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Research Article

CHARACTERIZATION AND QUANTIZATION OF HEPCIDIN FROM HUMAN AND MOUSE SERUM AND SECRETION MEDIUM FROM HUMAN LIVER CELL LINE, HU.7 BY MALDI-TOF MASS SPECTROMETRY

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Abstract: Hepcidin, an antimicrobial peptide, is expressed in liver cells. The synthesis and secretion of hepcidin depends on iron status and inflammation. Hepcidin levels in blood circulation and in urine are key indicators of iron homeostasis. There are surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF-MS) assays available to measure hepcidin levels from urine and serum samples. In the present work, the hepcidin from urine and secretion medium of liver cell line, HU.7 was analyzed by MALDI-TOF-MS. Hepcidin peptides were enriched from 10µl of acidified samples over ZipTipSCX, and bound peptides were directly eluted over MALDI-TOF-MS plate containing adrenocorticotropic hormone (ACTH) as an internal standard and analyzed in the mass range of 1500-3800 m/z. We observed a single polypeptide peak at 2791 m/z in mouse serum, which was identified as hepcidin and its sensitivity towards LPS treatment was further assayed. There was two-fold increase in hepcidin level in mouse serum due to LPS treatment. A physiological identification of serum hepcidin was accomplished by MALDI-TOF-MS/MS analysis. The majority of fragments present in mouse serum hepcidin were similar to synthetic 25-mer hepcidin. From these results, we concluded that hepcidin peptide levels can be identified and determined in mice serum by MALDI-TOF-MS procedure. For human subject, we chose the effect of dietary iron on hepcidin secretion. When a person was subjected to high iron diet, a single peak at 2753.6 m/z of hepcidin was observed in serum that increased by 59% after dietary iron as expected. There were several peaks of hepcidin in human urine, identified at 3077(28-mer), 3003(27-mer), 2711(25-mer), 2564(23-mer), 2436(22-mer), 2280 (21-mer), and 1912 (17-mer).

Keywords: Hepcidin; iron homeostasis; MALDI-TOF-MS; anemia; iron overload disease; human liver cell line

Introduction

Hepcidin, an antimicrobial peptide, is predominantly expressed in liver and has been purified and characterized from human blood and urine (Lee and Beutler, 2009; Nemeth and Ganz, 2006). The transcript of hepcidin encodes an 87 amino acid peptide with 24 amino acid putative leader sequence and 25-amino acid secretory mature peptide with 4-disulfide bonds which is processed by ER-proteases from the carboxylic end of the precursor hepcidin (Jordan *et al.*, 2009). The physiological significance of hepcidin in iron homeostasis was realized from the following results. Loss of hepcidin gene and CCAAT/enhancer binding protein, α-gene, resulted in an iron overload in mice. When the hepcidin gene was over-expressed, animal showed severe anemia and intravenous injection of hepcidin peptide has also reduced mucosal uptake of iron. In the meantime, it was discovered

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that ferroportin as an iron transporter in enterocytes, can bind hepcidin and hepcidin binding down regulates the cell surface expression of ferroportin, resulting in a decrease in iron export. From these studies, it became clearer that hepcidin directly plays an important role in iron homeostasis (Ganz, 2011; Lee and Beutler, 2009; Nemeth and Ganz, 2006).

Hepcidin expression is rapidly increased in response to iron stores and inflammation (Collins *et al.*, 2008; Mena *et al.*, 2006). In order to understand the mechanism of regulation of hepcidin expression and secretion and the role of hepcidin in iron homeostasis, biosynthetic characterization and development of techniques for quantitation of hepcidin from serum and urine and from secretion of human liver cells would be beneficial. There have been attempts to develop enzyme-linked immunosorbent affinity (ELISA) assay (Ganz *et al.*, 2008; Koliaraki *et al.*, 2009), surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) (Campostrini *et al.*, 2011; Tomosugi *et al.*, 2006; Ward *et al.*, 2008), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS (Anderson *et al.*, 2011), and tandem mass spectrometry (LC-MS/MS) (Bansal *et al.*, 2009; Murao *et al.*, 2007; Murphy *et al.*, 2007) based methods to determine the hepcidin concentrations in urine and blood samples (Bozzini *et al.*, 2008). These procedures are still limited in their potentials. We made an attempt to develop a rapid, simple, and versatile procedure to determine hepcidin from serum and from secretion medium by MALDI-TOF-MS. The present procedure has an advantage over earlier SELDI-TOF-MS procedure because we could also perform the MALDI-TOF-MS/MS of sample to confirm the authenticity of hepcidin molecule present in serum and urine samples. Therefore, the present procedure has great versatility and potential to use in characterization and quantitation of hepcidin in urine and serum samples of patients and secretion from Hu.7, Hepatocyte derived carcinoma cell line, a human cell line.

Material and Methods

Mice - The mice strain used was FVB/n. All animal care was performed in accordance with the "Guide for the care and use of laboratory animals" (Institute of Laboratory Animal Resources, Commision of Life Sciences, National Research Council, 1996) and studies performed under IACUC-approved protocol (1410).

Mice on normal dietary iron - Mice were on diets supplemented with 250ppm iron until they were euthanized. Blood samples were collected and serum was stored at -80°C for subsequent quantitation of hepcidin.

Endotoxin treatment of mice - Mice were treated with lipopolysaccharide (LPS) 1µg/g intraperitoneally or saline after overnight fasting. Six hours after LPS treatment the animals were bled for blood serum. The serum samples were frozen at -80°C before use for MALDI-TOF-MS analysis of hepcidin.

Supplementation of dietary iron in human - Individual took two tablets of ferrous fumarate, (Walgreen) each containing 27 mg Fe; 24 hr after the tablets the blood serum and urine samples were collected for hepcidin determination. As a control, blood serum and urine samples were collected before iron ingestion. The samples were frozen at -80°C.

Enrichment of hepcidin - Urine or serum (10µl) was mixed with equal volume of 10% acetic acid. To compare the heating effect on the recovery of hepcidin, acetic acid mixtures were also heated around 90°C for 30 s in a heating block. The mixture was incubated on ice for 30 min and centrifuged in Eppendorf centrifuge at top speed for 5 min in cold room (4°C). The clear supernatant as acid soluble fraction was carefully saved. The acidic precipitate was further washed with 20µl 5% acetic acid and clear supernatant was recovered after centrifugation at top speed in Eppendorf centrifuge at room temperature for 2 min. Both acid soluble fractions were pooled and diluted with 40µl water. The samples were dried on speed vac and stored at -20°C or -80°C until use.

Partial purification of hepcidin over ZipTipSCX - A dried acid soluble fraction was solubilized in 10µl 100 mM NH_4HCO_3, pH 8.0 buffer. If pH of samples was not around pH 8.0, it was adjusted. ZipTipSCX was equilibrated with 10µl 20 mM NH_4HCO_3, pH 8.0 buffer using four pipetting

stroke. Unbound material was washed out using 20 mM NH_4HCO_3, pH 8.0 buffer using five pipetting stroke. Bound hepcidin was eluted with 2µl 60%acetonitrile in 0.1% formic acid and analyzed by MALDI-TOF-MS or MS/MS.

Calibration of MALDI-TOF-MS - ACTH fragment, 18-39 amino acid in length, was used for calibration. Four different runs in the concentration range of 0.1 ng to 100 ng were carried out. The intensity counts as a function of concentration were plotted. The correlation between intensity counts and concentration of ACTH was linear between 1-100 ng and there was minimum variation between runs at different dates suggesting that ACTH could be used for quantitation of hepcidin as an internal control. Internal standard was added before MALDI-TOF and not during enrichment and purification.

Results and Discussion

Characterization of hepcidin peptides

Hepcidin plays an important role in the regulation of iron homoeostasis in mammals (Ganz and Nemeth, 2006). We identified hepcidin peak from the mouse serum in MALDI-TOF-MS showing mass over charge range of 1500-3800. First, we compared the MALDI-TOF-MS profile of synthetic 25-mer hepcidin with mouse serum hepcidin because mammalian secretory, 25-mer hepcidin are highly conserved and considered as biologically active hepcidin (Farnaud *et al.*, 2008). Figure 1A shows that the synthetic human 25-mer hepcidin has predominant peak at 2788 m/z which corresponds to a peak at 2791 m/z for mouse serum hepcidin (Figure 1B). These results suggest that mouse serum contains a 25-mer hepcidin.

Earlier it was reported that LPS stimulates transcription of the HAMP gene, which encodes hepcidin, which appears to function through the mediation of the inflammatory cytokine IL-6 (Lee *et al.*, 2004). Wang *et al.* (Wang *et al.*, 2008) reported that intravenous administration of LPS regulates the expression of hepcidin mRNA and protein in the rat. Recently, it was shown (Tjalsma *et al.*, 2011) that hepcidin expression was stimulated in 3 mouse strains (C57Bl/6, DBA/2 and BABL/c) with intravenous iron and LPS. We analyzed the mice serum before and after LPS treatment to identify hepcidin peak because LPS has been shown to increase the synthesis and secretion of hepcidin in mice. Figures 2A and B show that a peak at 2791 m/z in mouse serum treated with LPS was 38% higher than the mouse serum treated with saline. Therefore, major peak at 2791m/z was identified as a 25-mer hepcidin in the mouse serum and its secretion was enhanced by LPS, as expected. Taken together from these results, we concluded that MALDI-TOF-MS could be used to study the correlation between hepcidin level and iron status of the mice under different physiological conditions.

The identification of hepcidin peptide in the mice serum was further confirmed by MALDI-TOF-MS/MS of synthetic 25-mer hepcidin (2788 m/z) and compared with mouse serum 25-mer hepcidin (2791 m/z). Fig. 3 shows that the majority of fragments present in mouse serum hepcidin of 2791 m/z are similar as present in synthetic 25-mer hepcidin (2788 m/z). These results suggested that 25-mer hepcidin, 2791 m/z is an authentic hepcidin.

Characterization and quantization of hepcidin in mouse and human serum

In order to quantitate the hepcidin level in human serum, an internal standard curve was generated using ACTH fragment 19-39 for MALDI-TOF-MS profiles (Figure 4A). The major peak for ACTH was observed at 2465 m/z corresponding to 21-mer peptide. The intensity at 2465 m/z ACTH was determined for different concentration of ACTH in the range of 0.1-100 ng. Standard curve in duplicate was repeated twice. The results are summarized in Figure 4. There was no significant difference between several measurements and showed linear correlation between 0.1-100 ng ACTH and intensity counts. Therefore, we used 0.02-1 ng ACTH as an internal standard in determining the hepcidin level in serum and urine samples. As a proof of the procedure, hepcidin level was determined from the mouse serum using ACTH as an internal standard. A typical spectrum of mouse serum hepcidin and 0.02 ng ACTH is shown in Figure 4B. The peaks at 2465.5 m/z for ACTH and at 2791.5 m/z for 25-mer hepcidin were observed. The ratio of

hepcidin over ACTH was used to calculate the concentration of hepcidin. The hepcidin level from nine normal mice serum was around 29.15+2.95 ng/ml serum. Our results are in agreement with earlier reported methods (Murphy *et al.*, 2007).

Hepcidin plays significant role in the regulation of iron homeostasis (Ganz and Nemeth, 2011). Therefore, understanding the role of iron in the regulation of hepcidin expression is clinically relevant (Fleming and Ponka, 2012; Ganz, 2004). Earlier report suggests that alcohol reduces and iron increases liver hepcidin synthesis (Harrison-Findik *et al.*, 2007). Therefore, we measured the effect of dietary iron on hepcidin secretion in humans. MALDI-TOF-MS profile of human serum showed a single peak around 2753.6 m/z corresponding to a 25-mer hepcidin (Figure 5A). When a person was subjected to high iron diet, hepcidin level in the serum was increased. Using ACTH standard, we quantified the hepcidin levels in human serum and results are shown in Figure 5B. There was a 2.5 fold increase in hepcidin level in human serum after dietary iron. Recently, a method was developed and validated to measure the concentrations of hepcidin after dietary iron supplement ingestion in human serum and urine (Hwang *et al.*, 2011). We observed a positive correlation with dietary iron and hepcidin level in serum. This study provides an experimental evidence for the effect of dietary iron on hepcidin gene expression.

It was suggested that hepcidin gene expression is up-regulated by high dietary iron and down-regulated when iron availability is low (Mazur et al., 2003). We also determined the levels of hepcidin in human urine before (Pre) and after (Post) iron ingestion. The results are shown in Figures 6A and 6B. The MALDI-TOF- MS profile of human urinary hepcidin showed several major and minor peaks corresponding to m/z of 3077 (28-mer), 3003(27-mer), 2901 (26-mer), 2711(25-mer), 2564 (23-mer),2436(22-mer), 2280 (21-mer) and 1912 (17-mer). These peak intensities were affected, due to iron ingestion, suggesting they are iron responsive peptides. They may represent different processed hepcidin. The smallest hepcidin of 17-mer represents a peptide with four disulfide bonds, without any additional N- or C-terminal amino acid residues. The smaller mass of hepcidin seen in urine was not present in serum. The quantization results shown in Figure 6C suggested a 45% increase in urinary hepcidin (25-mer). This observation suggests that hepcidin gene expression has been related to iron status in humans (Hadley *et al.*, 2006). An increased hepcidin mRNA expression was observed in animal models of iron overload, including mice that were fed excess iron (Pigeon *et al.*, 2001). Earlier report suggest that urinary hepcidin concentrations significantly correlated with hepatic hepcidin mRNA concentrations, indicating that hepcidin quantification in urine is a valid approach to evaluate hepcidin expression (Detivaud *et al.*, 2005). Therefore, the urine hepcidin measurement is directly related to the synthesis of hepcidin by liver cell during course of iron ingestion. Detection of hepcidin concentration with high accuracy in the urine will provides a novel diagnostic tool for large clinical research studies (Anderson *et al.*, 2010).

Characterization of hepcidin from Hu.7 cell secretion medium

Since the liver is major site for hepcidin expression, therefore, human liver cell line Hu.7 was established as experimental conditions in which hepcidin mRNA expression can be measured in the presence of inducing agents (Verga Falzacappa *et al.*, 2007), including bone morphogenetic protein (BMP6), which increases hepcidin expression and reduces serum iron in mice (Andriopoulos *et al.*, 2009). MALDI-TOF-MS profiles of secretion medium of Hu.7 cells are shown in Figure 7A. There were several peaks of 2903 m/z (26-mer), 2792 m/z (25-mer), 2488 m/z (23-mer) and 2404 m/z (22-mer), respectively, for hepcidin and a peak of 2465 m/z for ACTH (21-mer) standard peptide. After BMP treatment of Hu.7 cells, the secretion of hepcidin peptides was found to be increased (see Figure 7B). These results suggested that hepcidin could be determined from the secretion medium of Hu.7 cells and BMP treatments induce hepcidin secretion in the culture medium as expected. In separate experiments, we quantitated the amount of hepcidin secreted in the medium of Hu.7 cells using different sample preparation procedure for MALI-TOF-MS. We found that acid solublization,

combined with heating, was relatively better in the recovery of hepcidin than acid solublization alone (Table 1). These observations suggest that BMP-6 causes a dramatic increase in the hepcidin expression.

Table 1
BMP-induced hepcidin secretion from Hu.7 cells

Cell Treatment	**Secretion medium of Hepcidin ng/ml*	
	Acid solublization	*Acid solublization and heating*
Control	0.30±0.030	0.68±0.112
BMP-treated	1.40±0.07	2.36±0.118

*Hepcidin was quantified using ACTH as a standard peptide. These results are average of three independent measurements. Average deviation was 5-10%

Due to the enormous clinical application of hepcidin, various assay platforms and related

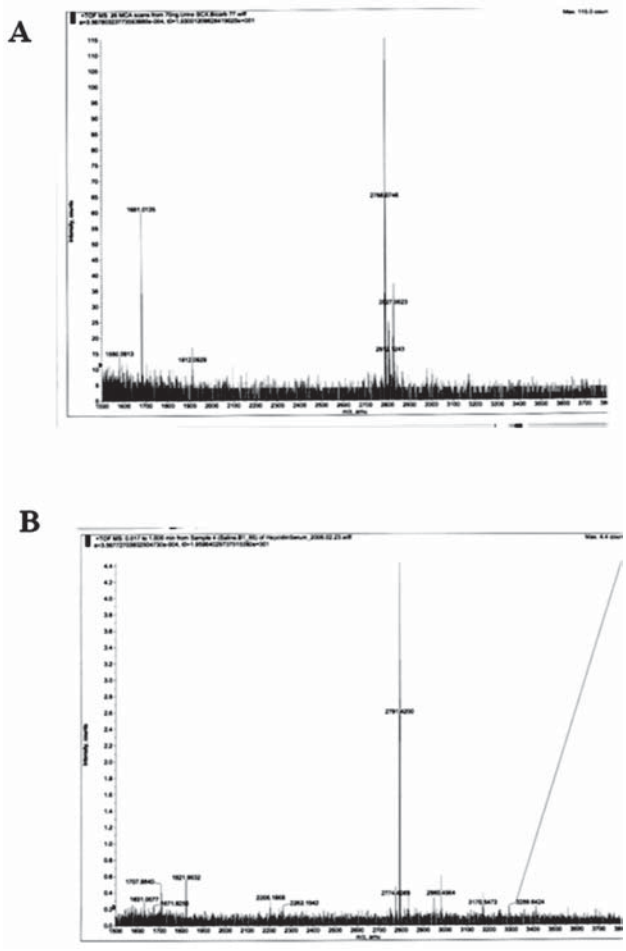


Figure 1: **MALDI-TOF/MS profile of (A) synthetic 25-mer hepcidin and (B) mouse serum hepcidin. Both these sample showed identical TOF spectra**

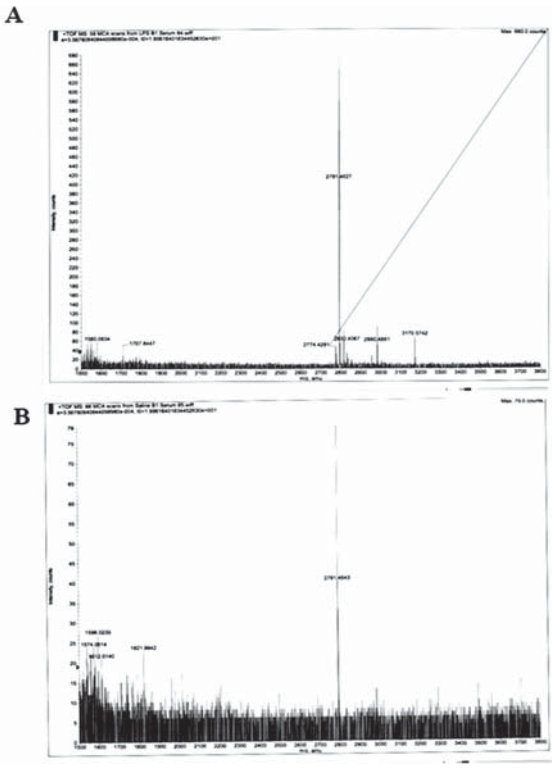


Figure 2: **MALDI-TOF/MS profile of mouse serum hepcidin (A) before and (B) after treatment with LPS. We observed 38% increase in hepcidin due to LPS treatment in mouse serum**

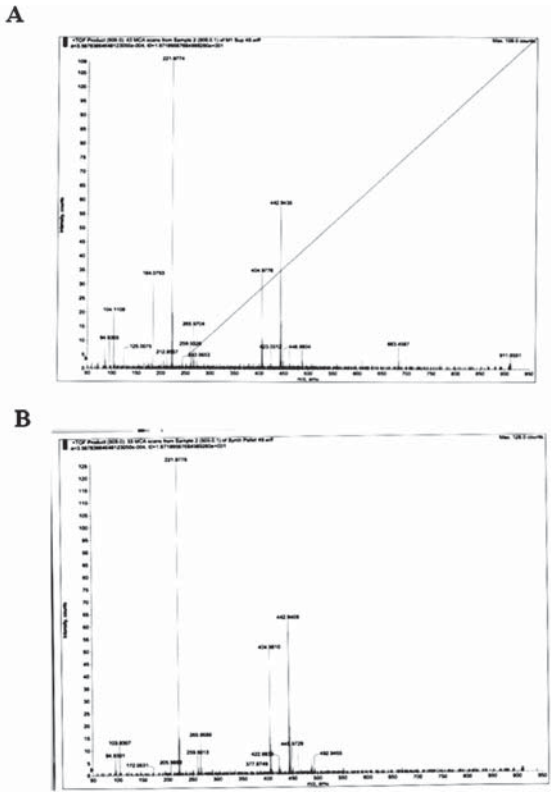


Figure 3: **Peptide mass finger prints of (A) synthetic 25-mer hepcidin and (B) mouse serum hepcidin. Both these sample showed identical fragments on their Mass spectrum**

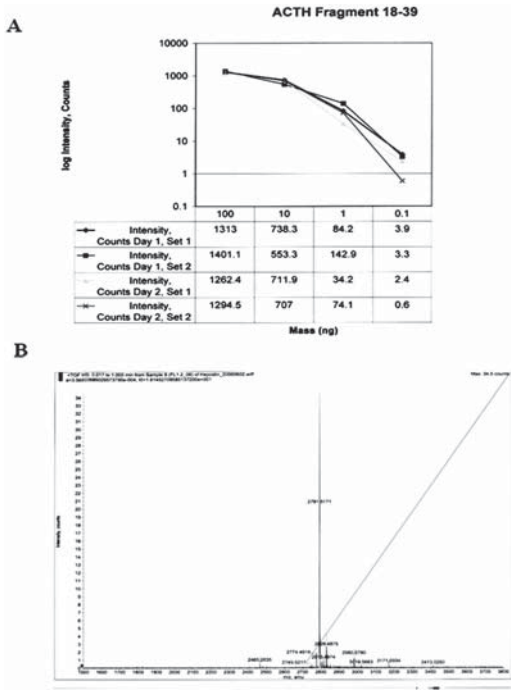


Figure 4: **(A) An internal standard curve was generated using ACTH fragment 19-39 for MALDI-TOF-MS profiles. There was no significant difference between several measurements and showed a linear correlation between 0.1 – 100 ng ACTH and the intensity counts. (B) Typical spectrum of hepcidin and 0.02 ng ACTH. The major peak for ACTH was observed at 2465 m/z corresponding to 21-mer peptide**

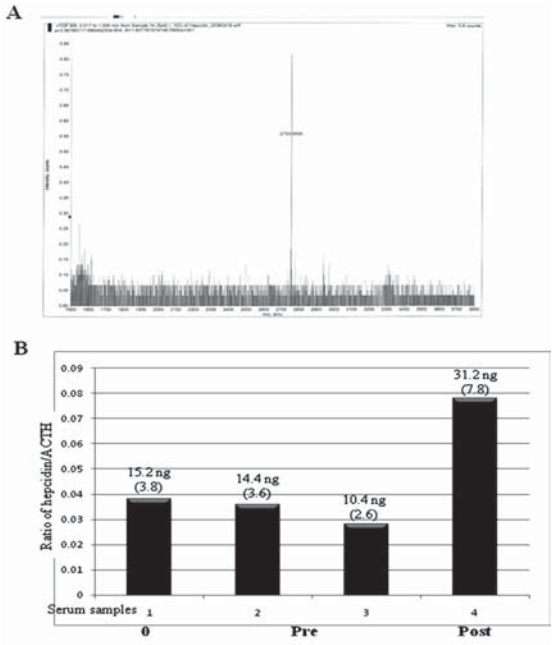


Figure 5: **(A) MALDI-TOF/MS profile of human hepcidin. A single peak at 2753.6 m/z corresponding to a 25-mer hepcidin was observed. (B) Hepcidin level in human serum after iron ingestion was measured by ACTH standard. There was a 2.5 fold increase in hepcidin level in human serum after dietary iron ingestion**

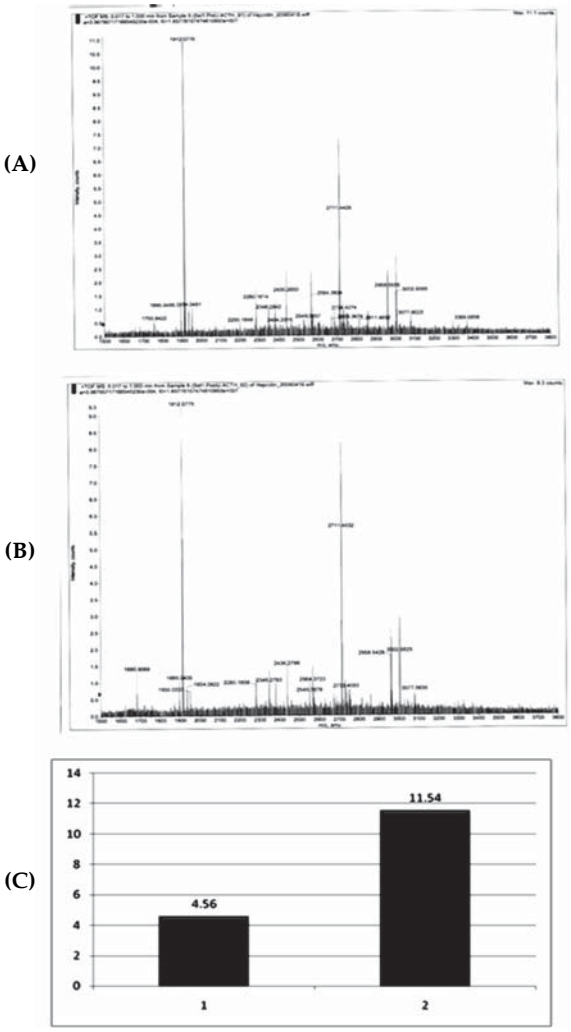
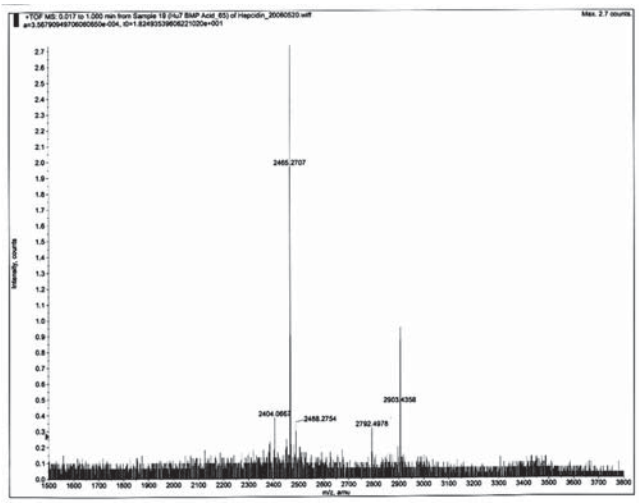


Figure 6: **Peptide mass fingerprints of hepcidin from human urine (A) before iron ingestion and (B) after iron ingestion. The peak intensities were affected due to iron ingestion, suggesting they are iron responsive peptides. (C) Level of urine hepcidin measured before and after iron ingestion. A 45% increase in urinary hepcidin was observed before/after iron ingestion**



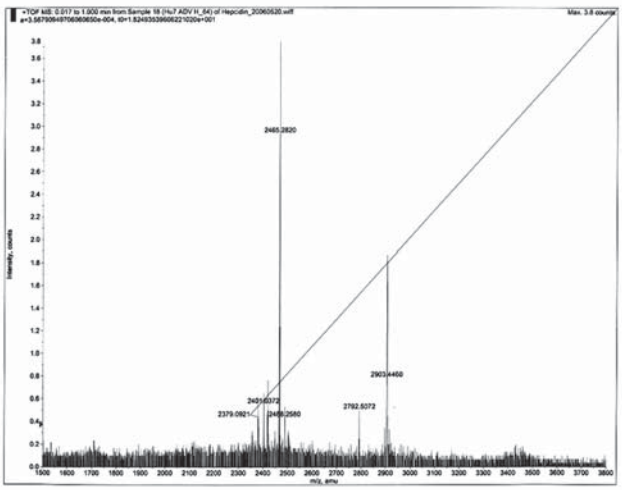


Figure 7: **MALDI-TOF/MS profile of hepcidin from Hu.7 liver cell line (A) before and (B) after treatment with BMP6. After BMP treatments of Hu.7 cells, the secretion of hepcidin peptides increased dramatically**

studies help the potential utility of hepcidin as a diagnostic parameter for diseases related to iron imbalance (Kroot *et al.*, 2012). Recently, a novel high-throughput MALDI-TOF based assay for the quantification of hepcidin in human plasma was reported (Anderson *et al.*, 2011). While earlier reports have focused on detection of hepcidin in urine, serum, or plasma (Bansal *et al.*, 2009; Bozzini *et al.*, 2002; Murao *et al.*, 2007; Tomosugi *et al.*, 2006), we tried here to determine hepcidin concentration in the urine, serum and Hu.7 cell line. Our approach to enrich hepcidin from secretion medium, urine and serum before MALDI/TOF is different from others, which offers significant sensitivity, precision, and throughput.

Conclusion

We observed that mouse serum contains 25-mer hepcidin and its sequence is identical to the synthetic hepcidin. Furthermore, when mouse serum was treated with LPS a 38% increase in serum hepcidin level was observed. Moreover, 2.5 fold increase in hepcidin level in human serum was observed after intake of dietary iron. Our approach to enrich hepcidin from secretion medium, urine and serum before MALDI TOF is different from earlier report. We further identified a smaller peptide (17-mer) seen in urine, which was not present in serum. On intake of dietary iron 45% increase in urine hepcidin was observed. We also observed that on BMP treatment the secretion of hepcidin peptide was dramatically increased in HU.7 cell line. MALDI-TOF MS could be used to study the correlation between hepcidin level and iron status under different physiological condition.

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Abbreviations

MALDI-TOF-MS, Matrix assisted laser desorption ionization time of flight mass spectrometry; SELDI-TOF-MS, surface-enhanced laser desorption ionization time of flight mass spectrometry; ACTH, Adrenocorticotropic hormone.

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