High benzoic acid production in a grape-based Ayurvedic medicinal wine (asava)

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Fermentative production of benzoic acid is seldom reported and the present report serendipitously expressed it, though in small quantity (~30 mg/l). Lipoxygenases (LOXs) catalyse the stereo-specific insertion of molecular oxygen into arachidonic acid, leading to hydroxy derivatives as end products. Serendipitously found LOX inhibitor was benzoic acid, produced in Ayurvedic-fermented medicated wine, pippalyasava. The present study reports the significance of the polyphenol derivatives in a traditional Ayurvedic therapeutic preparation where LOX inhibition might have a role, besides its importance in fermented benzoic acid production.

Keywords: Ayurvedic-fermented wine, benzoic acid, inflammation, LOX inhibitors, polyphenol metabolites.

THE lipoxygenases (LOXs), a family of non-haeme iron-containing dioxygenases that catalyse the stereospecific insertion of molecular oxygen into arachidonic acid[1], are classified with respect to their positional specificity of arachidonic acid oxygenation. They are referred to as 5-, 8-, 12- and 15-LOX[2,3]. The biological properties of human lipoxygenases (hLOXs) have been extensively studied due to their involvement in several diseases such as bronchial asthma, allergic rhinitis, inflammatory skin diseases, rheumatoid arthritis (5-hLOX)[4], cancer, osteoporosis, cardiovascular diseases (15-hLOX)[5,6], breast cancer and psoriasis (12-hLOX)[7,8].

Treating of different inflammatory conditions[9–11] involves the use of agents such as aspirin-like nonsteroidal anti-inflammatory agents, glucocorticoids, methotrexate and cyclophosphamide. These agents generally produce side effects, and are not suitable for chronic inflammatory symptoms[12,13]. Hence, new agents, free of side-effects for treating inflammatory conditions are needed.

The present search for new LOX inhibitors has led to the serendipitous identification of benzoic acid produced in Ayurvedic-medicated wine, pippalyasava. The present study reports the significance of the polyphenol derivatives reports in a traditional Ayurvedic therapeutic preparation, where LOX inhibition might have a role. Since the fermentative production of benzoic acid is industrially significant[14], we report here the details of its serendipitously observed presence in the Ayurvedic fermentation[15].

The LOX inhibition assay was performed with the methanolic extract of pippalyasava[15]. The inhibitory activity was measured by a modified spectrophotometric method[16]. LOX (EC 1.13.11.12) type I-B (Soybean), linoleic acid and all other compounds were purchased from Sigma (Sigma-Aldrich, UK) and used without further purification.

Enzyme solution of 0.93 μM and substrate, linoleic acid solution of 0.32 mM were prepared in 0.2 mM borate buffer of pH 9.0. Purified compound was prepared at a concentration of 20 μM. The assay mixture was made of 50 μl of LOX and 360 μl of the substrate. The final volume was made up to 2 ml with 1.59 ml borate buffer. The activity of LOX was measured by the formation of hydroperoxy eicosatetraenoic acid which was monitored at 234 nm on a spectrophotometer, HITACHI U 2900.

Thin layer chromatography was performed using silica gel 60 F254 plates (200 μm, Merck, Germany) and CAMAG-UV visualizer. Column chromatography was conducted on silica gel 60 (60–120 mesh). HPLC was carried out on Shimadzu instrument using Phenomenex C18 250 × 4.6 mm column. The mobile phase used was acetonitrile : 1% glacialacetic acid in water (9 : 1) and detection at UV 280 and 343 nm. NMR spectral analyses and automated single crystal diffractometer (Bruker Kappa Apex II. ShellX structure solution package) were carried out for the structure elucidation.

^{13}C-NMR were recorded on BRUKER NMR spectrometer with $CDCl_3$ as internal standard. ^{13}C NMR spectra were measured at 400 MHz. IR spectra in KBr disk were recorded on Shimadzu 8900 FTIR spectrophotometer. LC separation was performed on a Waters AQUITY UPLC system (Waters Corp., Milford, USA) using AQUITY BEH C18 (100 mm × 2.1 mm × 1.7 μm) column. Injection volume 10 μl and isocratic elution by acetonitrile and 0.1% formic acid in water in the ratio 90 : 10 at a flow rate of 0.4 ml/min were given.

The Ayurvedic fermented herbal wine, pippalyasava was analysed for the presence of LOX inhibitor. A fraction responsible for the LOX inhibitory activity (Figure 1) was separated. The compound separated from the LOX inhibitory fraction and analysed by single crystal X-ray diffraction method, was benzoic acid (see Supplementary data I online). The NMR spectra (see Supplementary data II, III and IV online) peaks at 7.5 and 7.6 indicated the aromatic moiety, and in between 8 and 9 showed the phenolic OH proton. The FTIR spectra (see Supplementary data V online) peak in 3000 and above range represented the C–H linkage. Peak at 1701 indicated the carboxylic acid. The MS data also supported these findings (see Supplementary data VI; Figure S1 online). Peak at 77 showed

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the breakage peak of benzene ring. Peaks at 105 and 122 showed the corresponding breakage of CO and OH groups. NMR and IR analyses also showed that the compound responsible for the LOX inhibition was benzoic acid, a known LOX inhibitor[17].

For finding out whether the compound benzoic acid is a degradation product of fermentation or not, we compared the HPTLC fingerprint (see Supplementary Data VII online) of methanolic extract of starting materials of pippalyasava with the methanolic extract of the fermented pippalyasava. The HPTLC fingerprint confirmed that the compound benzoic acid was a fermentation product.

The starting materials of pippalyasava, prior to fermentation, did not show the presence of benzoic acid, as evidenced from LC MS analysis (see Supplementary data VI, Figure S2 online).

In short, the results showed the fermentative production of benzoic acid by Ayurvedic fermentation protocol, which is significant industrially as well as medicinally. Fermentative production of benzoic acid is seldom reported[14]. The Ayurvedic fermentative protocols consist of highly complex input materials and microbial consortia[15]. Apart from the evidences presented above for the fermentative production of benzoic acid, at present, we are unable to present its line of production because of the complex nature involved in the process *per se*. However, the present report shows the fermentative production of benzoic acid. Fermentation of pippalyasava produced an average of ~30 mg/l benzoic acid. This is six times higher than an earlier reported benzoic acid production in wine without adding any specific substrate[18].

This study is related to the fermentative production of benzoic acid which is industrially very significant. Also, since it may be present in fermented herbal medicines, such fermented products may deliver higher therapeutic effects mediated through LOX inhibition. In the recent past, the anti-inflammatory properties of natural products or ingredients of traditional medicines and health foods and their specific compounds have been extensively studied for their therapeutic significances. However, the solubility of such compounds, in most cases is limited. Owing to their poor solubility, it is questionable whether their bioavailability could be accounted for their pharmacological effects. However, the polyphenols in health foods or drinks are readily metabolized to phenolic acids and aldehydes by the microflora of the intestines. This increases the possibility that such metabolites, rather than the original natural products or food ingredients, might be responsible for their anti-inflammatory properties[19]. However, in the case of fermented herbal medicines, the fermenting organisms may mimic the role of gut flora to the extent of modifying the compounds present in polyherbal drugs to deliver better soluble metabolites and enhance their therapeutic potentials[20,21]. The anti-inflammatory property of Ayurvedic therapeutic products, particularly medicated wines, might be partly due to the derivatives of polyphenols present in them. The presently expressed production of benzoic acid by fermentation may be significant only in therapy. However, the principle involved, together with the microorganisms, could be exploited in the industry also for fermentative production of benzoic acid.

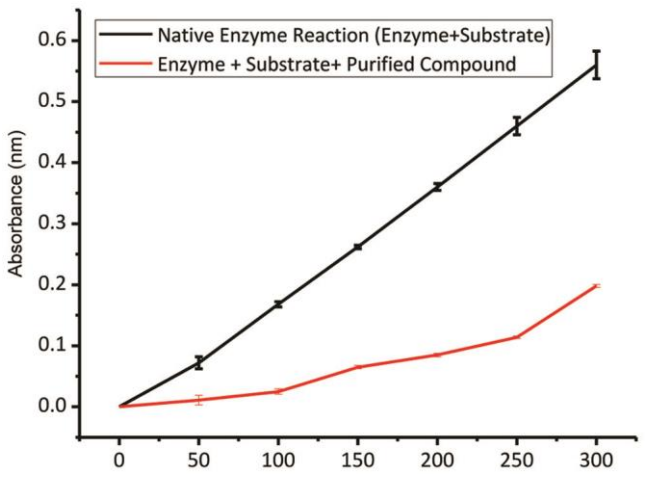

Figure 1. Graph showing the lipoxygenase inhibitory effect of purified compound (benzoic acid, average of six sets of experiments) from methanolic extract of 'Pippalyasava'.

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In silico characterization of ferritin-1 chloroplast targeting peptide encoding sequence in chickpea and pigeonpea

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Chloroplast is an important organelle and a hub of photosynthetic reactions that occur in all plants. Majority of the chloroplast proteins are encoded by the nucleus and then transported into the chloroplast. The chloroplast transport is aided by leader sequence, chloroplast targeting peptide (cTP) that directs the proteins into the chloroplast. We identified the genes encoding chloroplast targeting proteins from chickpea (*Cicer arietinum*) and pigeonpea (*Cajanus cajan* and *Cajanus cajanifolius*) genomes. All the sequences were aligned, conserved motifs were identified and their phylogeny was deciphered. In this study, structural and functional features of chloroplast transit peptides of Ferritin-1 and its putative role in chloroplast targeting have been examined. The study would impart better understanding of targeting heterologous proteins to chloroplasts in pulses.

Keywords: Chloroplast targeting peptide, domains, ferritin-1, motifs, transgenes.

THE chloroplast is the site of photosynthesis that supports the life of most of the living organisms. Chloroplast targeting has emerged as an important tool for heterologous expression of transgenes in plants. The targeting of transgenes into the chloroplast by using a suitable strategy with an efficient expression cassette containing all the required elements has been the need of the day. Majority (90%) of the 3000 different proteins necessary for fully functional chloroplasts are known to be encoded by nuclear DNA. These proteins are synthesized in their precursor forms with an amino-terminal signal peptide called the transit peptide[1]. A signature peptide sequence (chloroplast target peptide) drives protein into chloroplast and gets cleaved off after reaching target destination, delivering the protein to its destined cellular compartment[2]. Chloroplast targeted expression of genes such as anthranilate synthase for increasing tryptophan production in maize has been achieved using novel constructs encoding chloroplast transit peptide[3]. Looking into the importance of cTP sequences in localizing transgenes, this study is based on the isolation of three cTP candidate sequences from chickpea and pigeonpea genome and their structural characterization.

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Table 1. Ferritin 1 transit peptide sequences of different crops retrieved from Uniprot database and the Ferritin 1 chloroplast transit peptide sequence isolated from chickpea and pigeonpea

Peptide name	Uniprot ID	Transit peptide sequence
Ferritin 1 – *Glycine max*	P19976	MALAPSKVSTFSGFSPKPSVGGAQKNPTCSVSLSFLNEKLGSRNLRVCA
Ferritin 3 – *Vigna unguiculata*	O65100	MALSCSKVLTFSLSSVVGGDDAKKKLSLCSSSSLSASVNGGGSRNMRVCAAASN
Ferritin 1 – *Pisum sativum*	P19975	MALSSSKFSSFSGFSLSPVSGNGVQKPCFCDLRVGEKWGSRKFRVSA
Ferritin – *Malus baccata*	Q94FY2	MALAPSKVSTFSGFSPKPSVGGAQKNPTCSVSLSFLNEKLGSRNLRVCA
Ferritin – *Phaseolus vulgaris*	P25699	MALAPSKVSPFSGFSLSDGVGAVRNPTCSVSLSFLNKKVGSRNLGVSA
Ferritin 1 – *Arabidopsis thaliana*	Q39101	MASNALSSFTAANPALSPKPLLPHGSASPSVSLGFSRKVGGGRAVVVA
Ferritin 1 – *Brassica napus*	Q96540	MASKALSSFTAKPAVSLLPHGVSSSASPSVMSLSFSRHTGGRGVVAA
Ferritin 1 – *Nicotiana tobaccum*	Q8RX97	MLLKAAPAFALLNTQGENLSPLFSSSKSFSPKNGNRFVVSASKAT
Ferritin 1 – *Zea mays*	P29036	MMLRVSPSPAAAVPTQLSGAPATPAPVVRVAAPRGVASPSAGAACR
Ferritin 1 – *Cajanus cajan* (Asha)		MALFSSSKVSSFSGFSLSPISGNNLKKPTLSFCSCLRVNEKWGSRKLSVCAATTVPLT GVIFEPFEEVKKDNLAVPIVP
Ferritin 1 – *Cajanus cajanifolius* (ICP 15629)		MALFSSSKVSSFSGFSLSPISGNNLKKPTLSFCSCLRVNEKWGSRKLSV CAAILSSFNWAIYLNHLRRIRRIILLFQLCX
Ferritin 1 – *Cicer arietinum* (DCP 92-3)		MALFSSSKVSSFSGFSLSPISGNNLKKPTLSFCSCLRVNEKWGSRKLSV CAATTVPLTGVIFEPFEEVKKDNLAVPIVP

Chloroplast targeting proteins along with the predicted transit peptide sequences were retrieved from Uniprot (http://www.uniprot.org/). Heterologous transit peptide coding sequences of chloroplast targeted proteins from chickpea and pigeonpea genomes were retrieved from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The sequences obtained were translated and the peptides predicted for the presence of putative plastid targeting sequence using Predotar[4] (https://urgi.versailles.inra.fr/predotar/test.seq). The sequences were also subjected to cleavage site predictions using ChloroP[5] (http://www.cbs.dtu.dk/services/ChloroP/) and TargetP[6] (http://www.cbs.dtu.dk/services/TargetP/). Sequence alignment and phylogeny analysis was performed using Bioedit and MEGA6 (ref. 7) using Neighbour-Joining algorithm and a 1000 bootstrap value. Conserved motifs in cTPs were identified using MEME[8] (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). The presence of secondary structures was predicted using the Jpred4 (ref. 9) (http://www.compbio.dundee.ac.uk/jpred4). The analysis of residues found within the peptide sequence was done using the Phyre2 (ref. 10) (http://www.sbg.bio.ic.ac.uk/phyre2).

Most proteins involved in biosynthetic pathways of chloroplast are encoded by nuclear genes, made in the cytoplasm and localized in the chloroplast. Precise targeting of these proteins is essential for rendering their biosynthetic function. An N-terminal extension called the chloroplast targeting peptide (cTP) helps in this precise targeting of the proteins into the chloroplast. Bacterial genes when expressed in plants require to be attached to these cTP encoding oligos at the 5′ region. On average, the length of plastid transit peptides is 50 amino-acids but it varies between 13 and 146 amino acids[11]. The transit peptide encoding sequences from chickpea and pigeonpea translate to about 61 amino acids lying within the expected range (Table 1).

The chloroplast targeting peptide encoding sequences from chickpea and pigeonpea ferritin 1 gene were found to be highly conserved and closely associated with cTP from *Pisum sativum*. No sequence variation was found among the cTPs from wild and cultivated pigeonpea genotypes (Figure 1 *a*). The Predotar software has predicted the presence of chloroplast targeting sequence in our submitted sequence with a probability of 0.63–0.69 showing a 70% likelihood of the isolated sequences to be targeted into chloroplast (see Supplementary Information Table S1 online). ChloroP 1.1 predicted presence of cTP in the sequences with a significant score of 2.75 for peptide cleavage site (CS) (Table 2). A score above 2 for CS represents the presence of authenticated CS in the test sequence. TargetP 1.1 predicted chloroplast localization of the isolated sequences with a high reliability class score of 2 (see Supplementary Information Table S2 online). The prediction of cTPs with above software individually was found to be of low accuracy than using a combination of cTP predictors as demonstrated in *Arabidopsis*[12] with 2,450 proteins.

Initially, the transit peptide was thought to be enriched by serine and threonine[13], but later they found the most abundant residue to be serine in *Arabidopsis* and alanine in rice. Serine has been found abundantly in the chickpea and pigeonpea ferritin 1 gene transit peptide and there are no threonine residues. The ferritin gene cTPs contain a conserved 'homology block' Gly-X-Arg- XXX-Val close to the cleavage or processing site (Figure 1 *b*). This study has found many residues of serine and few of phenylalanine with no arginine in the N-terminal region of the transit peptide sequence. A low content of arginines, together with a high abundance of proline and serine in the N-terminal portion, would suggest a chloroplast targeting domain[14]. The three motifs identified in the study correlate with the common structure and modular organization of cTPs as described earlier[15]. The first motif

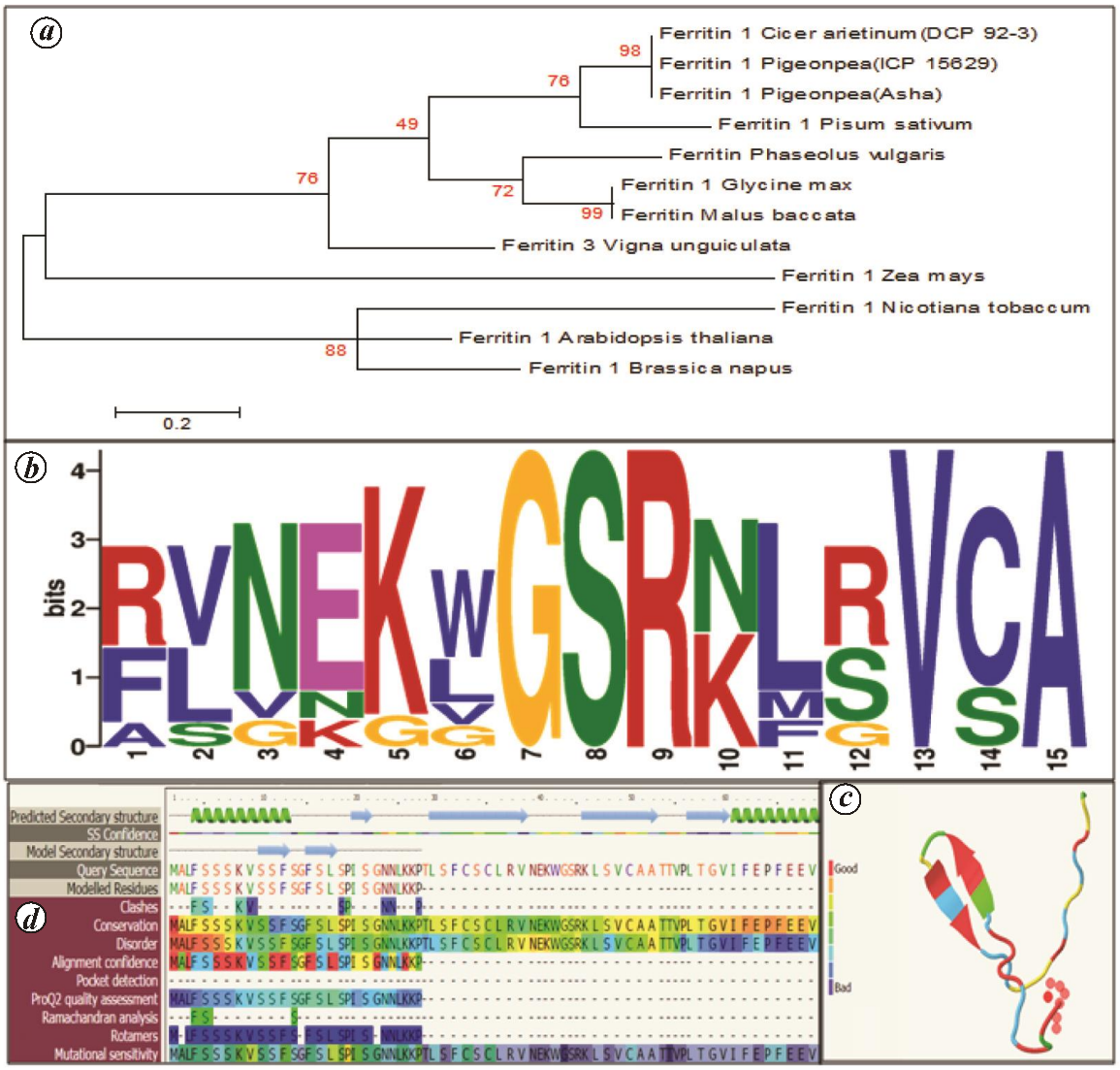

Figure 1. Sequence characterization of chloroplast targeting peptide. ***a***, Phylogenetic tree representing the relatedness of cTP from different crops including legumes constructed using neighbour–joining method; ***b***, Conserved 'homology block' found in the motif predicted at the cleavage site of cTP; ***c***, Protein structure representing the alignment confidence (AC) of cTP with modelled protein (red colour represents a good AC and blue colour represent bad AC); ***d***, Protein folding characteristics defined based on predicted secondary structures.

constitutes N-terminal residues beginning with M/A- and terminating with G/P, a second central motif lacking acidic residues but enriched in S/T and a C-terminal motif enriched in arginine have been observed. One α-helix and three β-sheet structures were predicted in the C-terminal region of the sequence beyond the position of 45 amino acids. The analysis of secondary structures depicted that out of 79 residues, 45 (57%) are part of repeating secondary structures (helices and beta-sheets) and the remaining residues are involved in making H-bonds, to turns, bends or irregular structures. Absence of membrane interacting structures with a minimal content of regular secondary or higher order structures suggest cTPs to be flexible peptides with relative tolerance to deletions and insertions in their central region. The ferridoxin 1 transit peptides contain two discontinuous alpha helical domains and are amphipathic in nature determined by the presence of hydroxylated amino acids (Ser, Thr) in accordance with the cTP properties reported. The Phyre2 analysis of the transit peptide sequence showed a N-terminal similarity hit by a transferase protein in the PDB database (Figure 1 *c*). The modelled residues were found to be present in the allowed regions of Ramachandran plot with a fairly good alignment confidence representing the reliability of pair-wise query-template alignment. A high degree of disordered regions were found in the

Table 2. ChloroP prediction of Ferritin 1 transit peptide sequences: *Y* represents presence of cTP (chloroplast transit peptide) of 61 amino-acids in the peptide sequence with a significant cleavage site (CS) score

	ChloroP prediction				
Peptide name	Length	Score	cTP	CS-score	cTP-length (amino acid)
Ferritin 1_Cp* (DCP 92-3)	236	0.55	Y	2.75	61
Ferritin 1_Pp* (Asha)	236	0.55	Y	2.75	61
Ferritin 1_Pp* (ICP 15629)	238	0.55	Y	2.75	61

*Cp – Chickpea, Pp – Pigeonpea.

N-terminal with ordered peptides in the C-terminal region of the cTP. This shows that the N-terminal regions are highly flexible and are distinct from irregular loop structures that are static in solution. Such flexible regions hinder protein crystallization supporting the role of cTPs in protein transport and not in the formation of functional domain of proteins. The modelled peptides were found to be devoid of active sites represented by none of the pockets detected in the functional analysis (Figure 1 *d*). The cTP sequence also rendered a moderate to low mutational sensitivity showing that the cTPs are mostly conserved for their function in protein transport into chloroplast[10].

Previous studies have shown that ribulose bisphosphate carboxylase small subunit promoter-transit peptide sequence (*rbcS-tp*) system facilitates the accumulation of the cry1A protein in chloroplast at a level equivalent to 2% of the total soluble proteins in transgenic crops[16]. The cTP sequences isolated from chickpea and pigeonpea genomes in this study could be used for targeting the genes with novel traits into the chloroplast representing a high-level expression system. Further, foreign proteins expressed at very low levels or toxic to the cytoplasm can be localized to intracellular compartments of chloroplast by fusing with cTP. It can also facilitate the genome editing of chloroplast genome and targeting of different cellular proteins into the chloroplast with higher and stable expression.

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