Sso1,the yeast homolog of t-SNARE(Fig. 4).

For further analysis, purification with Ni-NTA chromatography was performed. Therefore, the supernatant of disrupted cells of CONI and CONII were applied to the Ni-NTA column. As indicated in Fig. 4 (A), in the elution profile of CONI one peak can be observed, which represents the expression of A1AT as recombinant fusion protein tagged with tandem histidine residues. In the elution profile of CONII (Fig. 4 (B)), two peaks were detected. The presence of two peaks can be related to the adsorption difference of A1AT protein, and the cytoplasmic domain of v-SNARE which both co-expressed proteins fused with six histidine residues.

A1AT quantification and biological activity

ELISA analysis determined the intracellular and extracellular amount of A1AT in CONI and CONII . *Via* quantitative ELISA, A1AT level in cell culture supernatant and inside yeast cell were determined and presented in Table 3. As indicated in Table 3, the intracellular A1AT concentration of CONII is 10 times bigger than CONIand A1AT secretion in this construct decreased by 45%. These results suggest that A1AT vesicle fusion and secretion might have been inhibited partially because of the competition of v-SNARE on secretory vesicle membrane with co-expressed soluble domain of v-SNARE in forming subsequent SNARE complex. Hence, high amount of A1AT vesicles, as a secretory protein, accumulated inside the cell after passing through the whole secretory pathway.

The biological activity of A1AT in CONI and CONII was measured *via* TIC method [9]. As demonstrated in the table 4, the percentage of intracellular A1AT, which was biologically active, is higher than extracellular one. Although the high proportion of trapped A1AT in CONII were biologically active, this percentage for intracellular A1AT in CONI is less significant. This can be explained by the possible aggregation and degradation process as mentioned before (Fig. 3); however, the main causes of this destabilization were unknown.

Conclusion

In this study, co-expression of the soluble domain of Snc1, the yeast homolog of Synaptobrevin (v-SNARE)and A1AT in *P. pastoris* was examined. Our results demonstrate that in the co-expression system, secretion of A1AT into the fermentation broth declined by 45% compared to the yeast which lacked v-SNARE expression cassette in the vector. The competition between v-SNARE proteins exist on A1AT secretory vesicle membrane and recombinant cytoplasmic domain of v-SNARE protein to from SNARE complex, as a driving force of fusion and secretion, has been considered the main reason for this decline. *Via* this co-expression, biologically active A1AT accumulated inside the yeast after post-translation modification. As a result, the stability of A1AT is less likely to be threatened during the fermentation process and early clarification conditions. Isolation methods of post-Golgi A1AT vesicles, such as multiple centrifugation steps or other novel methods need to be further investigated.

Conflict of interests

The authors confirmed that there are no conflicts of interest.

References

[1] T. CABEZ6N, M. D. WILDE, P. HERION, R. LORIAU and A. BOLLEN, "Expression of human a1-antitrypsin cDNA in the yeast," *Proc. Natl. Acad. Sci.,* pp. Vol. 81, pp. 6594-6598, 1984.

[2] V. Levina, D. Weiwen, A. S. Knaupp, D. Kaiserman, M. C. Pearce, L. D. Cabrita, P. I. Bird and S. . P. Bottomley, "Expression, purification and characterization of recombinant Z a1-Antitrypsin—The most common cause of a1-Antitrypsin deficiency," *Protein Expression and Purification,* vol. 68, pp. 226-232, 2009.

[3] E. Karnaukhova, Y. Ophir and B. Golding, "Recombinant human alpha-1 proteinase inhibitor: towards therapeutic use," *Amino Acids,* vol. 30, pp. 317-332, 2006.

[4] D. T. Moir and D. R. Dumais, "Glycosylation and secretion of human alpha-1-antitrypsin by yeast," *Gene.,* vol. 56, pp. 209-217, 1987.

[5] K.-S. Kwon and Y. Myeong-Hee, "Effect of glycosylation on the stability of a -antitrypsin toward urea," *Biochimica et Biophysica Acta.,* vol. 1335, p. 265–272, 1997.

[6] H. A. Kang, S. W. Nam, K.-S. Kwon, B. H. Chung and M.-H. Yu, "High-level secretion of human a, -antitrypsin from," *Journal of Biotechnology,* vol. 48, pp. 15-24, 1996.

[7] Y. Durocher and M. Butler, "Expression systems for therapeutic glycoprotein production," *Current Opinion in Biotechnology,* vol. 20, pp. 700-707, 2009.

[8] H. A. KANG, J.-H. SOHN, E.-S. CHOI, B. H. CHUNG, M.-H. YU and S.-K. RHEE, "Glycosylation of Human á1-Antitrypsin in Saccharomyces cerevisiae and Methylotrophic Yeasts," *Yeast ·,* vol. 14, p. 371–381, 1998.

[9] I. M. Tamer and Y. Chisti, "Production and recovery of recombinant protease inhibitor a1-antitrypsin," *Enzyme and Microbial Technology,* vol. 29, pp. 611-620, 2001.

[10] R. K. Bretthauer, "Genetic engineering of Pichia pastoris to humanize," *TRENDS in Biotechnology,* vol. 21, 2003.

[11] G. P. L. Cereghino, J. L. Cereghino, C. Ilgen and J. M. Cregg, "Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris," *Current Opinion in Biotechnology,* vol. 13, pp. 329-332, 2002.

[12] J. L. Cereghino and J. M. Cregg, "Heterologous protein expression in the methylotrophic yeast," *FEMS Microbiology Reviews,* vol. 24, pp. 45-66, 2000.

[13] A. Hasannia, A. S. Lotfi, F. Mahboudi, A. Rezaii, F. Rahbarizadeh and A. Mohsenifar, "Elevated expression of human alpha-1 antitrypsin mediated by yeast intron in Pichia pastoris," *Biotechnol Lett,* vol. 28, pp. 1545-1550, 2006.

[14] S. Arjmand, E. Bidram, A. . S. Lotfi, M. Shamsara and S. . J. Mowla, "Expression and Purification of Functionally Active Recombinant Human Alpha 1-Antitrypsin in Methylotrophic Yeast Pichia pastoris," *Avicenna Journal of Medical Biotechnology,* vol. 3, 2011.

[15] S. Arjmand, A. S. Lotfi, M. Shamsara and S. J. Mowla, "Elevating the expression level of biologically active recombinant human alpha 1-antitrypsin in Pichia pastoris," *Electronic Journal of Biotechnology,* vol. 16, no. 1, 2013.

[16] N. C. Walworth and P. J. Novick , "Purification and Characterization of Constitutive Secretory Vesicles from Yeast," *The Journal of Cell Biology,* vol. 105, pp. 163-174, 1987.

[17] Z. Su, Y. Ishitsuka, T. Ha and Y. K. Shin, "The SNARE complex from yeast is partially unstructured on the membrane. , 16(7):1138-46," *Structure,* vol. 16, no. 7, pp. 1138-1146, 2008.

[18] P. Strop, S. E. Kaiser, M. Vrljic and A. T. Brunger, "The structure of the yeast plasma membrane SNARE complex reveals destabilizing water-filled cavities.," *The Journal of biological chemistry,* vol. 283, no. 2, pp. 1113-1119, 2008.

[19] D. Fasshauer, "Fasshauer D (2003).Structural insights into the SNARE mechanism. (BBA)," *Biochimica et Biophysica Acta,* vol. 1641, pp. 87-97, 2003.

[20] Y. Aikawa, K. L. Boswell and T. F. Martin, "A second SNARE role for exocytic SNAP25 in endosome fusion.," *Molecular biology of the cel,* vol. 17, no. 5, p. 2113–2124, 2006.

[21] A. Forsmark, G. Rossi, I. Wadskog, P. Brennwald, J. Warringer and L. Adler, "Quantitative Proteomics of Yeast Post-Golgi Vesicles Reveals a Discriminating Role for Sro7p in Protein Secretion," *John Wiley & Sons A/S,* vol. 12, p. 740–753, 2011.

[22] E. Hardy, E. Martı´nez, D. Diago, R. Dıaz, D. Gonza´lez and L. Herrera, "Large-scale production of recombinant hepatitis B surface antigen from Pichia pastoris," *Journal of Biotechnology,* vol. 77, pp. 157-167, 2000.

[23] M. Bradford, "A rapid, and sensitive method for quantitation of microgram quantities of protein utilising the principle of dye-binding," *Anal Biochem,* p. 72:248, 1976.

[24] J. J. Sedmak and S. E. Grossberg, "A rapid sensitive, and versatile assay for protein using Coomassie brilliant blue G250.," *Anal Biochem,* vol. 79, pp. 544-552, 1977.

[25] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature,* vol. 227, pp. 680-685, 1970.

[26] A. A. Dietz, H. M. Rubinstein and L. Hodges, "Measurement of AIpha1-AntitrypsininSerum, by Immunodiffusionand by EnzymaticAssay," *Clinical Chemistry,* vol. 20, no. 3, 1974.

Tab. 1.

The time of methanol induction in each fermentation process and consequent biomass weight and OD value of each yeast strain

Trial	Methanol induction time (h)	OD	Biomass concentration (mg/ml)	Total Biomass(gr)
X33	73	81	201	1234.43
CONI	36	57.75	119	662.93
CONII	14	48.2	122	641.13

Tab. 2.

The total protein (TP) concentration of CON Ⅰ and CON Ⅱ in the pellet and supernatant via Bradford's method

Trial	Intracellular TP (mg/ml)	Extracellular TP (mg/ml)
X33	6.7	0.1
CONI	8.6	0.3
CONII	11.9	0.1

Tab. 3.

The total protein (TP) concentration of CON Ⅰ and CON Ⅱ in the pellet and supernatant via Bradford's method

Trial	Intracellular AAT (mg/ml)	Intercellular AAT/TP	Extracellular AAT (μg/ml)	Extracellular AAT/TP
X33	0	0	0	0
CONI	0.092	0.01	0.876	2.92e^-3
CONII	1.2	0.1	0.386	3.86e^-3

Tab. 4.

The percentage of biologically active expressed A1AT in CON Ⅰ and CON Ⅱ via TIC method

Trial	Percentage of intracellular biologically active AAT	Percentage of extracellular biologically active AAT
CONI	61%	38%
CONII	86%	41%

Fig. 1. (A) Construction of A1AT expression in pPICZα A vector (CON Ⅰ). pPICZα vector set for expression of A1AT in secretion mode. (B) Construction of A1AT and v-SNARE co-expression in pPICZα A vector (CON Ⅱ). pPICZα vector set for co-expression of A1AT and cytoplasmic domain of v-SNARE in secretory and intracellular mode, respectively.

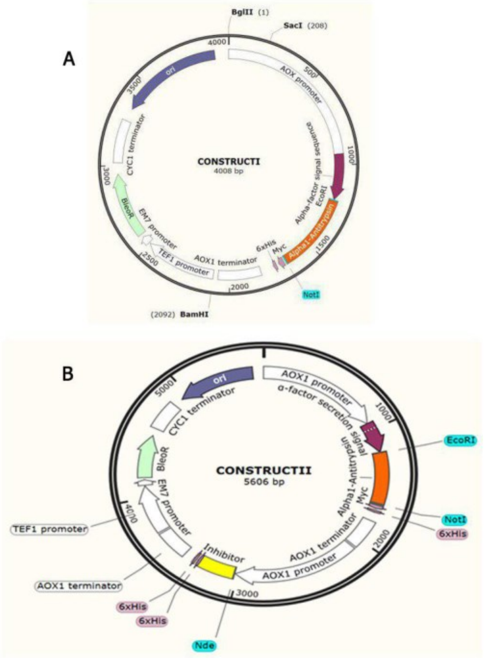

Fig. 2. Western blot analysis of supernatant cultures of X33, CON Ⅰ and CONⅡ samples. The supernatants of samples containing the soluble secretory AAT were subjected to reducing SDS-PAGE (12.5% polyacrylamide gel) and transferred to a PVDF membrane. Lanes 1,2 and 3 represent samples from the supernatants of cell cultures of X33, CONI and CONII, respectively.

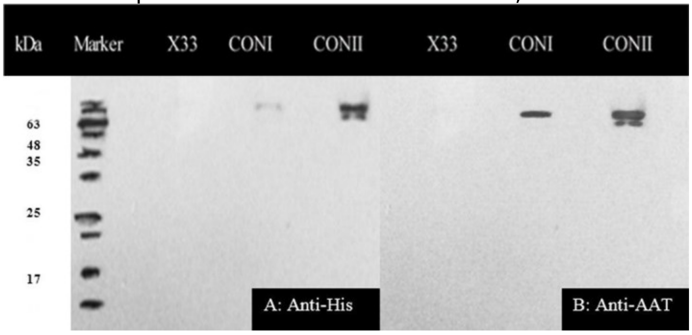

Fig. 3. Western blot analysis of cell-free lysate of X33, CON Ⅰ and CONⅡ samples.The samples of the cellfree lysate, obtained from centrifugation of disrupted cells, were subjected to reducing SDS-PAGE (12.5% polyacrylamide gel) and transferred to a PVDF membrane. Lanes1, 2 and 3 represent samples from the supernatant of the cell-free lysate of X33, CONI and CONII, respectively.

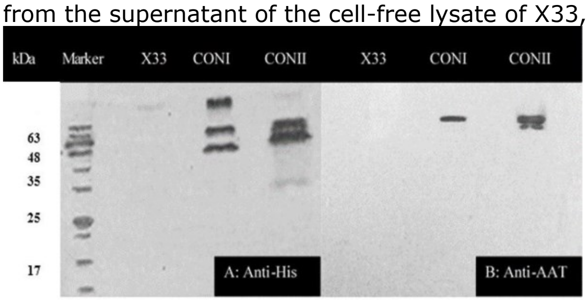

Fig. 4.Western blot analysis of cell-free lysate of X33,CONI and CONII samples.The samples of cell-free lysate, obtained from centrifugation of disrupted cells ,were subjected to reducing SDS-PAGE(12.5% polyacrylamide gel)and transferred to a PVDF membrane and blotted with Anti-Snc1(the yeast homolog of Synaptobrevin,v-SNARE).

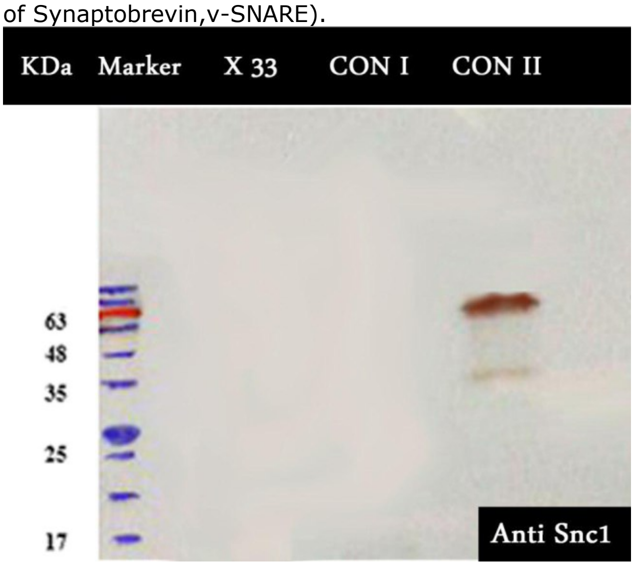

Fig. 5. (A) The Ni-NTA chromatogram for supernatant CONI cell-free lysate The Elution peak belongs to expressed A1AT with six histidine tag in P. pastoris. (B) The Ni-NTA chromatogram for supernatant CONII cell-free lysate Two elution peaks demonstrate co-expression of recombinant A1AT and the cytoplasmic domain of v-SNARE both tagged with six histidine residues.