ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Motif Analysis of Avian Liver Expressed Antimicrobial peptide 2 (LEAP2)

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ABSTRACT:
Liver-expressed antimicrobial peptide 2 (LEAP2) is the second blood-derived peptide identified from human blood and shows antimicrobial activity and predominant expression in the liver. It is a cysteine-rich antimicrobial peptide characterized by the presence of a four cystein conserved motifs and two intracellular disulphide bridges. *In silico* analyses of avian LEAP2 sequences were performed in the study. Mature peptide regions were found to be highly conserved in comparison to pro-domain and signal sequence. Majority of the avian samples analyzed possessed RQRR motif at the cleavage site between pro-region and mature region. Beyond microbial killing, LEAP2 of *Aquila chrysaetos canadensis* has been found to play additional roles in signaling pathway, oocyte, follicular development, cytokine activity and in apoptosis regulation. Higher conservation in the amino acid sequences of LEAP2 among birds suggests the significant role of this molecule in avian evolution.

KEYWORDS: LEAP2*, Motif, Antimicrobial, Pro-domain, Signal sequence*

INTRODUCTION:
Antimicrobial peptides (AMPs) are crucial elements of the host non-specific innate immune system which plays a major role as natural antibiotics by providing first line defense against microbial incursion[1,2]. AMPs are generally short, cationic, amphipathic and structurally diverse molecules that are widely present in nearly all life forms. These molecules display extensive and strong antimicrobial activity against microbial assault primarily by destroying membrane integrity of the invading pathogens[3,4]. In addition, AMPs aid in host defense via immuno modulatory functions by activating innate and adaptive immune cells; hence they are also known as host defense peptides (HDPs)[5]. Majority of the AMPs are encoded by specific genes, and numerous structurally diverse HDPs usually present in a single species[6]. Different groups of AMPs reported in birds include avian β-defensin (AvBD), cathelicidin, liver-expressed antimicrobial peptide 2 (LEAP2) and NK-lysin which endow them with tremendous innate immunity[7].

Received on 25.01.2018 Modified on 26.02.2018
Accepted on 29.03.2018
Research J. Pharm. and Tech 2018; 11(6): 2496-2502.
DOI: 10.5958/0974-360X.2018.00461.4

Liver-expressed antimicrobial peptide 2 (LEAP2) is the second blood-derived peptide identified from human blood and shows antimicrobial activity and predominant expression in the liver. This peptide belongs to cysteine-rich family of AMPs characterized by the presence of a four cystein conserved motif and two intracellular disulphide bridges[8,9]. LEAP2 has been characterized from few organisms like human, guinea pig, fishes, monkey, pig, mouse, cow, and chicken[8,10,11,12]. In chicken, LEAP2 gene is reported to be localized on chromosome 13 and the gene structure includes three exons and two introns[13]. Like any other cationic HDP, LEAP2 also possess a signal peptide, a pre-propeptide and an active mature peptide[14]. The RXXR motif at cleavage site between prodomain and mature region of LEAP2 is conserved throughout vertebrates[15].

Chicken LEAP2 (cLEAP2) gene encodes a 76 amino acid prepropeptide which is enzymatically processed by furin family of propeptide convertase to release mature peptide of 40 amino acids. Primary structure analysis of cLEAP2 specified the presence of RLKR motif ahead of the cleavage site separating the proregion and the mature peptide. The expression of cLEAP2 in different epithelial tissues suggests its role in preventing the microbes from interacting with epithelial surfaces and

tissue invasion. cLEAP2 exhibits antimicrobial activity against *Salmonella spp., Streptococcus spp*. and *Staphylococcus spp*.[10,16]. Studies suggest that in chicken, human and several species of fishes LEAP2 gene express several splicing variants as a result of alternative splicing[10, 17, 18]. It is possible that LEAP2 gene employs splicing as a major mechanism of its regulation. Howard *et al* (2010) reported that alternative splicing of human LEAP2 in diverse gastro-intestinal tissues generates splice variants predicted to be both secretory and intracellularly located peptides. The occurrence of intracellular variants of LEAP2, devoid of signal sequence recommends that LEAP2 may also have additional functions other than antimicrobial activity.

Multiple sequence alignment (MSA) is an indispensable tool in biological sequence analysis that aids to establish evolutionary distance and phylogenetic relationship among organisms. The detection of similar motifs in evolutionarily linked proteins would help in the prediction of structurally or functionally significant positions[19,20,21]. In the present study, we analyzed the LEAP2 protein sequences of birds including chicken (already characterized) which were retrieved from NCBI to reveal the similarities and differences of amino acid residues of LEAP2 across the group. Elucidation of the possible functional attribute of LEAP2 was also performed using BLAST2GO software.

MATERIALS AND METHODS:

The protein sequences of 50 avian Liver expressed antimicrobial peptide 2 were retrieved from NCBI [22].Redundancy of the sequences was removed by using PERL and AWK script. To analyse the potential cleavage site of the signal peptides of these retrieved sequences SignalP [23]was employed. Multiple sequence alignments and comparison of the avian LEAP2 sequences were performed with Bio-Edit version 7.1.9. [24]. Molecular weight, Isoelectric point and cationicity of the mature peptides were calculated using EMBOSS PEPSTAT[25]. Functional elucidation of retrieved sequences was carried out using BLAST2GO software [26].

RESULTS AND DISCUSSION:

Most of the avian LEAP2 sequences analysed using SignalP were predicted to have a 22 residue secretory signal peptide as previously reported in humans [8]. Out of the 50 sequences, signal peptides were found to be missing in sequences of 2 birds, *Anas platyrhynchos* (XP_021131249.1) and *Anser cygnoides domesticus* (XP_013031265.1) which possess extra residues at the N terminal when compared to other selected organisms. After removal of the extra N terminal region, Signal P predicted the presence of signal peptide. Further analysis is needed to investigate the role of these extra sequences.

Multiple sequences alignment result showed the presence of 4 conserved cystein residues which are known to form 2 disulphide bridges in the mature peptide sequence. In chicken, it has been reported that cLEAP2 mature peptide consists of 40 amino acids[13]. Except *Leptosomus discolor* (XP_009946223.1), all others possess mature peptide of 40 amino acids. Studies suggest that LEAP2 genes from different vertebrates are structurally similar, but distinct. Several conserved residues were also observed. Generally LEAP2 is known to possess a conserved motif, RXXR at the cleavage sites between pro-petide and mature peptide[15]. In human LEAP2 gene, acidic region DDSE and basic amino acid region RKRR were reported at positions 56-59 and 66-69 respectively[8]. Bioedit alignment result showed that almost 37 sequences possess RQRR motif before the mature peptide (Table 1). Other motifs observed are RFRR, RQKR, RLPR, RLKR, RHRR, RARR, RPRR and RRRR. In *Tinamus guttatus* (accession number: XP_010211017.1), this motif could not be identified. Like DDSE motifs in humans, DNSE was observed in almost all the selected birds except four (XP_011598055.1, XP_010211017.1, XP_008493727.1, and XP_008934879.1). RKNR motif was also found to be conserved in most cases. It has been reported that the initial 6 amino acid residues; “MTPFWR” at the N terminus of the mature peptide were highly conserved in birds and mammals[27]. These six residues were found to be identical in all the subjects selected for this study without any variation. Multiple sequence analysis revealed extremely conserved nature of avian LEAP2. Mature region are more conserved than signal peptide and pro-region. Higher conservation in the primary structure of LEAP2 among vertebrates suggests that LEAP2 may also have additional functions other than defense[17].

Zhang *et al.,* (2004) reported the physiochemical properties like molecular weight (MW), isoelectric point (PI) and cationicity of mature LEAP2 from various avian species including chicken. These physiochemical characteristics of the mature portion of the selected avian species were found to be almost similar to chicken (MW-4593 Da, PI-8.95 and cationicity-4). The mature peptide of *Leptosomus discolors* (XP_009946223.1) only showed variation as mentioned in **Table 2**. The similarity in features such as cationicity, isoelectric point and molecular weight of mature LEAP2 of birds with other species suggest that functionally they will be equally effective as antimicrobial agents[15]

Table 1: Various RXXR motif distribution in avian LEAP2 sequences

SL No	Number of sequences	Regions observed	Accession Number
1	37	RQRR	OWK52248.1, XP_021131249.1, XP_010397908.1, XP_009284363.1, XP_015497221.1, XP_009089803.1 XP_008926289.1, XP_017678407.1, XP_005053749.1 XP_014803370.1, XP_014743787.1, XP_005418876.1 XP_005523754.1, XP_005493117.1, XP_013801351.1 XP_013031265.1, XP_009329739.1, XP_010307126.1 XP_010289282.1, XP_010196885.1, XP_010183881.1 XP_010080867.1, XP_010170654.1, XP_010156579.1 XP_009999524.1, XP_010013007.1, XP_009946223.1 XP_009916431.1, XP_009888985.1, XP_009814466.1 XP_009696346.1, XP_009575282.1, XP_009462624.1 XP_009502312.1, XP_009486894.1, XP_009082681.1 XP_008493727.1
2	2	RERR	XP_005512492.1, OPJ87903.1
3	3	RQKR	BAU36332.1, XP_019476046.1, XP_009645163.1
4	1	RLKR	NP_001001606.1
5	1	RLPR	XP_011598055.1
6	1	RHRR	XP_010122767.1
7	2	RARR	XP_010136939.1, XP_009930730.1
8	1	RPRR	XP_009961794.1
9	1	RRRR	XP_008934879.1

Table 2: Characteristics of mature LEAP2 of birds

Sequ. ID	Residues	Molecular weight	Charge	Isoelectric Point
OWK52248.1	40	4446.24	4	8.9858
XP_021131249.1	40	4593.37	4	8.9856
XP_005512492.1	40	4577.37	4	8.9856
XP_010397908.1	40	4446.24	4	8.9858
OPJ87903.1	40	4577.37	4	8.9856
BAU36332.1	40	4593.37	4	8.9856
XP_019476046.1	40	4593.37	4	8.9856
XP_009284363.1	40	4607.4	4	8.9856
XP_015497221.1	40	4446.24	4	8.9858
NP_001001606.1	40	4593.37	4	8.9856
XP_009089803.1	40	4446.24	4	8.9858
XP_008926289.1	40	4460.26	4	8.9858
XP_017678407.1	40	4460.26	4	8.9858
XP_005053749.1	40	4446.24	4	8.9858
XP_014803370.1	40	4593.37	4	8.9856
XP_014743787.1	40	4446.24	4	8.9858
XP_005418876.1	40	4446.24	4	8.9858
XP_005523754.1	40	4446.24	4	8.9858
XP_005493117.1	40	4446.24	4	8.9858
XP_005440736.1	40	4566.31	3	8.5364
XP_013801351.1	40	4629.45	4.5	8.9795
XP_013031265.1	40	4593.37	4	8.9856
XP_011598055.1	40	4634.45	6	9.0718
XP_009329739.1	40	4607.4	4	8.9856
XP_010307126.1	40	4593.37	4	8.9856
XP_010289282.1	40	4607.4	4	8.9856
XP_010211017.1	40	4531.29	4	8.9856
XP_010196885.1	40	4579.34	3	8.5342
XP_010122767.1	40	4563.39	4	8.9777
XP_010183881.1	40	4619.46	4	8.9856
XP_010080867.1	40	4563.35	4	8.9856
XP_010170654.1	40	4607.4	4	8.9856
XP_010156579.1	40	4607.4	4	8.9856
XP_010136939.1	40	4607.4	3	8.5342
XP_009999524.1	40	4588.35	3.5	8.5378
XP_009961794.1	40	4607.4	4	8.9856
XP_010013007.1	40	4538.29	3	8.5342
XP_009946223.1	78	9210.72	11	11.793
XP_009930730.1	40	4617.48	4	8.9856
XP_009916431.1	47	5428.48	5	8.8124

XP_009888985.1	40	4593.37	4	8.9856
XP_009814466.1	40	4607.4	4	8.9856
XP_009696346.1	40	4607.4	4	8.9856
XP_009645163.1	40	4607.4	4	8.9856
XP_009575282.1	40	4607.4	4	8.9856
XP_009462624.1	40	4593.37	4	8.9856
XP_009502312.1	40	4633.48	4	8.9856
XP_009486894.1	40	4593.37	4	8.9856
XP_009082681.1	40	4444.22	4	8.9942
XP_008493727.1	40	4621.43	4	8.9856

Table 3: Functional Elucidation of LEAP2 Sequence Results

Sequ name	Description	length	hits	E-value	sim mean	# GO	GO Ids	GO Names
XP_009916431.1	Liver-expressed antimicrobial peptide 2	77	20	1.60195E-49	93.15	1	P:GO:0042742	P:defense response to bacterium
OWK52248.1	liver-expressed antimicrobial peptide 2	77	20	1.84393E-28	88.9	1	P:GO:0042742	P:defense response to bacterium
XP_021131249.1	liver-expressed antimicrobial peptide 2	114	20	1.06631E-64	90.5	1	P:GO:0042742	P:defense response to bacterium
XP_005512492.1	liver-expressed antimicrobial peptide 2	74	20	1.70864E-22	99.75	1	P:GO:0050829	P:defense response to Gram-negative bacterium
XP_010397908.1	liver-expressed antimicrobial peptide 2	81	20	2.12718E-28	83.05	1	P:GO:0042742	P:defense response to bacterium
OPJ87903.1	liver-expressed antimicrobial peptide 2	74	20	1.46763E-22	99.85	1	P:GO:0050829	P:defense response to Gram-negative bacterium
BAU36332.1	liver-expressed antimicrobial peptide 2	76	20	1.67363E-31	87.4	1	P:GO:0050829	P:defense response to Gram-negative bacterium
XP_019476046.1	liver-expressed antimicrobial peptide 2	76	20	1.8047E-48	86.3	1	P:GO:0050829	P:defense response to Gram-negative bacterium
XP_009284363.1	liver-expressed antimicrobial peptide 2	77	20	4.02913E-49	89.8	1	P:GO:0042742	P:defense response to bacterium
XP_015497221.1	liver-expressed antimicrobial peptide 2	77	20	6.95285E-41	89.2	1	P:GO:0042742	P:defense response to bacterium
NP_001001606.1	liver-expressed antimicrobial peptide 2	76	20	7.17729E-40	87.6	1	P:GO:0050829	P:defense response to Gram-negative bacterium
XP_009089803.1	liver-expressed antimicrobial peptide 2	81	20	3.44668E-28	84.65	1	P:GO:0042742	P:defense response to bacterium
XP_008926289.1	liver-expressed antimicrobial peptide 2	75	20	8.42495E-40	88.2	1	P:GO:0042742	P:defense response to bacterium
XP_017678407.1	liver-expressed antimicrobial peptide 2	75	20	6.81385E-30	86.65	1	P:GO:0042742	P:defense response to bacterium
XP_005053749.1	liver-expressed antimicrobial peptide 2	81	20	1.04405E-26	84.85	1	P:GO:0042742	P:defense response to bacterium
XP_014803370.1	liver-expressed antimicrobial peptide 2	77	20	2.08735E-32	91.55	1	P:GO:0042742	P:defense response to bacterium
XP_014743787.1	liver-expressed antimicrobial peptide 2	77	20	1.06563E-28	87.35	1	P:GO:0042742	P:defense response to bacterium
XP_005418876.1	liver-expressed antimicrobial peptide 2	81	20	3.40908E-28	84.35	1	P:GO:0042742	P:defense response to bacterium

XP_005523754.1	liver-expressed antimicrobial peptide 2	77	20	6.80182E-41	89.2	1	P:GO:0042742	P:defense response to bacterium
XP_005493117.1	liver-expressed antimicrobial peptide 2	81	20	3.40908E-28	83.95	1	P:GO:0042742	P:defense response to bacterium
XP_013801351.1	liver-expressed antimicrobial peptide 2	87	20	3.53699E-45	89.15	1	P:GO:0042742	P:defense response to bacterium
XP_013031265.1	liver-expressed antimicrobial peptide 2	114	20	6.50757E-65	90.45	1	P:GO:0042742	P:defense response to bacterium
XP_011598055.1	liver-expressed antimicrobial peptide 2	103	20	3.29E-031	76.65	16	F:GO:0008083 P:GO:0043408 F:GO:0005160 P:GO:0060283 P:GO:0060395 P:GO:0042981 P:GO:0048468 C:GO:0005576 F:GO:0005125 P:GO:0042742 P:GO:0060016 P:GO:0001541 C:GO:0005615 P:GO:0001556 P:GO:0030509 P:GO:0010862	F:growth factor activity P:regulation of MAPK cascade F:transforming growth factor beta receptor binding P:negative regulation of oocyte development P:SMAD protein signal transduction P:regulation of apoptotic process P:cell development C:extracellular region F:cytokine activity P:defense response to bacterium P:granulosa cell development P:ovarian follicle development C:extracellular space P:oocyte maturation P:BMP signaling pathway P:positive regulation of pathway-restricted SMAD protein phosphorylation
XP_009329739.1	liver-expressed antimicrobial peptide 2	77	20	1.05951E-48	90.4	1	P:GO:0042742	P:defense response to bacterium
XP_010307126.1	liver-expressed antimicrobial peptide 2	77	20	9.22163E-42	93.05	1	P:GO:0042742	P:defense response to bacterium
XP_010289282.1	liver-expressed antimicrobial peptide 2	77	20	4.75076E-49	94	1	P:GO:0042742	P:defense response to bacterium
XP_010211017.1	liver-expressed antimicrobial peptide 2	115	20	3.12441E-62	96.95	1	P:GO:0050829	P:defense response to Gram-negative bacterium
XP_010196885.1	liver-expressed antimicrobial peptide 2	76	20	1.00829E-48	90.7	1	P:GO:0042742	P:defense response to bacterium
XP_010122767.1	liver-expressed antimicrobial peptide 2	77	20	3.85595E-49	91.2	1	P:GO:0042742	P:defense response to bacterium
XP_010183881.1	liver-expressed antimicrobial peptide 2	77	20	6.16177E-41	90.95	1	P:GO:0042742	P:defense response to bacterium
XP_010080867.1	liver-expressed antimicrobial peptide 2	77	20	1.64268E-39	92.3	1	P:GO:0042742	P:defense response to bacterium
XP_010170654.1	liver-expressed antimicrobial peptide 2	77	20	1.84185E-41	91.45	1	P:GO:0042742	P:defense response to bacterium

XP_010156579.1	liver-expressed antimicrobial peptide 2	74	20	2.58738E-46	91.4	1	P:GO:0042742	P:defense response to bacterium
XP_010136939.1	liver-expressed antimicrobial peptide 2	77	20	3.87192E-42	87.4	1	P:GO:0042742	P:defense response to bacterium
XP_009999524.1	liver-expressed antimicrobial peptide 2	75	20	5.62652E-48	90.2	1	P:GO:0042742	P:defense response to bacterium
XP_009961794.1	liver-expressed antimicrobial peptide 2	77	20	1.02273E-36	89	1	P:GO:0042742	P:defense response to bacterium
XP_010013007.1	liver-expressed antimicrobial peptide 2	77	20	5.92284E-43	91.05	1	P:GO:0042742	P:defense response to bacterium
XP_009946223.1	liver-expressed antimicrobial peptide 2	116	20	3.50195E-51	87.55	1	P:GO:0042742	P:defense response to bacterium
XP_009930730.1	liver-expressed antimicrobial peptide 2	77	20	1.48928E-48	90.4	1	P:GO:0042742	P:defense response to bacterium
XP_009888985.1	liver-expressed antimicrobial peptide 2	77	20	6.92732E-35	90.85	1	P:GO:0042742	P:defense response to bacterium
XP_009814466.1	liver-expressed antimicrobial peptide 2	77	20	5.66349E-49	92.25	1	P:GO:0042742	P:defense response to bacterium
XP_009696346.1	liver-expressed antimicrobial peptide 2	77	20	3.22138E-29	91.2	1	P:GO:0042742	P:defense response to bacterium
XP_009645163.1	liver-expressed antimicrobial peptide 2	77	20	1.29984E-49	91.95	1	P:GO:0042742	P:defense response to bacterium
XP_009575282.1	liver-expressed antimicrobial peptide 2	77	20	9.73546E-35	91.55	1	P:GO:0042742	P:defense response to bacterium
XP_009462624.1	liver-expressed antimicrobial peptide 2	77	20	1.68645E-41	92.95	1	P:GO:0042742	P:defense response to bacterium
XP_009502312.1	liver-expressed antimicrobial peptide 2	77	20	1.85361E-31	90.05	1	P:GO:0042742	P:defense response to bacterium
XP_009486894.1	liver-expressed antimicrobial peptide 2	75	20	1.14462E-36	88.25	1	P:GO:0042742	P:defense response to bacterium
XP_009082681.1	liver-expressed antimicrobial peptide 2	77	20	2.15331E-27	85.55	1	P:GO:0042742	P:defense response to bacterium
XP_008493727.1	liver-expressed antimicrobial peptide 2	77	20	1.29028E-24	93.6	1	P:GO:0050829	P:defense response to Gram-negative bacterium

Several AMPs execute additional physiological functions apart from their primary role in providing immunity against microbial pathogenicity.LEAP1,the first blood derived defense peptide from the liver has been recognized to play a major role in iron homeostasis in mammals[28,29]. Howard *et al.,* 2010 reported that human LEAP2 are not involved in iron homeostasis, cell proliferation and chemotaxis. Accurate functional annotations may unearth new functions that facilitate to identify protein pathways or cascades which will eventually lead to improved understanding of protein-protein interactions in organisms. BLAST2GO results revealed that avian LEAP2 plays a significant role in defense response (Table 3). Moreover, *Aquila chrysaetos canadensis*, LEAP2 (XP_011598055.1) exhibits molecular functions beyond microbial killing viz., growth factor activity, cytokine activity and transforming growth factor beta receptor binding. This peptide is also found to be involved in biological processes such as SMAD protein signal transduction, regulation of apoptosis, cell development, regulation of MAPK cascade, negative regulation of oocyte development, ovarian follicle development, granulose cell development, BMP signaling pathway and positive regulation of pathway restricted SMAD protein phosphorylation.

In conclusion, the present insilico study suggests the significance of avian LEAP2 in innate immune system. The role of avain LEAP2 in protecting host against microbial invasion is required to be further investigated and validated. Advance studies on the structure–activity relationship of LEAP2 peptides will help us to comprehend their biological functions more evidently. Prediction of splice variants of each LEAP2 and functional elucidation of variants should be conducted.

ACKNOWLEDGEMENT:

The first author, Shalini K acknowledges the Council of Scientific and Industrial Research (CSIR), for the financial assistance.

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