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Recombinant Expression of an Antimicrobial Peptide Hepcidin in *Pichia pastoris*

Farzana Rashid, **Ijaz Ali**, **Imran Sajid**, **Salwa Elamin** and **Qipeng Yuan***
State Key Laboratory of Chemical Resource Engineering, School of Life Sciences and Technology, Beijing University of Chemical Technology, Beijing 100029.China (FR, SE, QY) . Institute of Zoology, Chinese Academy of Life Sciences, Beijing , China (IA) and Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan (IS)

Abstract.- Expression and purification of small peptides have always been problematic due to enzymatic degradation and many other technical problems. We report cloning and expression of a low molecular weight human antimicrobial peptide 'hepcidin' (Hepc, 20 amino acids) in pPIC9K transformed into *P. pastoris* GS115. The study reveals that active hepcidin peptide can be successfully expressed in this methylotrophic yeast. The BMMY medium was found to be optimal for the hepcidin protein expression and growth of the recombinant strains. Hepcidin protein expressed in recombinant strains was about 3 mg/L. Peptide expression was verified by Western blotting and ELISA assay. Recombinant hepc 20 was purified through Reverse-Phase HPLC column and characterized by Mass Spectrometry and amino acid sequencing. It also exhibited antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis.*

Key words: Hepcidin 20, antimicrobial peptide, iron, *Pichia pastoris,* secretory expression.

INTRODUCTION

Antimicrobial peptides are amphiphilic, positively charged molecules (Segrest *et al*., 1990) which recently emerged as novel antimicrobial agents for use in therapeutics, animal drugs, and food preservatives (Wei *et al*., 2005). The antimicrobial peptide, hepcidin (a small cysteine-rich cationic peptide synthesized in hepatocytes) has recently been isolated from human plasma and urine is a key regulator of intestinal iron absorption (Krause *et al*., 2000; Sima *et al*., 2003). Iron is an essential element for living organisms as it is required in many physiological functions. Therefore, iron balance must be maintained in the body which can be attained by the regulation of its absorbance and storage (Koliaraki *et al*., 2008). Circulating hepcidin affects iron absorption from the intestine through the interaction with the iron exporter ferroportin (Nemeth *et al*., 2004) and mediates iron retention within the macrophages. Human urine contains two dominant forms of hepcidin; comprised of 20 and 25 amino acids respectively differing only by N-terminal truncation. The 84-amino acid hepcidin pre-propeptide contains a typical endoplasmic reticulum targeting signal sequence and a consensus cleavage site for pro hormone convertases. In humans, the pre-propeptide is encoded by a single gene whose major site of expression is the liver (Sima *et al*., 2003; Pigeon *et al*., 2001). Although close hepcidin homologues have been identified in vertebrates ranging from fish to mammals, hepcidin does not appear to be related to any other previously known peptide family (Howard *et al*., 2002). Recently, information concerning the proteins involved in the iron absorption and in the regulation of iron homeostasis has been uncovered (Kulaksiz *et al*., 2004) who investigated inherited defects associated with iron disorders, both in humans and mice. Kulaksiz and coworkers (2004) investigating the presence and cellular localization of hepcidin in the liver and developed a noninvasive assay to analyze its regulation in patients with hereditary haemochromatosis (HH), chronic renal insufficiency (CRI), and renal anemia (RA). To make antimicrobial peptides more economically viable, researchers have found ways to mass-produce antimicrobial peptides using recombinant means such as insects/ baculovirus, *Escherichia coli* (Cipakova *et al*., 2004) and yeast based systems (Reichhart *et al*., 1992). To elucidate the biological

* Corresponding author: yuanqp@mail.buct.edu.cn
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function of hepcidin further and its use for other research, recombinant hepcidin was expressed and prepared in *Escherichia coli* using DNA recombinant technology.

Zhang *et al*. (1998) suggested that hepcidin may be medically applied in the treatment of various iron homeostasis disorders. When it was separated from urine its native concentration was very low (Park *et al*., 2001), and it is not easy to chemically synthesize the hepcidin (Klüver *et al*., 2002). The expression of tagged hepc 25 is also reported in *Pichia pastoris* strain X33. The problem associated with, to obtain high yields of active recombinant proteins from genes cloned in *E. coli* led to the development of expression systems for other organisms because the biological activity of the hepcidin protein expressed in *E .coli* is not perfectly understood.

To our knowledge, the expression and preparation of recombinant untagged Hepc 20 in eukaryotes (especially in *P. pastoris* GS115 using pPIC9K vector) has not yet been reported and no standard guidelines on this topic are available so far. The objective of this research is to investigate the suitability of the *P. pastoris* expression system as a means of recombinant expression of Hepcidin 20 in a biologically active form. The rationale to use this expression system in our research is based on the previously outlined features of this particular expression system which allows efficient secretory expression of complex recombinant proteins with correct intra- and inter-molecular disulphide bonds that do not require additional *in vitro* unfolding and refolding strtagies (Hasslacher *et al*., 1997; Hollenberg and Gellissent, 1997). A major challenge here is that we aim to express a small peptide (hepcidin) which is, indeed, a very small peptide (2.2 kD). The secretory expression in *P. pastoris* and activity analysis have been investigated in this report for its pharmaceutical use and advanced research work. Hopefully, the work will contribute to the futuristic research work in these disciplines such as control of body iron homeostasis.

MATERIALS AND METHODS

E. coli (BL21), DH5α, *P. pastoris* GS115 and plasmid pPIC9K were purchased from Invitrogen (California, USA). HEPC12-S and HEPC71-P were purchased from (Alpha Diagnostics, San Antonio, TX) as Rabbit anti-human antibody and synthesized human hepcidin respectively. Goat-anti- rabbit sec-antibody (AP conjugated) and the antibiotic, Geniticin (G418) were from Sigma. The composition of different media used for the cultivation of *P. pastoris* under different conditions are mentioned in Table I.

Table 1: Composition of different media.

Media	Composition
YPD	10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose
BMGY, BMMY	10 g/L yeast extract, 20 g/L peptone, 100 mmol/L Potassium phosphate, 13.4 g/L Yeast Nitrogen Base (YNB), 4×10^{-5} biotin, 10 g/L glycerol , pH 6.0. For BMMY, add 5 mL/L filter sterilized methanol instead of 10 g/L glycerol.
MD, MM plates	13.4 g/L YNB, 4×10-5 biotin, 20 g/L dextrose, 15 g/L agar. For MM, add 5 mL/L filter sterilized methanol instead of 20 g/L dextrose
RDB	20 g/L dextrose, 13.4 g/L YNB, 4×10-5 biotin, 0.05 g/L filter sterilized L-glutamic acid, L-lysine, L-leucine, L-isoleucine, L-methionine (each amino acid).
RDB G418 plates	Add 15 g/L agar to RDB and 0.5-3 mg/mL G418 in different plates.

Construction of pPIC9K-H expression plasmid

Standard recombinant DNA techniques (Sambrook *et al*., 1989) were used for plasmid construction. According to the codon preferences in yeast, a 99-bp fragment containing the coding sequence of enterokinase recognition site along with hepcidin was synthesized and inserted into pMD18-T (Takara, Dalian) with an upstream *EcoR* I site and a *Not* I site immediately downstream of inframe stop condons (TAATGA). The resulting plasmid was confirmed by PCR and DNA sequencing. The hepcidin fragment was amplified using the upstream primer (F: 5′-ATGAATTCGACGATGACGATAAGA-3′) containing and *EcoR* I site, and the reverse primer (R : 5′-ACGCGGCCGCTCATTAAGTCTT-3′) with *Not* I site. The primers facilitated the sub cloning of hepcidin protein coding sequence into

pPIC9K expression vector. The PCR product was digested with *EcoR* I and *Not* I and ligated into the pPIC9K expression vector digested with the same enzymes. The resulting pPIC9K-H was verified by restriction enzymes analysis and DNA sequencing.

Transformation of P. pastoris *and selection of* His^+Mut^+ *transformants*

P. pastoris was transformed by electroporation. The competent cells were prepared as described earlier (Invitrogen, 2002). Transformation was performed using a BioRad Gene Pulser II. Parameters used were 2.5 KV/cm, 50 μF and 400Ω. RDB-G418 agar plates containing transformed cultures were incubated at 29°C for 3-4 days. The His^+ and G418 resistant transformants were picked in a regular pattern on MM and MD plates and incubated at 29°C for 2-3 days. The His^+Mut^s (methanol utilization slow) transformants were differentiated from His^+Mut^+ (methanol utilization plus) via comparison of patch growth rate on MM and MD plates.

PCR analysis of P. pastoris *recombinant transformants*

The hepcidin peptide sequence was under the control of *AOX*1 promoter. The primers used were 5′*AOX1*: 5′-GACTGGTTCCAATTGACAAGC-3′; 3′*AOX1*: 5'-GCAAATGGCATTCTGACATCC-3′. The gene specific forward and reverse primers, HF: 5′- GCGGTTGATCTTCCAG -3′ and HR: 5′-ATTACGCCAAGTTTGC -3′, respectively were used to confirm the transformation. Genomic DNAs used for PCR were isolated as described in Multi-copy Pichia expression kit. PCR amplification was performed as follows: Initial denaturation at 94°C for 2 min, followed by 25 cycles (each at 94°C for 30s, 60°C for 30s, 72°C for 30s) and a final extension at 72°C for 10 min.

Shake-flask cultivation of P. pastoris *and secreted expression of hepcidin*

The recombinant *P.pastoris* strains confirmed by PCR analysis were grown in BMGY medium as described by Invitrogen (2002) with a little modification. A 100 mL/L of methanol was added to a final concentration 0.5% every 24 h to maintain induction. Samples were withdrawn every 24h to analyze expression level and cell density.

Optimization of cultures

The highly expressed recombinant strain initially judged by BMGY/ BMMY media was first grown in different media and then induced in relevant media. At last the absorbance and final methanol concentration were optimized during the induction phase. Samples were withdrawn every 24h to analyze expression level and cell density of the recombinant strains.

Estimation and western blot analysis of protein

As mentioned above the samples taken were centrifuged, supernatant were mixed with Tricine-SDS-PAGE sample buffer and separated on the gel (Schaggar *et al*., 1987). For protein estimation, Coomassie-stained Tricine-SDS-gels were analyzed by GelDoc analyzer (BioRad). Further protein samples was electroblotted onto a membrane (Amersham Pharmacia Biotech Hoefer TE 70 Series Semiphor Semi-Dry transfer Units, 0.8 mA/ cm^2 for 30 min). The membrane was blocked with (50 g/L fat free milk/TN buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.0) and probed with a Rabbit Anti-Human HEPC (dilution 1:1000 in 50 g/L fat free milk/TN buffer; 20-25^{o}C, 1h), followed by a goat-anti-rabbit IgG coupled to AP(dilution 1:10000 in 50 g/L fat free milk/TN buffer; 20-25^{o}C, 1h). Bound antibodies were detected using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue tetrazolium (NBT) in 100mmol/L NaCl, 5mmol/L $MgCl_2$, 100mmol/L Tris-HCl buffer, pH 9.5 as substrates.

Purification and characterization of recombinant peptide

To separate hepcidin, the peptide was first precipitated from the supernatant culture (60 hrs induced) according to the p*I* value using DNAMAN software (Lynnon BioSoft). Recombinant peptide was then purified by gel-filtration chromatography using a Sephadex G-25 column (1.0cm × 100cm), and eluting with 0.025M NaCl and 0.01M HCl at a flow rate of 0.3 ml/ min.The cleavage reaction with enterokinase was carried out at 37°C as described by Zhang *et al.* (2005). Then the hepcidin was further purified by reverse–phase (RP)

chromatography. The 10 uL of sample was charged to RP-HPLC (Shimadzu, Kyoto, Japan) with a Vydac C18 column (218TP54). The HPLC column was equilibrated with 0.1% TFA and eluted with CH3CN/0.1% TFA. The initial concentration of CH3CN was 15%, and the final concentration was 40% with a flow rate of 1 mL/min for 30 min. We collected the eluate that contained Hepcidin (each fraction was analyzed by ELISA, see below). The final yield of hepcidin was estimated using the values of the areas of the peaks. MS analyses were carried out using a Waters Ultra Performance LC-ES/MS with Waters Quattro Premier XE, USA. Fractions containing hepcidin were concentrated by lyophilization and stored at -20^{o}C. Furthermore, the purified hepcidin was characterized by N-terminal protein sequencer (Shimadzu PPSQ-21) to confirm whole amino acid sequences of the peptide.

ELISA assay

Fractions that contain recombinant peptide were dispensed into the microtitre plate at less than 2 µg/ml (Koganesawa *et al*., 2002) and read the absorbance (*A* 405nm).

Antimicrobial activity of recombinant protein

The antimicrobial activity of the protein expressed in *P. pastoris* was checked against *S. aureus, Bacillus subtilis* and *E. coli* BL21 (DE3) by agar diffusion assay. Bacterial cells in mid-logarithmic phase were plated onto 2×YT medium agar plate and 25µg recombinant hepcidin was added. Plates were incubated at 37° C for one day until circular antibacterial zone appeared. Water was used as an additional negative control.

RESULTS AND DISCUSSION

Screening of transformant phenotype

More than thirty geneticin-resistant (G418^{R}) *P. pastoris* colonies each having plasmid pPIC9K-H (Fig. 1) were cultivated on MD and MM plates. They were all His^{+}Mut^{+} because they all had the same growth rate. PCR amplification of Hepcidin was carried out with Hepc gene specific primers. Twenty transformants were selected each had the alcohol oxidase (*AOX*1) gene (promoter at the 5′ end of the hepc gene) and Hepc gene. The hepcidin protein coding sequence was correctly integrated into the *P. pastoris* genome in the positive recombinants via a single crossover (Fig. 2, lanes 1 and 4).

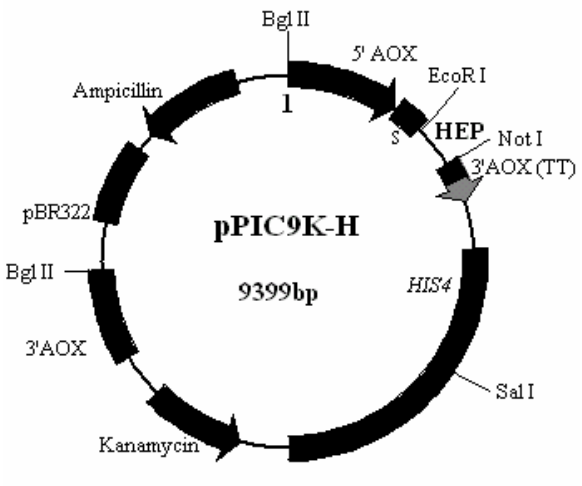

Fig. 1. Construct of expression plasmid pPIC9K-H under the control of *AOX*1 promoter (5′*AOX*1: promoter fragment; 3′ *AOX*1 (TT): transcription termination, HIS4 ORF: a select marker, HEP was inserted between 5′ *AOX*1 and 3′*AOX*1 (TT), Kanamycin: multi-copy select marker). The coding sequences for hepcidin gene were amplified from pMD-18T plasmid by PCR using primers (HEP-FP and HEP-RP) incorporating 5′ *Eco*R I and 3′ *Not* I, and subcloned into the pPIC9K.

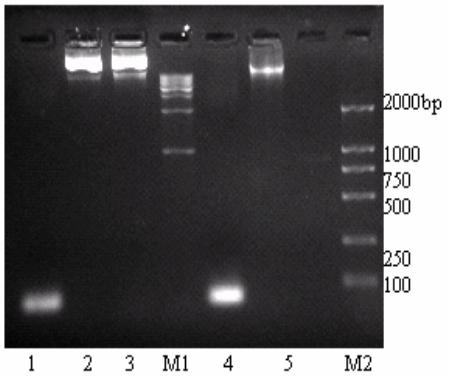

Fig. 2. Integration of Hepc gene into the *P. pastoris* GS115 and plasmids. Lanes 2 and 3, plasmids pPIC9K and pPIC9K-H, respectively; lane 1 and 4, PCR product of pPIC9K-H; lane 5, PCR product of *P. pastoris* GS115 without transformation; M1, DNA marker DL 15000.

Expression of hepcidin

A single recombinant colony (His^+Mut^+) resistant to G418 (1mg/ml) was used for expression study in shake flask. After the cell growth on BMGY with same conditions of growth as mentioned above, the cells were fed with a final concentration of 0.5% methanol every 24 hrs. The strains transformed by parent plasmid pPIC9K were taken as control. Tricine-SDS-PAGE showed that heterologous hepcidin peptide was successfully induced and expressed in *P. pastoris* (Fig. 3).

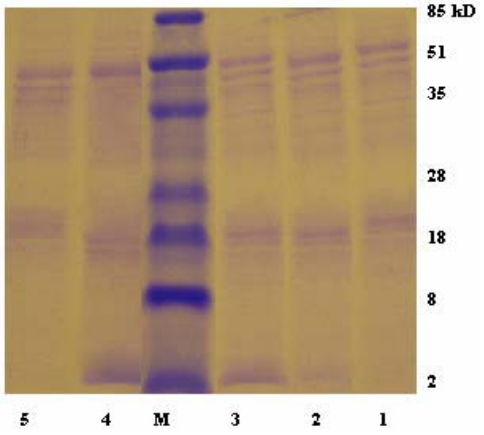

Fig. 3. Expression analysis of Hepc20 peptide from recombinant yeasts on Tricine SDS-PAGE; lane M, protein marker (2–85kD); lane 1-4, proteins from the best strain (0, 24, 48 and 60h, respectively); lane 5, proteins from control strain (60 h).

Optimization of culture and expression conditions have already been reported (Rashid *et al*., 2008). It was presented that the recombinant strains reached stationary phase after 48 hrs of induction, the expression level also reached the maximum. BMMY medium was optimal for hepcidin expression. Moreover, yeast extracts and peptone are rich in peptides, amino acids, vitamins and trace elements. These compounds could enhance the biomass and energy for foreign protein synthesis (Cregg *et al*., 1993).

Effect of the final concentration of methanol induction on yeast growth and hepcidin protein expression

We used the simple feeding technique for monitoring methanol concentration in the shake flask which worked well without any complicated measuring devices as described by Liu *et al*. (2004). All methanol taken up by the cells is oxidized to formaldehyde in a coupled reaction involving alcohol oxidase (*AOX*) and catalase (*CAT*) in peroxisomes. To find an optimum methanol concentration for induced expression, different percentage volume of methanol were added to the media every 12 hrs to decrease the resulting concentration shifts in the BMMY medium. The hepcidin expression level was observed to be maximized at 0.5% of methanol, increasing the methanol concentration from 0.5 to 2.0% resulted in reduced expression of hepcidin. The expression was almost same at the methanol feeding of 1.5 and 2.0%, but was decreased to about 50% at 2% methanol as compared to the methanol feeding of 0.5%. Therefore, the methanol feeding of 0.5% was optimal for yeast growth and expression of hepcidin peptide. Slight decrease in the growth and expression at methanol concentration above 0.5% was probably due to the limited carbon or the toxic effect of accumulated methanol (Boettner *et al*., 2002). So feeding with 0.5% methanol was considered as an optimal feeding strategy (Fig. 4). These reactions use molecular oxygen as an ultimate electron acceptor. Foreign protein synthesis needs abundant energy, so it is important to maintain a relatively high dissolved Oxygen (DO) for expressing foreign protein in *P. pastoris* (Rashid *et al*., 2008). DO of the culture was also maintained.

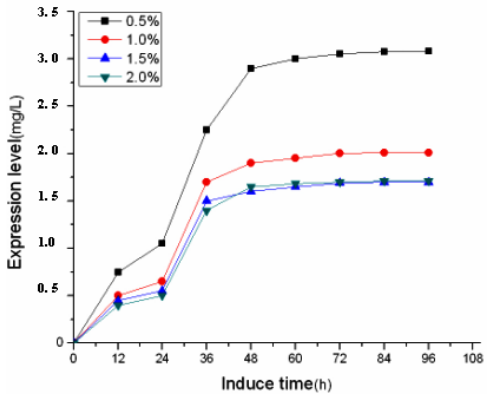

Fig. 4. Effect of percent concentrations (0.5, 1.0, 1.5, and 2.0) of methanol on the expression of protein.

Expression of peptide and western blot

The recombinant hepcidin protein was detected by Tricine-SDS-PAGE after 48 h of induction. Protein expression was evident when cells entered the logarithmic growth state and peaked when cell growth reached a steady state and became more or less constant thereafter. The magnitude of expression level reached 3 mg/L. The maximum cell OD of 4.2 at 600 nm was achieved. The optimal harvesting time of cells was 48-60 h after induction.

Western blotting with rabbit-anti- HEPC was carried out, synthetic HEPC 71-P was used as control. A single positive reaction band of about 2.2 kD can be seen in Figure 5. The band of the same molecular mass could not be detected in the induced recombinant strain transformed with pPIC9K vector**.**

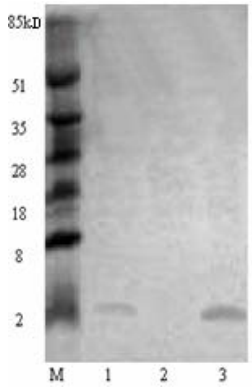

Fig. 5. Western blot analysis of peptide from recombinant yeast.1, peptide from the best strain; 2, Hepc negative strain; 3, human Hepc 20 (+ve control).

Purification, detection by ELISA and characterization of recombinant hepcidin

Recombinant peptide was purified from culture supernatants after a 60 hrs induction by a process involving precipitation at the p*I* of hepcidin (8.53) and Sephadex G-25 gel filtration chromatography (Fig. 6). The fractions containing hepcidin were further purified by RP-HPLC. The elution pattern of RP-HPLC using Vydac C18 is shown in Fig. 7. The elution time for the peak from the RP-HPLC column was the same as that of chemosynthetic hepcidin. In addition, the measured molecular weight accorded with the calculated molecular weight (2191.77Da) of hepcidin. Although hepcidin is shown as a single peak from RP-HPLC, hepcidin isomers with different disulfide bond connection may existed.

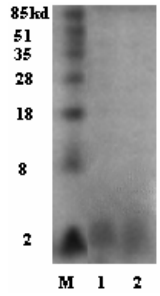

Fig. 6. Electrophoresis of the Hepc purified by Sephadex G-25 Lane 1; sample 1, lane 2; sample 2, M; marker

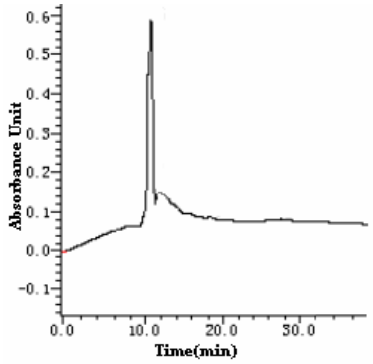

Fig. 7. Purification of hepcidin by Reverse-Phase Chromatography.

The immunochemical studies indicate that the recombinant protein produced was suitable for the detection of anti-Hepc in diagnostic assays. Each of the fractions was analyzed by ELISA to determine whether recombinant Hepc was contained in each fraction and confirmed that the material was indeed hepcidin. The results of ELISA shown in Table II, also indicate a positive signal for the presence of hepcidin in pPIC9K-H, whereas not in negative control (pPIC9K). The same results were observed in culture media collected at 48 h.

Furthermore the peak having a molecular weight of 2192.0 Da from LC-ESI–MS analysis was determined as recombinant hepcidin. The mass spectrum of recombinant hepcidin is shown in Figure 8. The mass spectrum of the main peak and the results of amino acid sequencing indicated that

the relative molecular mass and amino acid sequence of the recombinant product was identical to native hepcidin 20.

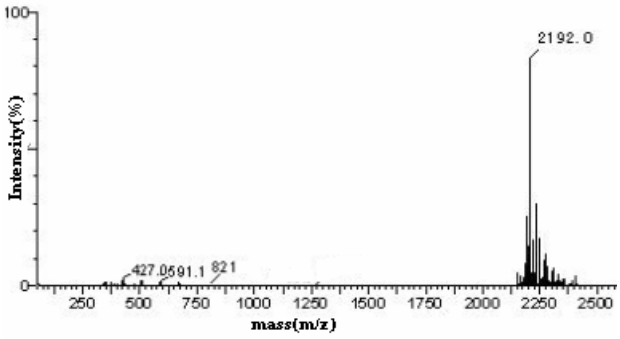

Fig. 8. Mass spectrum of recombinant Hepc purified by RP-HPLC. Peak in Fig. 7 was collected and measured. The main peak (2192.0 Da) corresponds to the molecular weight of Hepc (2191.77 Da).

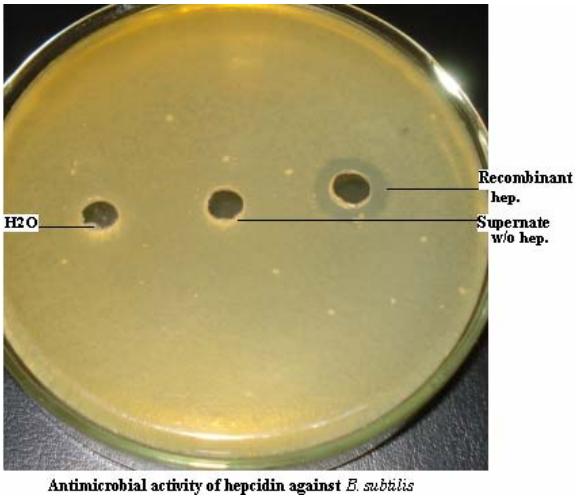

Fig. 9. Antimicrobial activity of recombinant protein against *B. subtilis*.

Antimicrobial activity of recombinant peptide

Moreover, through agar diffusion assay, the recombinant hepcidin also exhibited antibacterial activity against *S. aureus* (Fig. 8) and *B. subtilis* but was unable to inhibit the growth of *E. coli* BL21(DE3) as previously reported (Zhang *et al*., 2005).

Table II.- ELISA results showing the Absorbance at 405nm (mean value of triplicates). No.1, 2, 3: experimental samples containing recombinant Hepc, 4: -ve control without Hepc, 5: +ve control with HEPC 71-P.

Sample Nos.	1	2	3	4	5
Mean ELISA values (at 405nm)	0.309	0.281	0.299	0.132	0.399

It is concluded that shake flask expression of hepcidin would yield 3mg/ml of the hepcidin protein, a concentration greater than previously reported. Moreover, in the previous attempts hepcidin was either isolated from the urine samples or studied in the tagged form (Kulaksiz *et al*., 2004; Park *et al*., 2001), here we studied the protein in untagged form using *P. pastoris* GS115 and successfully expressed the active Hepc 20 having the antimicrobial activity against *S. aureus* and *B. subtilis*. This is the first success with secretory expression of the hepc 20 in *P. pastoris*. Its pharmaceutical use in the patients suffering from various types of iron disorders would be of great benefit like in control of body iron homeostasis, and other types of severe juvenile hemochromatosis.

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REFERENCES

BOETTNER, M., PRINZ, B., HOLZ, C., STAHL, U. AND LANG, C., 2002. High-throughput screening for expression of heterologous proteins in the yeast *Pichia pastoris J. Biotechnol.,* **99**: 51–56.

CIPAKOVA, I. E., HOSTINOVA, J., GASPERIK, AND VELEBNY, V., 2004. High-level expression and purification of a recombinant hBD-1 fused to LMM protein in *Escherichia coli. Prot. Express. Purific.* **37**: 207–212.

CREGG, J.M., VEDVICK, T.S. AND RASCHKE, W.C., 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology,* **11**: 905–910.

HASSLACHER, M., SCHALL, M., HAYN, M., BONA, R., RUMBOLD, K., LÜCKL, J., GRIENGL, H., KOHLWEIN, S.D. AND SCHWAB, H., 1997. High-level intracellular expression of hydroxynitrile lyase from the tropical rubber tree *hevea brasiliensis* in microbial hosts. *Prot. Express. Purific.,* **11**: 61–71.

HOLLENBERG, C.P. AND GELLISSENT, G., 1997. Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.,* **8**: 554–560.

HOWARD, N., HUNTER, D., FULTON, B., GANZ, T. AND HANS, J.V., 2002. The Solution Structure of Human Hepcidin, a Peptide Hormone with Antimicrobial Activity That Is Involved in Iron Uptake. *J. biol. Chem.,* **277**: 37597-37603.

INVITROGEN, *Instruction manual for multi-copy* Pichia *expression kit-version* F, 1999–2002.

KLÜVER, E., SCHULZ, A., FORSSMANN, W. G. AND ADERMANN, K., 2002. Chemical synthesis of β-defensins and LEAP-1/ hepcidin. *J. Pept. Res.,* **59**: 241–248.

KOGANESAWA, N., AIZAWA, T., SHIMOJO, H., MIURA, K., OHNISHI, A., DEMURA, M. AND HAYAKAWA, Y., 2002. Expression and purification of a small cytokine growth-blocking peptide from armyworm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris. Prot. Express. Purific.*, **25**: 416–425.

KOLIARAKI, V., MARINOU, M., SAMIOTAKI, M., PANAYOTOU, G., PANTOPOULOS, K. AND MAMALAKI, A., 2008. Iron regulatory and bactericidal properties of human recombinant hepcidin expressed in *Pichia pastoris. Biochimie,* **20**: 1–10.

KRAUSE, A., NEITZ, S., MAGERT, H. J., SCHULZ, A., FORSSMANN, W. G., SCHULZ-KNAPPE, P. AND ADERMANN, K., 2000. LEAP-1, a novel highly disulfide bonded human peptide, exhibits antimicrobial activity. *FEBS Lett.,* **480**: 147–150.

KULAKSIZ, H., GEHRKE, S. G., JANETZKO, A., ROST, D., BRUCKNER, T. AND KALLINOWSKI, B., 2004. Pro-hepcidin: Expression and cell specific localization in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. *Gut,* **53**: 735–743.

LIU, R. S., YANG, K. Y., LIN, J., LIN, Y. W., ZHANG, Z. H., ZHANG, J. AND XIA, N. S., 2004. High-yield expression of recombinant SARS coronavirus nucleocapsid protein in methylotrophic yeast *Pichia pastoris*. *Wld. J. Gastroenterol.,* **10**: 3602–3607.

NEMETH, E., RIVERA, S., GABAYAN, V., KELLER, C., TAUDORF, S., PEDERSEN, B. K. AND GANZ, T., 2004. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin, *J. clin. Invest.,* **113**: 1271–1276.

PARK, C. H., VALORE, E. V., WARING, A. J. AND GANZ, T., 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J. biol. Chem.*, **276**: 7806–7810.

PIGEON, C., ILYIN, G., COURSELAUD, B., LEROYER, P., TURLIN, B. AND BRISSOT, P., 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin is overexpressed during iron overload. *J. biol. Chem.*, **276**: 7811–7819.

RASHID, F., ALI, I. AND YUAN, Q. P., 2008. Expression studies of human antimicrobial peptide hepcidin. *J. Biotech.,* **136S**: S541–S547.

REICHHART, J. M., PETIT, I., LEGRAIN, M., DIMARCQ, J. L., KEPPI, E., LECOCQ, J. P., HOFFMANN, J.A. AND ACHSTETTER, T., 1992. Expression and secretion in yeast of active insect defensin, an inducible antimicrobial peptide from the fleshfly Phormia terranovae. *Invertebr. Reprod. Dev.,* **21**: 15–24.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T., 1989. *Molecular cloning: A laboratory manual*, 2nd ed, Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y.

SCHAGGAR, H. AND JAGOW, G.V., 1987. Trince–sodium dodecyl–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem*., **166**: 368–379.

SEGREST, J. P., LOOF, H. D., DOHLMAN, J. G., BROUILLETTE, C. G. AND ANANTHARAMAIAH, G. M., 1990. Amphipathic helix motif: classes and properties. *Prot. Struct. Funct. Gene.,* **8***:* 103–117.

SIMA, P., TREBICHAVSKY, I. AND SIGLER, K., 2003. Mammalian antibiotic peptides. *Folia Microbiol.* (Prague), **48**: 123–137.

SREEKRISHNA, K., BRANKAMP, R.G., KROPP, K.E., BLANKENSHIP, D.T., TSAY, J.T., SMITH, P.L., KOLIARAKI, V., MARINOU, M., SAMIOTAKI, M., PANAYOTOU, G., PANTOPOULOS, K. AND MAMALAKI, A., 2008. Strategies for optimal synthesis and secretion of heterologous protein in methylotrophic yeast *Pichia pastoris*. *Biochimie,* **20**: 1–10.

WEI, Q., KIM, Y. S., SEO, J. H., JANG, W. S., LEE, I. H. AND CHA, H. J., 2005. Facilitation of expression and purification of an antimicrobial peptide by fusion with baculoviral polyhedrin in *Escherichia coli. Appl. environ. Microbiol.,* **71**: 5038–5043.

ZHANG, L., FALLA, T., WU, M., FIDAI, S., BURIAN, J., KAY, W. AND HANCOCK, R. E., 1998. Determinants of recombinant production of antimicrobial cationic peptides and creation of peptide variants in bacteria. *Biochem. biophys. Res. Commun.*, **247**: 674–680.

ZHANG, W. H., YUAN, Q. P., ZHU, Y. AND MA, R., 2005. Expression and preparation of recombinant hepcidin in *Escherichia coli*. *Prot. Express. Purific.,* **41**: 409–416.

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