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Research paper

Discovery, synthesis, and *in vitro* evaluation of a novel bioactive peptide for ACE and DPP-IV inhibitory activity

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ABSTRACT

Biologically active or bioactive peptides are unique amino acid sequences found encrypted in food proteins. These peptides, upon hydrolysis, can exert positive physiological effects on human health, different from that of their native protein. These effects are brought about by their interaction with specific targets in the body, thereby, mimicking physiologically relevant peptides. Peptides are derived from food proteins, they are popular natural alternatives for the management of common metabolic disorders. In the present study, we aimed to identify bioactive peptide sequences (less than 3 kDa) from fat globule membrane protein (FGMP) hydrolysates of buffalo colostrum using a combination of empirical, computational and *in vitro* methods. The empirical approach aided in the identification of 89 FGMP peptides (*m/z*-415 to 2939) which were annotated and profiled for bioactivity. Few lead peptides were analyzed by molecular docking for the inhibitory potential of Angiotensin Converting Enzyme (ACE) and Dipeptidyl Peptidase-IV (DPP-IV). A heptapeptide (*m/z*-723.3) synthesized was found to inhibit ACE (IC_{50}: 74.27 μM) and DPP-IV (IC_{50}: 3.83 mM).

1. Introduction

Current interest in food science research is tremendous, chiefly due to the fact that food, in addition to providing basic nutrition, also endows physiological health benefits. Dietary proteins, in particular, are vital to maintaining a nutritional healthy state of an individual. In addition to being the principal source of amino acids, they are also the source of bioactive peptides [1]. Bioactive peptides are cryptides released due to hydrolysis of dietary proteins by means of gastrointestinal digestion or food processes [2]. They often exert their effect at the protein level by inhibiting metabolic enzymes, by influencing conformational changes in them, regulating genes directly or by interacting with transcriptional regulators. Likewise, peptides may directly interact and remove metabolites, thus, they also help to maintain homeostasis [2]. They share structural characteristics with endogenous peptides of the organism that function as hormones, neurotransmitters, or regulatory peptides, and hence, can interact with the same receptors and bestow functions beyond basic nutrition [3].

Some of the activities attributed to food-derived bioactive peptides include anti-hypertensive, anti-microbial, anti-oxidant, anti-carcinogenic, anti-inflammatory, opioid agonistic or antagonistic, and anti-viral properties, among others [4]. The immense scope of bioactive peptides makes them ideal candidates for the development of dairy formulations, nutraceuticals, functional foods, as well as therapeutics [3,5,6]. Moreover, they are presumably safer than conventional pharmaceuticals as they are derived from natural sources and produced by food-grade enzymes [2,7]. Their small size, specificity, selectivity, efficacy and minimal systemic toxicity gain them popularity over small molecule organic drugs [8]. However, they are not meant to replace pharmaceutical drugs but to complement disease-management by acting as natural prophylactic agents [9].

Lately, the potential of bioactive peptides to mitigate the pathologies of the metabolic syndrome has received a lot of interest [10–12]. The metabolic syndrome refers to the predisposition to hypertension, type II diabetes, deregulation of food intake and chronic inflammatory bowel disease [11]. In this context, the role of dietary peptide hydrolysates generated during digestion and their physiological implications on metabolic syndrome is the current hot spot in food science research [11,12]. Hence, numerous reports have characterized and evaluated bioactive peptides, especially for their potential effects on lipid metabolism, immunomodulatory, anti-thrombotic, anti-diabetic, analgesic, anti-oxidant and anti-

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inflammatory activities [10,13–16].

Among dietary proteins, milk/colostrum has been the best-mined resource for peptides with diverse functions. The unique nutritional and functional composition of milk marks it to be a commonly exploited source for bioactive compounds and functional food compounds [17]. Milk or colostrum is the primary source of nourishment to the newborn mammal. In addition to nutrition, it also bestows the infant with passive immunity, growth factors and beneficial components crucial for its development. The protein fraction of milk constitutes the most studied component for bioactivity, which, majorly comprises of casein, followed by whey and lastly, the proteins of the milk fat globule membrane or the mucins. Reports of bioactive proteins and peptides from milk have novel activities attributed to them, such as, anti-microbial, anti-oxidative, anti-thrombotic, anti-hypertensive, immunomodulatory and opioid properties [17–22], to name a few. Milk is an abundant source of anti-hypertensive peptides, which are usually derived from whey and casein fractions. The inhibitory effects of anti-hypertensive peptides on the Renin-Angiotensin-Aldosterone system (RAAS) either involved in the inhibition of ACE or renin [10,23]. Further, milk protein hydrolysates have also been extensively evaluated for their anti-diabetic potential with the hydrolysates showing inhibitory activity against key enzymes such as α-glucosidase and DPP-IV [24].

Of particular interest is the colostrum or early milk obtained from buffalo (*Bubalus bubalis*), which is known for its nutritional properties, as well as its compositional similarities to human colostrum [25]. It is characterized by higher content of solids, in addition to being a richer source of lipids, proteins, lactose, and minerals [26] with almost 11.42% more protein compared to bovine milk [27]. However, few studies have evaluated the bioactivities of proteins and peptides from buffalo colostrum [25,28–34]. In particular, the studies reporting bioactive peptides from FGMP are scarce. In the present study, we have attempted to identify novel multi-functional bioactive peptides from the FGMP of buffalo colostrum through a combination of both empirical and computational approaches.

2. Materials and methodology

2.1. Materials

Amicon ultracel-3K (Merck Millipore, Germany), C-18 solid phase extraction disks (3 Mempore), Pepsin, N-hippuryl-histidyl-leucine hydrate (HHL) (>98%, HPLC grade), sodium borate, boric acid, formic acid (~98%, MS grade), HCl, water & acetonitrile (≥99.93%, HPLC grade), pyridine (99.8%) and benzenesulphonyl chloride (99%) were purchased from Sigma-Aldrich (Now Merck). DPP-IV inhibitor screening assay kit was procured from Cayman Chemical, Ann Arbor, MI, USA. All the other reagents used were either of HPLC or analytical grade.

2.2. Methodology

2.2.1. Sample collection and isolation of fat globule membrane proteins

Colostrum was collected on the first day post-partum from healthy water buffaloes (*Bubalus bubalis*) in and around Mysore district, Karnataka, India. The samples were pooled and stored at −20 °C until analysis.

The colostrum and milk fat globule membranes (CFGM & MFGM) were extracted from cream and the total protein content of the fat globule membrane (FGM) was estimated as detailed by Brijesha and Aparna [25]. Briefly, the cream was used for the extraction of FGMPs, it was washed twice successively (4500×*g*, 10 min, 4 °C) with phosphate buffered saline (10 mM, pH 7.2) and distilled water. Subsequently, FGM was suspended in distilled water and crystallized for 20 h at 4 °C. The serum fraction was recovered from fat fractions by warming at 45 °C for 30 min. The total serum obtained was washed twice with distilled water (5000×*g*, 15 min, 4 °C) and acetone (1:4, v/v; 8000×*g*, 20 min, 4 °C) in order to remove the fat content, was freeze-dried and stored at −20 °C until further analysis.

2.2.2. Simulated gastric digestion of FGMPs

In vitro gastric digestion of FGM was performed with reference to the protocol put forth by Wu and Ding in 2002 [35] with minor modifications. Briefly, the FGMPs were dissolved in 3.5% KCl-HCl buffer (100 mM, pH 2), subsequently, pepsin was added (4%, w/w) and the assay tubes were incubated for 4 h at 37 °C. The reaction was terminated by boiling for 10 min followed by the addition of 2N NaOH. The assay tubes were then centrifuged (10,000×*g*, 30 min) and recovered through gel elution. The FGMPs hydrolysates were then filtered through the molecular weight cut-off (MWCO) membrane (3 kDa) and were desalted using C-18 solid phase extraction disks [36].

2.2.3. Identification of peptide profile of digested FGMPs sample using tandem mass spectrometry

The desalted and filtered FGMP hydrolysates (<3 kDa) were then analyzed by nano liquid chromatography-electrospray ionisation-tandem mass spectrometry (nLC-ESI MS/MS) through Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies) coupled to an Agilent 1260 Infinity capillary pump and 1260 Infinity nanoflow pump LC system as described by Brijesha and Aparna [25]. The samples were added on to a Polaris-HR chip-3 C18 reverse-phase separation column (150 mm × 75 μm, 3 μm, Agilent Technologies). The solvent system comprised of 0.1% formic acid (Solvent A) and acetonitrile (ACN: H_2O [90:10]) containing 0.1% formic acid (Solvent B). The peptides were analyzed using a linear gradient of 3%–97% solvent B for 90 min at a flow rate of 0.3 μl/min. The mass spectra were acquired in positive ion mode with the m/z-range from 100 to 3200. The data obtained was examined by the Mass Hunter software for LC/MS data acquisition, version: B.05.01. LC.

2.2.4. Cellular localization, functional annotation, and clustering of the identified FGMPs

The localization of identified proteins was investigated using Bologna Unified Subcellular Component Annotator (BUSCA) [37], a web-server that can predict protein subcellular localization and localization-related protein features. The identified protein sequences were uploaded onto the web server in FASTA format, followed by selection of taxonomic origin for the protein sequences; Eukarya-animal division was selected and submitted. Functional annotation of the proteins identified from nLC-ESI MS/MS was carried out by Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 [38] as described previously by Brijesha and Aparna [25].

The protein-protein interaction was analyzed and classified using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/) as described previously.

2.2.5. Profiling for bioactivity of generated peptides using BIOPEP database and in silico tools

The FGM peptide sequences with masses below 1 kDa were considered for assessment of their bioactive potential. Primarily, the peptides were analyzed for their biological activity by BIOPEP tool (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) using the "profiles of bioactivity" option in order to identify already reported bioactive peptide sequences. Subsequently, an *in silico*

assessment of the likelihood of a peptide sequence to be bioactive was carried out by means of software known as Peptide-Ranker (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php). It compares the given sequence against a multitude of bioactive peptide sequences already published in the literature.

2.2.6. Molecular docking of peptides to target proteins

Structure-based molecular docking study was then designed to the two target proteins selected on the basis of the results obtained in the previous *in silico* analyses. The entire study was carried out through Biologics suite of Schrödinger, the protocol of the same is described below.

2.2.6.1. Preparation of target protein. The X-ray crystallographic structure of human ACE (PDB-ID 1O86) chosen was selected for molecular docking and retrieved from RCSB-Protein Data Bank (www.rcsb.org; PDB). The structure comprised of ACE in complex with the well-known inhibitor, lisinopril having a resolution of 2 Å. Crystal structure of dipeptidyl peptidase-IV chosen for molecular docking analysis was with the ID-1WCY retrieved from the RCSB. This structure comprised of the human DPP-IV in complex with diprotin-A (Ile-Pro-Ile) with a resolution of 2.2 Å. The proteins were prepared through protein preparation wizard of Maestro software in the Biologics suite of Schrödinger. The preparation involved assignment of bond orders, missing hydrogens, missing loops, creating di-sulphide bonds, addition of missing chains and loops using Prime and deletion of water residues beyond 5 Å of the selected protein.

2.2.6.2. Preparation of ligands. The selected peptide sequences were sketched in Maestro 9.9 using 2D Sketcher, subsequently minimized using optimized potentials for liquid simulations (OPLS) 2005 force field and different conformations were generated using flexible sampling for optimum docking.

2.2.6.3. Generation of a suitable receptor grid. The receptor grid was specified around the active site region with reference to the binding site of inhibitor in both the protein complexes. The receptor grid scaling of Van der Waals radii was set at 0.8 and partial charge cut off value of 0.15. The side chains of the binding site residues were allowed to be flexible, to allow for interactions within this region.

2.2.6.4. Scoring through glide. Docking was performed using the Glide module with the prepared protein structures, to generate scores for each of the peptides that were sampled. In addition, the bound receptors, lisinopril and diprotin-A were scored in place for reference.

2.2.7. Synthesis of lead peptide

The lead peptide with potential bioactivity obtained from in silico data and glide score was synthesized through conventional 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase synthesis through a protocol adapted from Jimsheena [39]. Fmoc-L-Ile -2-chlorotrityl resin comprising of the first amino acid of the peptide in C-terminal was preloaded on an automatic peptide synthesizer (Model CS 136, CS Bio Co. San Carlos,CA).O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyl uronium hexafluoro phosphate and thioanisole, 1-hydroxybenzotriazole were used as peptide coupling and activating agents. Diisopropylethylamine was used as the neutralizing reagent in three-fold molar excess for three-fold molar excess of Fmoc-amino acids to activate the carboxyl group of the incoming amino acid. Before the removal of the resin, Fmoc was removed from the N-terminus of the assembled peptide using 20% (v/v) of piperidine in dimethyl formamide (DMF). Subsequently, the resin was washed with DMF, dichloromethane (DCM) and isopropanol. A concoction of cleaving as well as scavenging agents was prepared with the following composition TFA/ethanedithiol/water/phenol/thioanisol in a ratio of 8:0.25:0.5:0.75:0.5 (v/v) to cleave the peptide from chlorotrityl resin and remove the side-chain protecting groups in 50% TFA in DCM (v/v)/ethanedithiol/water/phenol/thioanisol 8:0.25:0.5: 0.75:0.5 (v/v) for 1 h at 25 ± 2 °C. The resin was filtered and ice-cold diethyl ether was added to extract the crude peptide precipitate and washed thrice with ice-cold diethylether. The residual ether was removed under pressure and the peptide was HPLC purified. The structure of the peptide product was confirmed by LC-MS.

2.2.8. Evaluation of ACE-inhibitory activity of the lead peptide

ACE activity was assayed by monitoring the release of hippuric acid from hippuryl-histidyl-leucine as previously described [40] in order to assess the inhibition by the lead peptide in vitro. One unit of ACE activity was defined as the amount of enzyme which released 1 μmole of HA per min at 37 °C and IC_{50} of the synthetic peptide was determined by plotting the log of peptide concentration on X-axis and percent inhibition on Y-axis.

2.2.9. Evaluation of DPP-IV-inhibitory activity of the lead peptide

The effect of the lead peptide on DPP-IV was analyzed *in vitro* by means of a fluorometric assay kit from Cayman Chemical. The assay was performed at 37 °C and carried out in duplicates, with the final volume of the assay being 100 μl/well. All reagents were equilibrated to room temperature before beginning the assay, except the enzyme. The synthesized peptide was dissolved in the assay buffer Tris-HCl 100 mM NaCl and 1 mM EDTA pH 8.0 at different concentrations. The substrate used was H-Gly-Pro-conjugated to amino-methyl coumarin and positive control inhibitor used was sitagliptin (100 μM). Fluorescence was monitored with the excitation wavelength of 360 nm and an emission wavelength of 460 nm. The graph was plotted with percent inhibition as a function of inhibitor concentration to determine the IC_{50} value in Graph Pad Prism (Version 7.04).

2.3. Statistical analysis

The data given in this paper are expressed as the mean ± SEM. The Statistical data was evaluated by using Graph Pad Prism software, Version: 7.04 (Graph Pad Software, Inc.). The IC_{50} value for the in vitro assays was determined by means of non-linear regression curve fit through the Graph Pad Prism software, Version: 7.04.

3. Results and discussion

3.1. Sample collection and isolation of fat globule membrane proteins

The total yield of the FGM isolated from milk and colostrum was found to be 2 and 8.4 g/L (dry weight) respectively. The protein concentration was estimated to be 500 and 800 μg/mg of FGM pellet.

The fat content in milk is dispersed in the form of small spherical globules enveloped by a membrane known as the MFGM [41]. This membrane prevents the enzymatic degradation and coalescence of the fat globules in milk which relatively constitute 1–4%. Often, research groups have focused mainly on casein and whey proteins for their bioactive potential [42–44]. Currently, the focus has shifted to FGMPs, with reports showing the anti-sarcopenia effect of human FGMP [44], while, buffalo FGMP exhibited anti-oxidant potential [25]. In this regard, no report has yet elucidated the

ACE-inhibitory and DPP-IV inhibitory potential of bioactive peptides derived from FGMP.

3.2. Simulated gastric digestion of FGMPs

Peptides derived from milk obtain their functionality only once they are released from their parent protein. Enzymatic hydrolysis, especially in vitro gastrointestinal digestion is a commonly used technique to generate the protein hydrolysates. In the current study, in vitro gastric digestion of proteins was chosen as the method for proteolysis, as, majority of the proteins of FGM are known to be hydrolyzed by pepsin [46]. The peptides thus obtained yielded 3.57 mg/ml (milk) and 2 mg/ml (colostrum) respectively. The lower yield of peptides in colostrum could be due to extensive glycosylation that is a characteristic of early milk.

In vitro gastric digestion primarily aimed at generation of peptides with putative bioactivity, and at the same time, it also helped to determine the occurrence and stability of the peptides. Although previous studies have focused on simulated gastric hydrolysis by means of simulated gastric fluid [46] and dynamic gastric model [47] of fat globule membrane proteins, only one study reported from our lab has focused on their bioactive peptides, on its antioxidant activity [25]. Bioactive peptides usually comprise of 2–20 amino acid residues, however, a majority of the known bioactive peptides are shorter than 10 amino acids [48], hence we focused on isolating peptides with mass less than 3 kDa for potential bioactivity.

3.3. Identification of FGMP peptides profile of pepsin-digested sample using tandem mass spectrometry

The FGMP hydrolysates were analyzed through nLC-ESI-MS/MS. The major proteins of the colostrum fat globule membrane such as xanthine dehydrogenase/oxidase and lactadherin were identified through the peptide profiles generated. The peptides identified varied from *m*/*z* of 415.234 (LIPWL) to a maximal mass of 2938.538 (SLSQSASGRKDSSAPPALEGKKGNWVPLSAW) in case of milk and (L) YIGGQDHF(Y) with 936.421 and (F)YPHVRLKAQTYE(L) with 1504.791 in case of colostrum. As many as 89 proteins were identified from both the samples. The data retrieved from nLC-ESI-MS/MS is detailed in Tables 1 and 2.

3.4. Cellular localization, functional annotation, and clustering of the identified FGMPs

For functional annotation of the proteins identified from nLC-ESI-MS/MS in DAVID software, the proteins were converted to gene IDs for annotation. The annotation was primarily categorized on the basis of cellular components, biological processes, molecular functions, INTERPRO, SMART, KEGG pathway, keywords, and their sequence features. Then, the list submitted was clustered based on co-occurrence, thereby grouping functionally related genes together. They were classified into 11 clusters based on biological similarity with enrichment scores ranging from 3.92 to 0.33. Each cluster was depicted in the form of a two-dimensional (2D) heat map as shown in Fig. 1 to indicate the corresponding gene-term associations positively reported (green) and those gene-term associations not reported yet (black).

The first cluster mainly depicted 27 secreted glycoproteins involved in signaling, also involving some of the FGM specific proteins such as butyrophilin, xanthine dehydrogenase among other proteins. The proteins that were annotated in this group were done so based on the presence of intra-chain disulfide bonds too. Representatives of the second gene cluster involved proteins with the Pleckstrin-homology domain and those associated with GTPase activities. The third cluster represented the maximal number of 45 proteins of extracellular, membrane and cytoplasmic origin. The fourth cluster depicted 13 proteins associated with mitochondrial and transit peptides. Similarly, the fifth cluster indicated 5 proteins involved with cell differentiation and development, whereas, the sixth cluster represented 8 proteins involved with the Golgi apparatus and the endoplasmic reticulum. The seventh cluster depicted 12 proteins associated with GTPase activity, binding, nucleotide phosphate binding, nucleoside triphosphate hydrolase, nucleotide binding group. The eighth cluster showed representatives involved in transcription, regulation, differentiation, and DNA-binding. The ninth cluster represented mainly lipoproteins, G-protein coupled receptors, which act as transducers, or receptors and possess lipid-binding regions and those that form an integral component of the plasma membrane, and/or possess an extracellular domain. The tenth and the eleventh clusters clustered genes associated with ATP-binding/nucleotide binding and the immunoglobulin-like domains/folds. The detailed functional annotation table is summarized in Supplementary Table 1. The annotation study thus allowed accounting for the proteins associated with the fat globule membrane of buffalo colostrum.

The protein-protein interaction was analyzed and classified using STRING database (http://string-db.org/) as indicated in Fig. 2B. The protein networks depicted by STRING involved nine major nodes (of high confidence), and five edges that represent associations between proteins that jointly contribute to a function. This network was predominated by rho GTPase-activating protein 15, rho-related GTP-binding protein rhoU, rho GTPase-activating protein 10; α-actinin-1 NHL repeat-containing protein 2; trifunctional purine biosynthetic protein adenosine-3 phosphoribosylamine-glycine ligase, 60S ribosomal protein L9 mitochondrial import receptor subunit; protein transport protein Sec61 subunit α isoform 1. In addition, they were categorized into biological process, molecular function and cellular component based on gene ontology (GO), which are represented in Supplementary Table 2-4. Biological process and molecular function-based classification categorized the genes, providing insights into their functionalities and, cellular components allowed for their localization in the cell. The majority were classified under biological processes, single-organism processes, cellular processes and regulation of biological processes. The molecular functions most commonly annotated involved “binding”, “ion-binding” and “organic cyclic compound-binding” functions among various others. A majority were found to be associated with the intracellular cytoplasmic and of membrane-bound origin.

3.5. Profiling for bioactivity of generated peptides using databases and in silico tools

The computational studies conducted involved the comparison of MS-generated peptide sequences to peptides with reported bioactivity using a database-based search tool such as the BIOPEP and Peptide Ranker. The bioactivities reported in BIOPEP for the given peptide data set was represented in the form of a pie-chart (Fig. 3). The most commonly recurring activities were found to be those of ACE and DPP-IV inhibitory. The Peptide Ranker scores predicted for the peptides that are most-likely to be bioactive are outlined in Table 3. On the basis of the results obtained through BIOPEP database and Peptide ranker, the best-performing peptides were chosen for subsequent studies.

In contrast to the empirical method for identification of bioactive peptides, the bioinformatic approach is both cost and time-effective alternative. The bioinformatic approach relies on the information curated in various databases, such as BIOPEP, PepBank and PeptideDB. Several studies have utilized the *in silico* approach

Table 1
Peptide hydrolysates of MFGMPs identified through n-LC-ESI-MS/MS.

Protein name	Accession no	Sequence	MH+ (Da)	Species	Score	SPI %	Z
Actin filament-associated protein 1-like 1	A6QQV9.1	(Y)LSDTTLEKKMAVASI(L)	1606.872 (2)	BOVIN	4.96	58	3
Actin filament-associated protein 1-like 1	A6QQV9.1	(Y)LSDTTLEKKMAVASI(L)	1606.872	BOVIN	4.85	60	3
Alpha-actinin-1	Q3B7N2.1	(L)WCQRKTAPYKNVNIQNFH(I)	2304.146	BOVIN	3.12	44.2	3
Alpha-1-syntrophin	Q0P5E6.1	(G)ISIKGGRENKmPIL(I)	1555.899 (2)	BOVIN	3.02	48.3	2
Alpha-1-syntrophin	Q0P5E6.1	(G)ISIKGGRENKmPIL(I)	1555.899	BOVIN	7.39	69.1	2
Ammonium transporter Rh type B	Q95M77.2	(T)LTFASVGGG(L)	808.42 (2)	BOVIN	3.19	56.2	1
Ammonium transporter Rh type B	Q95M77.2	(T)LTFASVGGG(L)	808.42	BOVIN	3.21	45.5	1
Angiopoietin-related protein 4	Q2KJ51.1	(G)LREHVERTRGQLGELERR(L)	2234.222	BOVIN	4.73	41.5	3
Atlastin-1	Q58D72.2	(L)IPW(L)	415.234	BOVIN	3.33	51.4	1
Autophagy protein 5	Q3MQ24.2	(G)IEPm(L)	489.238	BOVIN	4.06	53.8	1
BSD domain-containing protein 1	Q3SX22.1	(A)LYTKmVPAAVSHSE(F)	1532.778	BOVIN	4.72	42.2	3
Butyrophilin subfamily 1 member A1	P18892.2	(L)FTVRASV(I)	779.441	BOVIN	5.8	47.7	2
CAAX prenyl protease 2	A6H7A0.1	(A)LEHPQRRPLLAG(Y)	1386.796 (2)	BOVIN	3.36	44.3	2
CAAX prenyl protease 2	A6H7A0.1	(A)LEHPQRRPLLAG(Y)	1386.796	BOVIN	3.03	41.5	2
Calcium-binding mitochondrial carrier protein SCaMC-2	Q0V7M4.1	(G)FTQMIREGGARSL(W)	1465.758	BOVIN	3	44.1	1
Carboxypeptidase N catalytic chain	Q2KJ83.1	(H)ITRVYSIGRSVKGRH(L)	1728.998	BOVIN	3.63	48.8	2
CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase	Q6ZXC9.1	(S)LLPEVSPmKNRR(F)	1439.815	BOVIN	3.08	41.3	2
Colipase	A0JNQ7.1	(L)LVALAVAYAVPDPRG(I)	1511.858	BOVIN	5.06	54.6	3
Cone-rod homeobox protein	Q9XSK0.1	(S)YFSGLDPY(L)	961.43	BOVIN	4.85	48.4	1
C-type lectin domain family 3 member A	Q28008.1	(A)LFSQSAQGK(W)	965.505	BOVIN	3.3	44.3	1
Cytoplasmic tRNA 2-thiolation protein 1	Q0VC66.1	(L)LPPGAVVAVGASGGKDSTVLAHV(L)	2102.16	BOVIN	5.19	48.6	3
Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit STT3A	Q2KJI2.1	(K)LNPQQFEVL(F)	1087.578	BOVIN	5.39	61.6	2
D(1A) dopamine receptor	Q95136.2	(A)ISSPFRYERKMTPKAA(F)	1882	BOVIN	5.17	42.8	3
DDB1- and CUL4-associated factor 12	Q3MHH0.2	(L)FVVDVQTSQITKIPI(L)	1687.963	BOVIN	3.32	57.9	2
DnaJ homolog subfamily C member 3	Q27968.1	(D)IAAAKEVLSDPEMRKK(F)	1785.989 (2)	BOVIN	3.52	51.3	3
DnaJ homolog subfamily C member 3	Q27968.1	(D)IAAAKEVLSDPEMRKK(F)	1785.989	BOVIN	3.47	44.4	3
Evolutionarily conserved signaling intermediate in Toll pathway, mitochondrial	Q3SX05.1	(A)LRHMEPDLSARVTT(Y)	1625.843	BOVIN	4.29	50.4	3
Fatty acid-binding protein/FABP	P10790.2	(S)LVREMVDGKLI(L)	1272.734	BOVIN	4.98	53.2	3
Fatty acid synthase	Q71SP7.1	(G)IRKGVVQPLKRTVFPRTQAEDA(F)	2509.436	BOVIN	3.15	41.5	4
Ferritin	Q2YDI9.1	(F)LPKHISTSLV(F)	1094.657	BOVIN	4.89	58.6	2
GA-binding protein subunit beta-1	Q1RMI3.1	(A)LHWATEHNHQEVVE(L)	1728.809	BOVIN	4.79	40.8	3
Gap junction gamma-1 protein/alpha-7 protein	Q2HJ66.1	(N)IAVKPDQIQ(Y)	1011.583 (2)	BOVIN	7.79	81.3	2
Gap junction gamma-1 protein/alpha-7 protein	Q2HJ66.1	(N)IAVKPDQIQ(Y)	1011.583	BOVIN	7.47	76.4	2
Growth factor receptor-bound protein 7	Q1RMW5.1	(V)LSLCHVQKVKHY(L)	1511.815	BOVIN	4.85	45.7	3
Homeobox protein PKNOX1	Q2HJ84.1	(A)IYRHPLFPL(L)	1155.667	BOVIN	3.07	41.9	3
Interleukin-7/IL-7	P26895.1	(F)LNRASRKLRQ(F)	1241.755 (5)	BOVIN	5.26	61	2
Interleukin-7/IL-7	P26895.1	(F)LNRASRKLRQ(F)	1241.755	BOVIN	5.13	63.8	2
Interleukin-7/IL-7	P26895.1	(F)LNRASRKLRQ(F)	1241.755	BOVIN	4.98	61	2
Interleukin-7/IL-7	P26895.1	(F)LNRASRKLRQ(F)	1241.755	BOVIN	4.45	61.1	2
Interleukin-7/IL-7	P26895.1	(F)LNRASRKLRQ(F)	1241.755	BOVIN	4.02	62.9	4
Kinetochore protein Spc24	Q24JY3.1	(Q)LEAELQRASEEDAH(L)	1597.745 (2)	BOVIN	10.17	72.5	2
Kinetochore protein Spc24	Q24JY3.1	(Q)LEAELQRASEEDAH(L)	1597.745	BOVIN	5.5	54.2	2
Lactadherin	Q95114.2	(F)IQVAGRSGDKIF(I)	1290.717 (2)	BOVIN	4.51	47.3	3
Lactadherin	Q95114.2	(F)IQVAGRSGDKIF(I)	1290.717	BOVIN	3.05	45.3	3
Lactadherin	Q95114.2	(A)YRVAYGDDGVTWTE(Y)	1631.734 (3)	BOVIN	15.19	92.6	2
Lactadherin	Q95114.2	(A)YRVAYGDDGVTWTE(Y)	1631.734	BOVIN	13.21	92	2
Lactadherin	Q95114.2	(A)YRVAYGDDGVTWTE(Y)	1631.734	BOVIN	4.71	50.9	2
Lactadherin	Q95114.2	(W)LQIDLGSQKRVTG(I)	1414.801	BOVIN	8.92	55.6	3
Methionine–tRNA ligase	Q2T9L8.1	(G)LESLPPLRPQQNPV(L)	1587.885	BOVIN	3.01	59.6	2
Mitochondrial import receptor subunit TOM40B	A6QR22.1	(R)LCKDVFPAQMEGVKLVVNKV(L)	2274.235	BOVIN	4.15	41	4
Mitochondrial GTPase 1	Q4PS77.2	(Q)IPLSGRNPLFQETLG(L)	1641.896	BOVIN	4.02	56.5	3
Mitochondrial-processing peptidase subunit alpha	Q0P5M8.1	(V)LAATRL(L)	644.409	BOVIN	3.37	66.2	2
Myosin-10	Q27991.2	(A)LKVVRELQAQ(I)	1183.716	BOVIN	4.32	57.3	2
Myotubularin-related protein 2	A6QLT2.1	(N)IPDEELKRVASFRSRGR(I)	2016.11	BOVIN	5.38	45.1	3
NADH dehydrogenase [ubiquinone] complex I, assembly factor 7	Q2KHV5.2	(K)IKSTGPITVAEYMKEV(L)	1765.94	BOVIN	3.13	47.4	4
Neurogenic differentiation factor 6	Q08DI0.1	(Q)IKKPESFSKQIV(L)	1403.826	BOVIN	4.15	45	3
Neuropeptide Y receptor type 1	Q1RMU8.1	(I)LKQKEMRNVTNIL(I)	1586.905	BOVIN	3.62	48.1	3
Nuclear RNA export factor 1	Q1RMS5.1	(R)ISII(I)	445.302	BOVIN	4.08	46.5	1
NHL repeat-containing protein 2	A4IF69.1	(V)LDLETKTVSVFPV(F)	1447.804	BOVIN	3.65		2
Oxygen-regulated protein 1	Q8MJ05.1	(S)LEKAVFPENVTHHSVQSYVQR(W)	2468.268	BOVIN	6.29	40.9	4
Pentatricopeptide repeat-containing protein 2	Q3SZ55.1	(L)LNKRTVSRRTFQPLSQS(L)	2018.125	BOVIN	3.57	43.4	3

(*continued on next page*)

Table 1 (*continued*)

Protein name	Accession no	Sequence	MH+ (Da)	Species	Score	SPI %	Z
Perilipin-2/Adipophilin	Q9TUM6.1	(D)YLVNNTPLNW(L)	1233.626 (2)	BOVIN	8.67	60.6	2
Perilipin-2/Adipophilin	Q9TUM6.1	(D)YLVNNTPLNW(L)	1233.626	BOVIN	3.31	48.3	2
Probable ATP-dependent RNA helicase DDX4	Q5W5U4.1	(K)LTPVQKYSIPI(I)	1258.741	BOVIN	5.92	49.5	2
Probable RNA polymerase II nuclear localization protein SLC7A6OS	Q1JQE2.1	(G)LERAAENNVFQLVATVRSQEEPVQPL(L)	2937.543	BOVIN	4.17	44	4
Prostacyclin receptor/Prostaglandin I2 receptor	P79393.1	(S) LSQSASGRKDSSAPPALEGKKGNWVPLSA(W)	2938.538	BOVIN	4.95	40.9	5
Proteasome assembly chaperone 2	Q2NL24.1	(L)LTPSMQKSVQNKIQS(L)	1688.9 (4)	BOVIN	5.36	56.2	2
Proteasome assembly chaperone 2	Q2NL24.1	(L)LTPSMQKSVQNKIQS(L)	1688.9	BOVIN	5.31	61.9	2
Proteasome assembly chaperone 2	Q2NL24.1	(L)LTPSMQKSVQNKIQS(L)	1688.9	BOVIN	4.82	57.8	2
Proteasome assembly chaperone 2	Q2NL24.1	(L)LTPSMQKSVQNKIQS(L)	1688.9	BOVIN	4.32	56.6	2
Protein strawberry notch homolog 2	A0JND4.1	(M)LAVGPAmDGELPPHEAPPAGSVL(Y)	2225.127	BOVIN	4.89	60.2	2
Protein transport protein Sec24A	A6QNT8.1	(L)LHPFKDLVQLPVVTSST(I)	1881.048	BOVIN	4.9	44.9	3
Protein transport protein Sec61 subunit alpha isoform 1	Q5EA68.3	(L)LATRTDKVRALREAF(Y)	1746.997	BOVIN	3.92	49.5	3
Protein-lysine 6-oxidase	P33072.3	(L)LDASTQRRVAEGHKASFC(L)	2032.998	BOVIN	3.64	43.5	4
Putative helicase MOV-10	Q0V8H6.1	(M)LFPVASRGVPL(L)	1155.689	BOVIN	4.1	45.7	3
Pyrroline-5-carboxylate reductase 2	Q17QJ7.1	(A)LRKMGVNLTRSNKETVRHSDV(L)	2440.32	BOVIN	4.2	42.9	4
PH and SEC7 domain-containing protein 1	F1MUS9.1	(E)LGRVTAPCTPLRGPPSPR(I)	1932.06	BOVIN	4.34	43.9	3
P2Y purinoceptor 14	Q3SX17.1	(I)LFYVKE(F)	798.44	BOVIN	6.42	68.5	2
P3 protein	Q0V8N6.1	(S)IHETLHVPVSKI(L)	1372.795	BOVIN	3.23	48.6	3
Ras-related protein Rab-39B	Q17QU4.1	(V)FVLVGHKCD(L)	1074.54	BOVIN	6.32	60.7	2
Retinol-binding protein 1	P02694.4	(E)LHLEMRVEGVVCKQV(F)	1796.951 (2)	BOVIN	3.29	41.8	2
Retinol-binding protein 1	P02694.4	(E)LHLEMRVEGVVCKQV(F)	1796.951	BOVIN	3.27	54.4	2
Rho GTPase-activating protein 15	A4IF90.1	(I)YRVSGNLAT(I)	980.516	BOVIN	5.16	48.8	2
Rho GTPase-activating protein 10	Q08DP6.1	(F)LVHKLPEKNKEM(L)	1465.82	BOVIN	3.7	60.8	3
Rho-related GTP-binding protein RhoU	A5D7J5.1	(E)IRCHCPKAPII(L)	1364.729	BOVIN	3.71	49.4	2
Sclerostin	Q9BG79.2	(I)IPELGE(Y)	657.345 (2)	BOVIN	3.26	68.6	1
Sclerostin	Q9BG79.2	(I)IPELGE(Y)	657.345	BOVIN	3.11	64.5	1
Short palate, lung and nasal epithelium carcinoma-associated protein 2B	P79125.1	(L)IPITADVTVSLPF(L)	1372.772	BOVIN	3.13	47.6	2
SLAIN motif-containing protein 2	Q3MHV6.1	(N)LKSSSDRNPPLSPQSSIDSE(L)	2144.047	BOVIN	3.68	43.7	3
Sodium-dependent neutral amino acid transporter B(0)AT2	Q9XS59.1	(I)LPIPVVF(I)	784.497	BOVIN	3.31	45.1	1
Solute carrier organic anion transporter family member 3A1	Q8HYW2.1	(M)IGAmAQTPSVI(I)	1087.582 (2)	BOVIN	6.45	73.3	2
Solute carrier organic anion transporter family member 3A1	Q8HYW2.1	(M)IGAMAQTPSVI(I)	1087.582	BOVIN	5.63	69.5	2
Supervillin	O46385.2	(T)IEERKHLITVREDA(W)	1708.934	BOVIN	4.19	49.2	3
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1	Q2TBN1.1	(L)LMPPEPIIINHV(I)	1372.766	BOVIN	5.74	49.3	2
Tensin-4	Q32PJ7.2	(L)LRKEEPGAFIVRDSSS(Y)	1790.94	BOVIN	4.83	47	3
Transmembrane emp24 domain-containing protein 9	Q3T133.1	(E)IPDETmVIGNYRTQ(L)	1636.8	BOVIN	3.66	50.9	2
Transmembrane protein 158/Ras-induced senescence protein 1	A2VDX9.1	(N)ISVQRQmLSS(L)	1148.609	BOVIN	6.75	58.5	2
Transmembrane protein 163	A6QQX9.1	(L)IDMVPRVRQTRHYEmFE(-)	2207.085	BOVIN	3.51	42.5	4
Transmembrane protein 175	Q32PG7.1	(T)FPEVPLGIF(L)	1018.561	BOVIN	5.4	63.6	2
Transmembrane protein 171	Q58DS4.1	(S)LEPIQVTVGDAVI(I)	1353.762	BOVIN	5.24	48.7	2
Trifunctional purine biosynthetic protein adenosine-3	Q59A32.1	(I)LPLLKSDL(Y)	898.561	BOVIN	4.48	44.1	2
Tripeptidyl-peptidase 2	A5PK39.1	(V)IGVGAYVSPDMmVAE(Y)	1538.723	BOVIN	4.6	52	2
T-cell surface protein tactile/Cell surface antigen CD96	Q3MHP9.1	(Q)YQKEImQRPPPFKPPPPPIK(Y)	2388.326	BOVIN	3.5	48.3	2
T-cell-specific surface glycoprotein CD28/CD-antigen = CD28	Q28071.1	(S)IQVAENKILVKQSPM(L)	1697.962	BOVIN	3.49	43	3
Unconventional prefoldin RPB5 interactor	Q3B7M7.1	(S)LTNSELFNGQVNSPLN(Y)	1746.866	BOVIN	4.05	47.5	2
WD repeat-containing protein 6	A7Z052.1	(V)LAVEMPELEEAVGGAEL(L)	1756.867	BOVIN	4	52	2
Xanthine dehydrogenase/oxidase	P80457.4	(E)FMPLDPTQEPIFPPEL(L)	1870.93 (2)	BOVIN	5.34	50	2
Xanthine dehydrogenase/oxidase	P80457.4	(E)FMPLDPTQEPIFPPEL(L)	1870.93	BOVIN	3.64	44.8	2
Xanthine dehydrogenase/oxidase	P80457.4	(S)FTVPFLNQAGAL(I)	1277.689 (2)	BOVIN	4.74	41.5	2
Xanthine dehydrogenase/oxidase	P80457.4	(S)FTVPFLNQAGAL(I)	1277.689	BOVIN	4.65	52.1	1
Xanthine dehydrogenase/oxidase	P80457.4	(F)LSADDIPGSNETGL(F)	1388.654	BOVIN	4.65	44.6	2
Zinc finger protein Aiolos	A2VDW9.1	(A)LRPLVQTPPAPTSEMVPV(I)	1932.062	BOVIN	3.51	55.8	3
60S ribosomal protein L9	Q3SYR7.1	(I)LSNQTVDIPENVDIN(L)	1670.823	BOVIN	3.23	44.1	2

Table 2
Peptide hydrolysates of CFGMPs identified through n-LC-ESI-MS/MS.

Protein name	Accession no	Sequence	MH+ (Da)	Species	Score	SPI%	Z
Lactadherin	Q95114.2	(F)IQVAGRSGDKIF(I)	1290.717 (2)	BOVIN	3.65	47.4	3
Lactadherin	Q95114.2	(F)IQVAGRSGDKIF(I)	1290.717	BOVIN	3.57	41.3	3
Lon protease homolog 2	Q3SX23.1	(T)LRGLVLPVGG(I)	980.625	BOVIN	3.59	39.2	2
Septin-10	Q2KJB1.1	(F)YPHVRLKAQTYE(L)	1504.791	BOVIN	3.82	13.8	1
Xanthine dehydrogenase/oxidase;	P80457.4	(L)YIGGQDHF(Y)	936.421	BOVIN	4.67	48	2

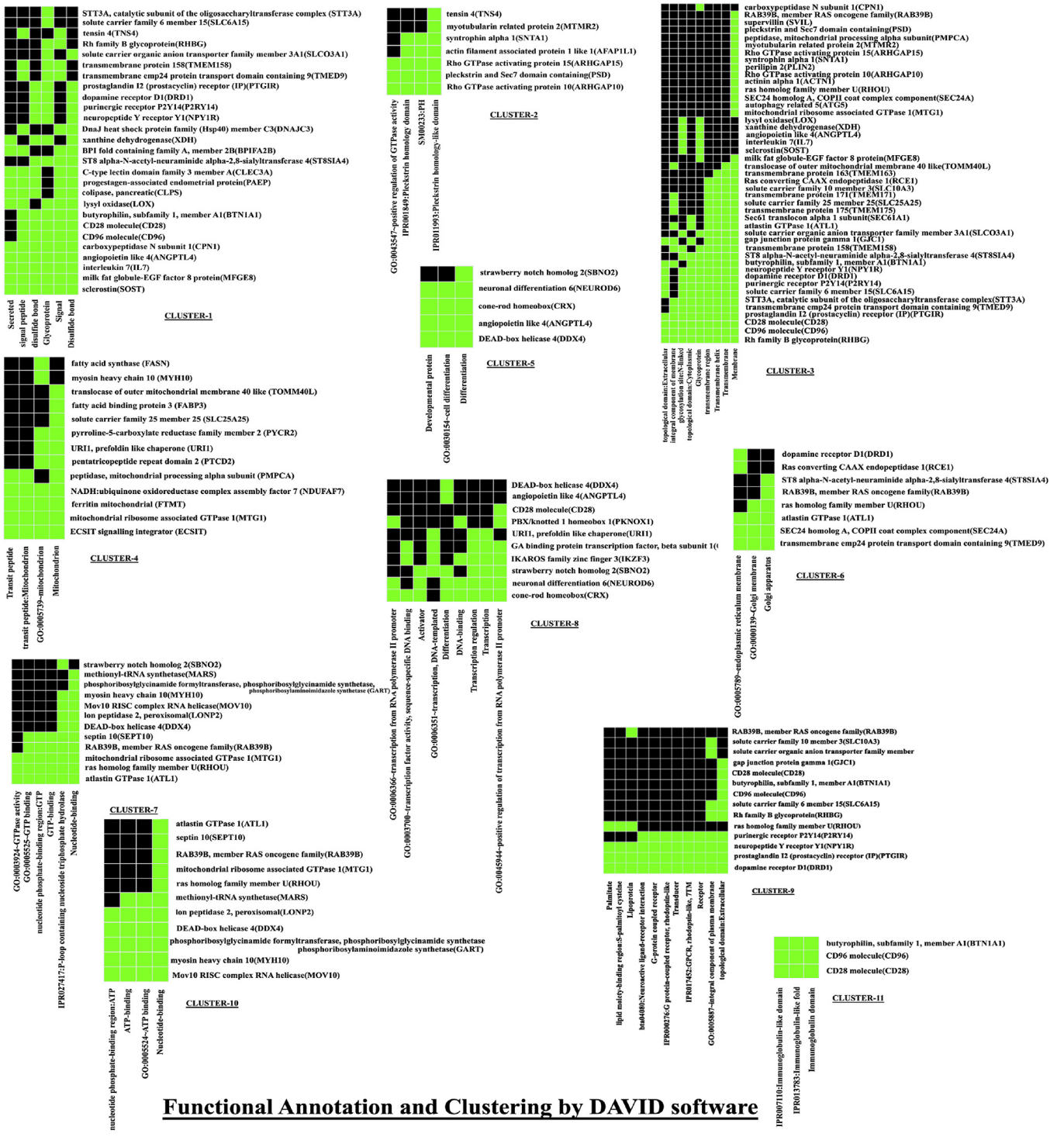

Fig. 1. Functional Annotation and clusters predicted by DAVID in the form of a 2D-heatmap. The gene-term associations positively reported are represented in green and those gene-term associations not reported yet are depicted in black. The localization of the identified proteins was investigated using BUSCA [37] as represented in Fig. 2A. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

using BIOPEP to predict bioactive peptide sequences [49–53]. In addition, bioactive peptide databases also assist in the identification of peptides with bioactivities, truncated/precursor peptide sequences or sequences presenting features of bioactivity previously reported after MS analysis of the hydrolysates [54]. PeptideRanker is particularly useful for screening a large dataset for bioactive peptide sequences as it ranks proteins according to their bioactive potential through the data reported/deposited in databases [55].

3.6. Molecular docking of selected peptides to the target proteins-ACE and DPP-IV

Two specific proteins ACE and DPP-IV were chosen for

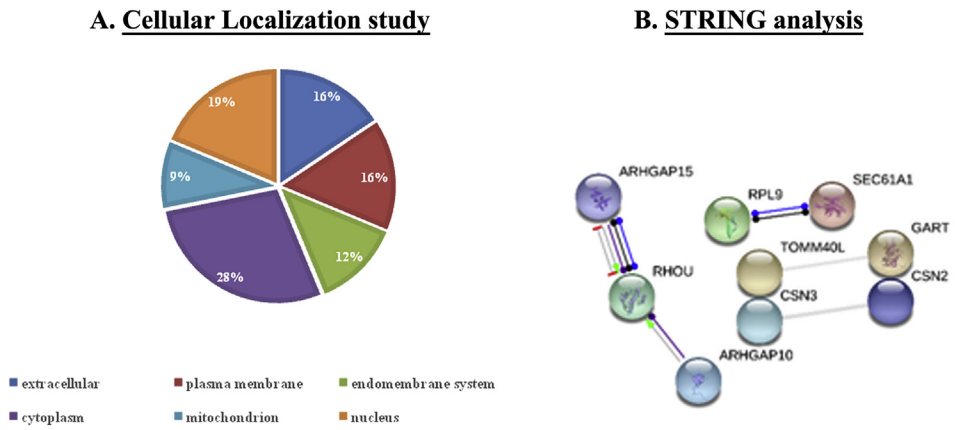

Fig. 2. A- Schematic representation of the localization of proteins; **B-** Major protein-protein associations predicted by STRING. The network of associations of the other proteins can be accessed at the web-link, https://version-10-5.string-db.org/cgi/network.pl?networkId=ltrODF9LQQwl.

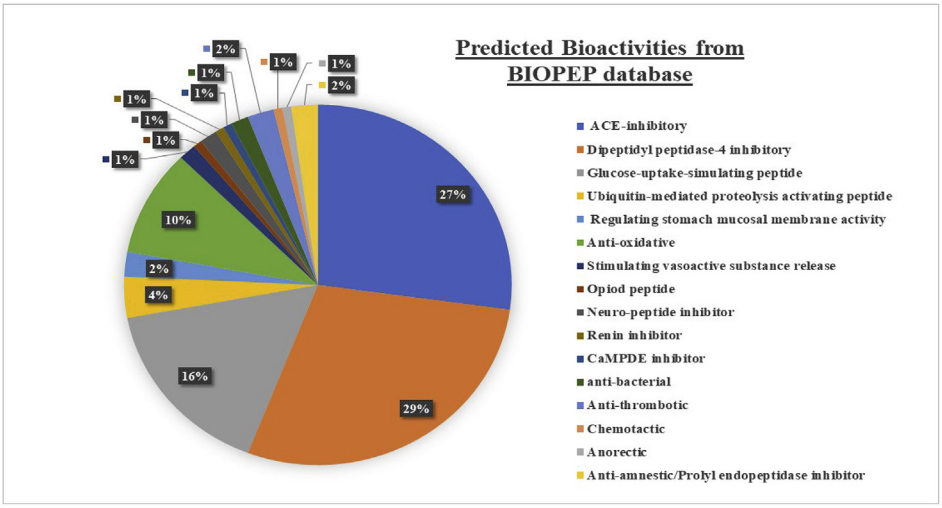

Fig. 3. A compiled summary of the varied bioactivities reported for the FGM peptide data in BIOPEP represented in the form of a pie-chart.

Table 3
The score results of peptides obtained in Peptide Ranker are given below.

Serial No.	Peptide Sequence	Score
1	LFGPPLL	0.93
2	SLPYPFI	0.92
3	SFTVPFL	0.88
4	GWLEPLL	0.86
5	FFVAPFPEVF	0.86
6	GIPLPLI	0.78
7	GLPGPPEL	0.74
8	RFPRPVY	0.69
9	GIEPmL	0.69
10	SYFSGLDPYL	0.68
11	CLYVPLI	0.66
12	AIKIPLL	0.64
13	FWAVELY	0.61
14	ALFSQSAQGKW	0.59
15	HWLPLEI	0.58
16	GLVVPPAGL	0.54
17	ILPIPVVFI	0.53
18	FLGPVPVY	0.5

molecular docking on the basis of the most common activities identified during the BIOPEP and Peptide Ranker analysis. The protein structures (1O86 for ACE and 1WCY for DPP-IV) were selected on the basis of the optimal resolution and the complexed inhibitor within. The docking analysis revealed that 20 peptides interacted with the target proteins, the peptide docking scores are outlined in Table 4. Upon analysis and consideration of the data obtained through BIOPEP, Peptide Ranker score (Rank of 0.78) and molecular docking score of ACE (−9.358), the heptapeptide GIPLPLI generated from pepsin & pancreatin digested peptidome seemed to be a promising lead for assessing the bioactivity *in vitro*. The interaction profile of the reference ligands-lisinopril and diprotin-A are compared to that of the heptapeptide (Supplementary Tables 5 and 6). Although another peptide GWLEPLL also scored higher than the selected peptide in both molecular docking and Peptide Ranker analysis, we opted for synthesis of GIPLPLI as it contained more proline residues than the former peptide since proline residues have been predicted to be relatively stable to gastrointestinal enzymes [56].

The heptapeptide's interaction within the active site of both ACE and DPP-IV alongside the inhibitors lisinopril and diprotin-A are given in Fig. 4A, B, 5A and 5B. In the case of ACE, both, the heptapeptide and lisinopril were found to form metal co-ordination bond with Zn701, in addition to forming hydrogen bonds with Tyr523 whereas, in case of DPP-IV, the lone common residue interacting with both the heptapeptide and diprotin-A was Arg125. In addition,

Table 4
The molecular docking scores obtained for the peptides that interacted with the target proteins computationally.

SLNO.	PEPTIDE SEQUENCE	M/Z	TARGET PROTEINS	
INHIBITORS			ACE	DPP-IV
			Lsn: -8.341	DP-A: -6.61
1	GWLEPLL	657.361	−10.345	−6.863
2	SFTVPFL	610.323	−7.568	−8.738
3	GIPLPLI	723.376	−9.358	−5.978
4	MIQLDLI	601.356	−8.832	−6.245
5	FWAVELY	617.329	−8.315	−6.786
6	GLPGPPEL	609.324	−7.982	−9.674
7	HWLPLEI	657.361	−5.958	−6.839
8	AIKIPLL	583.418	−5.911	−6.82
9	TLALPIL	526.36	−5.048	−6.254
10	SLPYPFI	636.339	-	−7.335
11	CLYVPLI	604.37	-	−6.872
12	RFPRPVY	615.361	-	−9.342
13	QIPTVNNL	657.357	-	−8.24
14	EIIESPLF	671.397	-	−6.744
15	VIVLPLL	554.391	-	−6.678
16	LLLVNPIW	668.434	-	−6.29
17	AYVPII	491.286	-	-
18	RISIII	445.302	-	−9.804
19	GIEPML	489.238	-	−7.173
20	VLAATRL	644.409	-	−6.91

diprotin-A also formed a salt bridge with the same residue.

Molecular docking is used to virtually screen peptide sequences with bioactivities involving target proteins of interest. It has often been used for predicting bioactive peptides that can specifically interact with proteins such as ACE [30,57,58], DPP-IV [59,60], renin [61] xanthine oxidase [59] inhibitory properties. Yet, docking simulations alone cannot ascertain the differences between the competitive and non-competitive binding interactions that affect bioactivity of the peptide, thus the predicted bioactivity has to be analyzed in vitro [57,59,62].

3.7. Synthesis of the lead peptide

The heptapeptide showing notable glide scores for both the ACE and DPP-IV was selected for synthesis through solid phase peptide synthesis. The peptide product (*m/z*-723.3) was confirmed by LC-MS for its structure (Supplementary Fig. 1).

3.8. Evaluation of ACE-inhibitory activity of the lead peptide

Hypertension or high blood pressure is the primary risk factor for the development of cardiovascular disease. Increase in the activity of ACE results in the rise of angiotensin-II levels, the prime vasoconstrictor responsible for the development of hypertension [63]. Hence, ACE is a renowned target of the RAAS for blood pressure regulation. Bioactive peptides with ACE-inhibitory activities are the most studied group among different bioactivities [64,65]. In addition, bioactive peptides are comparatively less prone to the ensuing side effects often associated with conventional small molecule drugs. Incidentally, the most common and potent ACE inhibitory peptides, Ile-Pro-Pro and Val-Pro-Pro, are derived from casein in milk [66]. Although molecular docking has significantly contributed to the target-based drug-discovery pipeline, other challenges such as the binding-site flexibility and prediction of key solvent molecules for interactions need to be addressed [67]. Hence, drawing comparable inferences between *in silico* and *in vitro* data becomes a challenging affair. Most of the reported competitive peptide inhibitors of ACE usually comprise of hydrophobic or aromatic amino acid residues such as proline, phenylalanine, tyrosine & tryptophan at the C-terminal and hydrophobic amino acids in the sequence, hence exploring the exact mechanism of inhibition of the present peptide could provide interesting results.

Based on the docking score and the interacting residues of the heptapeptide, the in vitro assay was planned to assess the lead peptide's effect on the activity of ACE. The heptapeptide inhibited the ACE-enzyme activity in a dose-dependent manner with an IC_{50} of 74.27 ± 3.75 μM (Supplementary Fig. 2). This peptide is presumably the most potent peptide candidate from FGMP reported so far. We have previously reported ACE inhibitory peptides from whey fraction with 300 ± 2 μM (IQKVAGTW- [31]) and 498 ± 2 μM (IIAMK- [30]).

Upon analysis of the data from the docking, the peptide was found to interact with Zn701, Tyr523 similar to lisinopril while His353, Glu162 & Lys511 residues also interacted with lisinopril and similarly, Ala354, Ala356 and Arg124 interacted with peptide. The glide scores of lisinopril and peptide being very close the interactions we presumed to be good enough to inhibit ACE. The *in vitro* data substantiates the inhibitory potential of peptide having IC_{50} which considered significant amongst known peptide inhibitors.

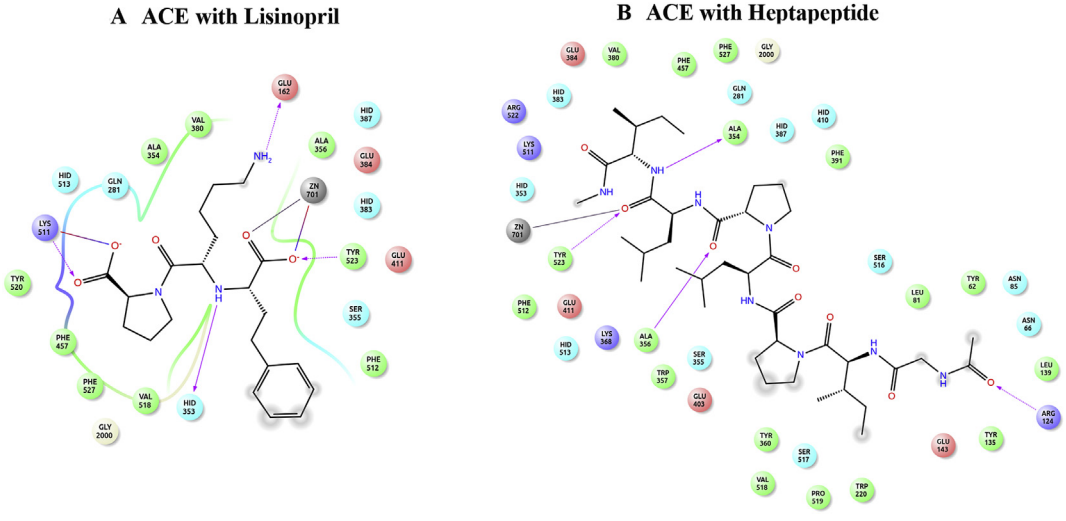

Fig. 4. Schematic representation of the active site of ACE with A-Lisinopril and B-Heptapeptide.

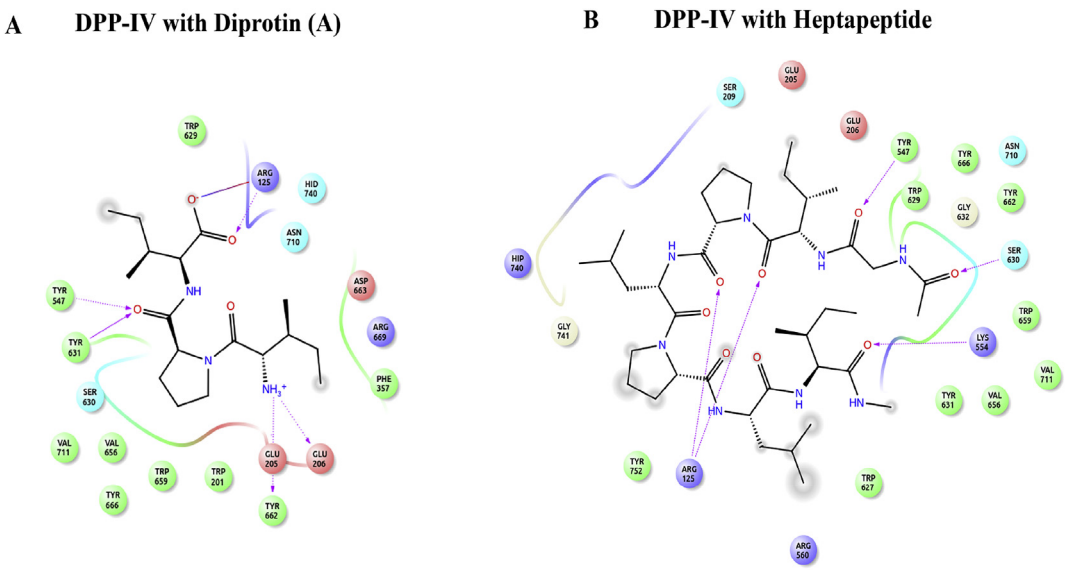

Fig. 5. Schematic representation of the active site of DPP-IV with A-diprotin-A and B-heptapeptide.

3.9. Evaluation of DPP-IV-inhibitory activity of the lead peptide

DPP-IV is a serine protease that is primarily known for its regulatory role involving the incretins-Glucose-dependent-insulinotropic peptide and glucagon-like-peptide-1 (GLP-1). Furthermore, DPP-IV is known to be responsible for more than 95% of the inactivation of GLP-1, hence, its inhibition serves as a key strategy to efficiently manage type II diabetes [68]. Usually, peptides rich in proline residues-dipeptides and tripeptides with N-terminal proline residues, commonly have DPP-IV inhibitory properties [69]. Especially peptides with proline or alanine residues in the penultimate position are known to be preferred substrates of DPP-IV [70] and diprotin-A is believed to be a substrate with a low turnover rate [71]. Recently, Nongonierma and Fitzgerald [72] assessed the susceptibility of milk peptides for DPP-IV and identified that the peptides, Ile-Pro-Ile-Gln-Tyr, Tyr-Pro-Tyr-Tyr, and Leu-Pro-Tyr-Pro-Tyr derived from casein functioned as substrate-type, Leu-Pro-Leu-Pro-Leu also from casein and CasH-protein hydrolysate was prodrug-type, while, WPH and LFH to be true inhibitors of DPP-IV. Therefore, in accordance with the activity predicted by BIOPEP and the molecular interaction of the heptapeptide's residues assessed by docking, prompted us to evaluate the heptapeptide for its DPP-IV-inhibitory activity. The heptapeptide showed inhibition of DPP-IV with an IC_{50} value of 3.83 ± 0.087 mM (Supplementary Fig. 3). The relatively high IC_{50} value may be attributed to the fact that the peptide scored low during the *in silico* studies, suggesting ACE inhibitors need not inhibit DPP-IV even though they share common preferential amino acid residues in the sequence. Although the peptide showed interactions with the key residues Arg125 & Tyr547 as did diprotin-A, the peptide failed to interact with Tyr631, Tyr662, Glu205 & Glu206 residues which could probably confer the enhanced inhibition potential of diprotin-A. While there are several studies reporting ACE and DPP-IV inhibitory properties of peptides derived from milk proteins separately, interestingly, a study carried out by Lacroix, Meng, Cheung, & Li-chan in 2016 [73] explored the probability of whey peptides to serve as dual inhibitors of ACE as well as DPP-IV. They found that while some peptides could inhibit both these enzymes, most could not, interestingly, similar to the scenario evidenced in the present study. Nevertheless, ACE-inhibition was found to be more effective when compared to DPP-IV inhibition, thereby, further studies are warranted to assess the role of amino acid residues with respect to both activities and understanding their role in efficacy of inhibition.

4. Conclusion

Milk proteins are known for their comprehensive bioactive properties, especially ACE & DPP-IV inhibition. Among the protein components present in milk, FGMP have been the least explored source for bioactivity. Hence, we focused on elucidating the bioactive potential of buffalo colostrum FGMP peptides after in vitro gastro-intestinal simulation digestion. As many as, 89 proteins identified by nLC-ESI-MS/MS were functionally annotated into 11 clusters on the basis of biological similarity and analysed for their localization. The protein-protein interaction network majorly revealed interaction of GTP-binding proteins involved in membrane and vesicular trafficking. The bioactivity profile revealed ACE and DPP-IV inhibitory potential to be most common among the listed peptides. Hence, the lead peptides were docked against the target proteins ACE and DPP-IV and on the basis of glide scores, the peptide GIPLPLI (m/z 723.3) was evaluated for *in vitro* ACE & DPP-IV inhibition. The heptapeptide was found to be a better inhibitor of ACE (74 μM) than of DPP-IV (3.83 mM). Currently, the authors are concentrating on identifying effective DPP-IV inhibitory peptides from the same source.

Declarations of interest

NONE.

competing interest

The authors declare no competing financial and/or personal interests

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
BUSCA	Bologna Unified Subcellular Component Annotator
CFGM	Colostrum Fat Globule Membrane
DAVID	Database for Annotation, Visualization and Integrated Discovery
DPP-IV	Dipeptidyl Peptidase-IV
FGM	Fat Globule Membrane
FGMP	Fat Globule Membrane Protein(S)
GLP-1	glucagon-like-peptide-1
GO	Gene Ontology
MFGM	Milk Fat Globule Membrane
PDB	Protein Databank
PDB-ID	Protein Databank- Identifier
RAAS	Renin-Angiotensin-Aldosterone system
STRING	Search Tool for the Retrieval of Interacting Genes/ Proteins

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.07.009.

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