"Mass Spectrometry-Guided Library preparation of Peptides as Metabolic enzyme Natural Inhibitors for the management of Obesity and Diabetes"

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Abstract

Goat whey protein (GWP) is recognized as a valuable source of bioactive peptides with significant health-promoting properties. In this study, GWP was enzymatically hydrolyzed using a combination of gastrointestinal enzymes—pepsin, trypsin, and chymotrypsin—to generate peptides. These peptides were identified through high-resolution liquid chromatography-mass spectrometry (HR-LC/MS), resulting in library of 2,883 peptides with lengths ranging from 6 to 44 amino acids. Among them, 40 peptides were predicted to exhibit high bioactivity scores (0.90–1) based on PeptideRanker analysis, with 28 of these being classified as nontoxic. Molecular docking simulations were employed to investigate the interactions of these peptides with pancreatic lipase and α-amylase to screening of inhibitors, these two enzymes critical in lipid and carbohydrate metabolism. Several peptides demonstrated strong binding affinities, suggesting their potential as enzyme inhibitors. Notably, peptides WPGIMR and WQDGSWQF showed the highest binding affinity for pancreatic lipase, while AAPFIWL and WQDGSWQF exhibited significant interactions with α-amylase. These results shed light on the molecular mechanisms underlying the inhibitory activities of whey protein-derived peptides. They highlight their potential applications as functional food

ingredients or natural therapeutic agents for managing metabolic disorders such as obesity and diabetes, advancing the understanding of whey protein hydrolysates in modulating key metabolic enzymes.

Keywords: bioinformatics, mass spectrometry-based peptide library, peptidomics, pancreatic lipase, α-amylase

1.0 Introduction

Milk proteins are significant sources of bioactive peptides, which can promote health benefits beyond their nutritional value (Korhonen & Pihlanto, 2006). These peptides, encrypted within protein sequences, require enzymatic hydrolysis, typically with enzymes like subtilisin or trypsin, to release their bioactive forms characterized by hydrophobic or positively charged C-terminal amino acids (Hernández-Ledesma et al., 2011; Power et al., 2013). Regarding the protein substrates, most of the studies dealing with the production of bioactive peptides from milk proteins employ cow milk as a raw material (Nagpal et al., 2011). Goat milk, with a higher proportion of β-casein and superior digestibility compared to cow milk, has shown potential for producing bioactive peptides with antihypertensive, antioxidant, and cholesterol-lowering properties (Ahmed et al., 2015; Park, 2009; Muro Urista et al., 2011).

The increasing prevalence of metabolic disorders such as obesity and type 2 diabetes has become a pressing global health issue. These conditions are often linked to the excessive intake of calorie-dense foods, which can disrupt normal lipid and carbohydrate metabolism, resulting in adverse health effects (Klein et al., 2022).

Enzymes like pancreatic lipase and **α**-amylase play critical roles in breaking down dietary fats and carbohydrates, making them important targets for therapeutic strategies to address these disorders (Aguiar et al., 2024; Kaur et al., 2021).

Pancreatic lipase is a key enzyme in the digestion of triglycerides, converting them into absorbable components. Inhibiting its activity can reduce triglyceride absorption and limit caloric intake, a primary contributor to obesity (Patil et al., 2017; Bello et al., 2017). Medications such as orlistat, which inhibit pancreatic lipase, have demonstrated their effectiveness in promoting weight loss and improving blood sugar regulation. However, their

use is often accompanied by side effects, including hyperoxaluria due to disrupted fat absorption, which underscores the need for safer, naturally derived alternatives (Buysschaert et al., 2016; Patil et al., 2017).

Similarly, α-amylase is responsible for initiating the digestion of starch by hydrolyzing internal α-1,4-glucosidic bonds, producing maltotriose, maltose, glucose, and dextrin. Rapid starch digestion can lead to sudden increases in blood glucose and insulin levels, which are significant risk factors for type 2 diabetes (Ramasubbu et al., 2003; Butterworth et al., 2011; Labes et al., 2008). Slowing carbohydrate digestion through α-amylase inhibition can help stabilize postprandial glucose levels and reduce the risk of insulin resistance and related complications (Dhital et al., 2017; Kato et al., 2017; Jayaraj et al., 2013). Recently, attention has shifted toward natural inhibitors of pancreatic lipase and α-amylase due to their potential to provide safer and more sustainable therapeutic options. Protein hydrolysates and peptide fractions from various natural sources have been shown to positively influence lipid and carbohydrate metabolism, offering anti-obesity benefits (Birari & Bhutani, 2007). These findings emphasize the importance of exploring natural bioactive compounds to develop innovative treatments for metabolic disorders. This research focuses on further investigating such natural alternatives to create effective management strategies for obesity and diabetes. Whey protein, a byproduct of cheese production, is a rich source of peptides with bioactive potential (Dinika et al., 2020; Verma et al., 2023). These peptides, released through enzymatic hydrolysis. The therapeutic potential of whey protein-derived peptides requires advanced proteomic tools to identify, characterize, and evaluate their functionality.

Recent advances in mass spectrometry and bioinformatics approaches have enabled the precise identification and functional prediction of bioactive peptides (Fabre et al., 2021). Additionally, molecular docking simulations provide insights into the binding interactions of these peptides with target enzymes, elucidating their mechanisms of action. In this study, gastrointestinal enzymes (pepsin, trypsin and chymotrypsin) for proteolytic enzymatic hydrolysis of goat milk whey protein were hydrolysed to release a diverse array of whey protein peptides (WPP). Further, the proteomic

characterization and molecular mechanism of WPP as inhibitors of pancreatic lipase and α-amylase.

A combination of HR-LC/MS-based peptide library preparation and identification. Bioinformatics tools, such as PeptideRanker and ToxinPred, was employed to predict bioactivity. Molecular docking simulations were conducted to assess the binding affinity and interactions of selected peptides with pancreatic lipase and **α**-amylase inhibitors. This work aims to uncover the therapeutic potential of whey protein-derived peptides in modulating lipid and carbohydrate metabolism, presenting an innovative approach to address the growing challenge of metabolic disorders. By elucidating the molecular interactions of these peptides with key enzymes, this study contributes to the development of functional foods and nutraceuticals for improving metabolic health.

2.0. Methods and material

WP-PTC hydrolysate was purified and sequence of peptides were identified using high-resolution liquid chromatography-tandem mass spectrometry (HR-LC/MS). Bioinformatics analysis, using tools like the Trans-Proteomic pipeline (TPP), potential peptides were identified. Then, bioactivity of peptides was predicted by peptide ranker tool, followed by prediction of physiological properties of peptides (molecular weight, length of peptides). Thereafter, selected bioactive peptides targeted to α-amylase and pancreatic lipase active site to analyse the interaction of peptides by molecular docking (Autodockvina).

2.1. Milk Collection

Goat milk was procured from Livestock Research Center at ICAR-National Dairy Research Institute, Karnal, India. These animals were raised under a consistent feeding and breeding regimen as part of a larger herd at the institute. The milk samples were collected and pooled before the experiment. The aim of pooling the samples was to minimize animal-to-animal variations.

2.2. Partial purification of casein and whey proteins

To isolate the casein and whey proteins raw goat milk was skimmed using a centrifuge at 4255×g for 20 min at 10 □C. Afterward, an acid precipitation was performed on the skimmed goat milk using 1N HCl at pH 4.6 to separate the whey and caseins. Separation involved centrifugation at

4255 ×g for 10 min at 4 □C, which was repeated thrice to ensure maximum removal of casein particles. The separated caseins proteins (CP) were dissolved in double-distilled water using 0.1 M NaOH which was converted to Na-caseinate. Afterward, they were lyophilized with a lyophilizer and stored at −20 □C for future experiments. To prevent the proteolytic degradation of milk whey, 0.01% phenyl methyl sulfonate was added. To isolate the whey protein (WP), the salting-out method was used, followed by dialysis using 3kDa cut off membrane.

2.3. Acetone precipitation

Dialyzed WP were then additionally precipitated by adding 3-fold chilled acetone and incubated at -20 □C for 14–16 h. Protein precipitation was followed centrifugation at 13,000 x g at 4 □C for 10 min, after that supernatant was discarded. The pellet was reconstituted with 1x PBS at pH 6.8. After that, the vacuum-sealed concentrator was used to dry the partially purified WP, which was then kept at -20 □C for upcoming tests.

2.4. Protein profiling by SDS-PAGE

Before conducting the SDS-PAGE, the concentrations of partially purified CP and WP were determined by the Lowry's method (Lowry et al., 1951). The molecular weight of the proteins was determined using SDS-PAGE, and the protein profile was confirmed. The separating gel, following Laemmli (1970) approach, was composed of 15%, and the stacking gel was made up of 4% polyacrylamide. Sample loaded in 5-10µg per well. After electrophoresis, the gels were stained with a solution of 0.005% Coomassie brilliant blue R-250 in a mixture of glacial acetic acid, methanol, and water in a ratio of 1:5:4. for 30 min. Following staining, the gels were fixed with 10% trichloroacetic acid (TCA) for further analysis. Gels were destained, and after that, gel were observed by image scanner system (E-Gel Imager).

2.5. In-solution digestion of whey protein by pepsin, trypsin and chymotrypsin combination

For in-solution digestion, whey protein (1mg/mL) were taken then precipitated using 3fold acetone or trichloroacetic acid (TCA). Furthermore, 45mM DTT (dithiothreitol) was dissolved in 50 mM NH_4HCO_3 (ammonium bicarbonate) to reduce disulfide bonds in the protein samples for 20 min. This was followed by the alkylation of cysteine residues using 10 mM IAA

172 (iodoacetamide) in 50 mM NH_4HCO_3 (ammonium bicarbonate) for 15 min.
173 Subsequently, digestion was carried out using pepsin (1:100) at pH 2.0. Pepsin
174 was inactivated by raising the pH to 7.5-7.8 using 0.1 mM NaOH. Then,
175 trypsin and chymotrypsin were added (1:100) and the mixture was incubated
176 at 37 □C for three hours. The reaction was subsequently halted with 10% TFA
177 (trifluoroacetic acid), and the peptides were further dried using vacuum
178 drying. The samples were then desalted using a zip tip and subjected to
179 peptide identification by HR-LC/MS Orbitrap (Ali et al., 2017).

180 **2.6. Electrospray ionization tandem mass spectrometry HR-LC/MS**
181 **orbitrap analysis**

182 The lyophilized peptides were reconstituted in 0.1% formic acid in LC-
183 MS-grade water and subjected to nano-LC (Thermo EASY-nLC; Thermo
184 Fischer Scientific) followed by identification in captive ion source i.e. Q
185 Exactive Plus- orbitrap mass spectrometer (MS) with high mass accuracy and
186 sensitivity. The peptides were enriched in nano-trap column and eluted from
187 nano-analytical column. The peptide elution was carried out using a linear
188 gradient of 5–45% acetonitrile at 300 μL/min flow rate in a total run time of
189 90 min keeping the solvent system as follows: solvent A, 100% water in 0.1%
190 formic acid; and solvent B, 100% acetonitrile in 0.1% formic acid. Positive ions
191 were generated by electrospray, and the orbitrap was operated in data-
192 dependent acquisition mode to automatically switch between MS and MS/MS
193 acquisition. Precursor ion orbitrap survey scan was acquired with a range of
194 350–2,000 m/z with resolution R =70,000 @ m/z 200. Q1 sequentially
195 selects six most intense precursor ions for fragmentation using collision-
196 induced dissociation for MS-MS analysis with a fixed cycle time of 3 sec along
197 with 2 min of release for exclusion filter. Data acquisition was conducted by
198 software, version 24.8; Thermo Fischer Scientific.

199 **2.7. Data processing and analysis**

200 Vendor-created .raw file format was analyzed with TPP search engine
201 which is based on comet algorithm, used for high confidence identification of
202 the *de novo* peptides library. Resultant .raw files were used for the
203 identification of proteins using the profiled inhouse combined goat database
204 (UniprotKb proteome database) along with usual contaminant proteins for
205 spectra examination. The parameter for MS/MS ion search contains digested

206 sites with two missed cleavages allowed, flexible modification on amino acids-
207 methionine for oxidation, N-terminal acetylation, Gln-pyro Glu, and Glu-pyro
208 Glu, while static modification of cysteine as carboxy amidomethylation or
209 propionamide. The peptide precursor ion tolerance was 20 ppm, with MS/MS
210 tolerance of 0.01 Da. The "ion score cutoff" was set to 30, thereby eliminating
211 the lowest quality matches and minimum peptide length as five amino acid
212 residues. To increase the confidence and remove the false-positive
213 identification, a 0.01% false discovery rate (FDR) was used at equal peptide
214 and protein levels.

215 **2.8. *In silico* prediction of length, bioactivity and molecular weight of**
216 **identified peptides**

217 *In silico* prediction of peptides with specific properties, such as
218 molecular weight and length, involves computational analysis. The bioactivity
219 of peptides is predicted using a peptide ranker. Furthermore, the
220 physicochemical properties of peptide sequences, molecular mass, and
221 including toxicity were determined through using the ToxinPred tool (Mooney
222 et al., 2012.

223 **2.9. Peptide binding interaction with pancreatic lipase and α-amylase**

224 After predicting the bioactivity of peptides, 28 peptides were chosen for
225 molecular interaction analysis with pancreatic lipase and alpha-amylase.
226 Docking simulations were utilized to gain a deeper understanding of the
227 interaction between the most effective bioactive peptides and the binding sites
228 of pancreatic lipase (PDB id: 1LPB) and α-amylase (1JFH). PyRx
229 (AutoDockVina) was employed to conduct the molecular docking
230 investigations. Discovery Studio 2022 software was used to evaluate docking
231 orientations and interactions with binding pocket residues.

232 **3.0. Result and discussion**

233 The graphical workflow provides a comprehensive overview of the
234 process for profiling and identifying anti-obesity and antidiabetic peptides
235 derived from whey protein hydrolysate peptides. The procedure begins with
236 the collection of goat milk. After the milk is collected, initial sample processing
237 is conducted to prepare the whey fraction for further processing. The next step
238 involves the purification of the whey protein. This is accomplished through a

combination of methods: salting out, acetone precipitation, and dialysis. Salting out uses high salt concentrations to precipitate proteins, which helps isolate the whey proteins from other soluble milk components. Acetone precipitation further purifies the proteins by selectively removing contaminants and concentrating the whey proteins. Dialysis is then employed to remove small molecular contaminants, such as salts or solvents, by using a semi-permeable membrane that allows only the passage of small molecules, leaving behind larger proteins. Once the whey protein is purified, it is subjected to enzymatic digestion using digestive endopeptidases, specifically pepsin, trypsin, and chymotrypsin. These enzymes break down the whey protein into smaller peptides by cleaving specific peptide bonds, producing a mixture of peptides with potential bioactive properties. These peptides are then purified using a Zip Tip C18 column, a solid-phase extraction method. Following the purification, the peptides are analyzed using high-resolution liquid chromatography-mass spectrometry (HR-LC/MS) for the generation of peptides library. This advanced analytical technique separates and identifies the peptides based on their mass-to-charge ratio and provides detailed information on their molecular structure and sequence. The peptide profiling is further refined through the use of an in-house proteome database of goat. The peptide sequences are compared against this database using the TPP (Trans-Proteomic Pipeline), a bioinformatics toolset that helps identify and quantify peptides and proteins by matching experimental data with theoretical sequences. This step ensures the accurate identification of peptides and enhances the confidence in the findings. Finally, the peptides library was subjected to bioinformatics analysis, where various computational tools was used to predict the bioactivity of the identified peptides, specifically focusing on their potential anti-obesity and antidiabetic effects. This process, as depicted in the graphical workflow (**Fig 3.0**), highlights the integrated approach used in profiling anti-obesity and antidiabetic peptides and emphasizes the importance of both experimental techniques and bioinformatics in identifying potential bioactive peptides.

3.1. Nano-HPLC chromatogram and total ions spectra of HR-LC/MS of whey protein hydrolysate

Whey protein hydrolysate peptides was selected for identifying peptide sequences. HR-LC/MS was employed to determine the amino acid sequences and generated library of peptides, with the results depicted in **Fig. 3.1 a & b**. Subsequently, peptides were identified using an in-house combined database on the Trans-Proteomic-Pipeline (TPP) software. Utilizing the database to confirm amino acid sequences, a library of 2,883 peptides were identified in the whey protein hydrolysate peptides (**Fig. 3.2. a, Supplementary-I).** Moreover, it's noteworthy that all peptides range from 6 to 44 amino acids in length, with the majority falling within the range of 8 to 13 residues **(Fig. 3.2. a).**

3.2. *In silico* prediction of bioactivity of peptides by Peptide ranker

Identification of peptides within whey protein hydrolysate were a crucial step in understanding its bioactive potential. In our investigation, a comprehensive analysis revealed the successful identification of a total of 2,883 peptides in library. Among these mostly peptide was observed to be bioactive in nature according to bioactivity score, but only we selected 40 peptides classified as bioactive peptides, characterized by a probability score ranging from 0.90 to 1, as determined by Peptide Ranker **(Fig. 3.2 b, Supplementary-II).** Sequence logos were constructed for peptides exhibiting bioactivity scores in the range of 0.90 to 1, indicating cysteine, proline, tryptophan, phenylalanine, methionine, and glycine as the predominant amino acid residues **(Fig. 3.2 c)**.

28 peptides predicted as nontoxic out of 40 by Toxinpred **(Fig. 3.2 d, Supplementary-II)**. This classification underscores the potential bioactivity of these peptides, suggesting their significance in various physiological processes or functional applications. One study proposed the *in-silico* prediction of novel peptides, potentially bioactive and bio-accessible from chicken hydrolysate (Xiao et al., 2022), while another study conducted by Coscueta, (2022) utilized a peptide ranker to predict bioactive peptides from casein hydrolysate.

The identification of these bioactive peptides within whey protein hydrolysate holds significant implications for its potential health benefits.

304 **3.3. Prediction of total molecular weight of bioactive peptides**

305 *In silico* prediction of molecular weight (MW) for peptides plays a
306 crucial role in various fields such as bioinformatics, pharmaceuticals, and
307 proteomics. Molecular weight is a key factor in determining the bioavailability
308 of peptides. Peptides with low molecular weights are more likely to be
309 absorbed and distributed effectively within the body (Romay et al., 2003). In
310 our study, MW predictions of peptides offer that all peptides fell within the
311 range of 500 to 2550 Da **(Fig. 3.3).** This range is significant as it
312 encompasses peptides of varying sizes, from small bioactive peptides to larger
313 protein fragments. Peptides within this MW range are of particular interest
314 due to their potential biological activities and therapeutic applications.

315 **3.4. Molecular docking analysis of the pancreatic lipase and alpha**
316 **amylase with selected bioactive peptides**

317 Molecular docking analyses using AutoDock Vina provided detailed
318 insights into the interactions of pancreatic lipase (PL) and alpha-amylase with
319 bioactive peptides derived from WP-PTC, highlighting their potential
320 therapeutic applications. Pancreatic lipase, essential for hydrolyzing dietary
321 fats, features an active site in the N-terminal domain comprising a catalytic
322 triad (S153, D177, H264) and key substrate-binding residues (F78, I79, H152,
323 F216, W253, R257). This site is regulated by a flexible flap that governs
324 enzyme activation by adopting open or closed conformations (Hermoso et al.,
325 1996).

326 Among the 28 bioactive peptides evaluated, binding energies ranged
327 from 121.9 to -6.5 kcal/mol, with WPGIMR and WQDGSWQF demonstrating
328 the strongest binding energies of -6.5 and -6.1 kcal/mol, respectively **(Fig.**
329 **3.4. a)**. WPGIMR interacted with PL via six hydrogen bonds and three pi
330 bonds, while WQDGSWQF showed even more robust interactions with eight
331 hydrogen bonds and eight pi bonds **(Fig. 3.4. b & c)**. These interactions
332 indicate strong structural stability within the enzyme's binding pocket,
333 suggesting that these peptides could serve as effective inhibitors or
334 modulators of pancreatic lipase activity.

335 Hirose, (2024) identified two anti-obesity peptides, LDQW and

336 LKPTPEGDLEIL, from whey protein using LC-MS, which showed promise in
337 combating obesity and metabolic disorders. Similarly, camel whey protein-
338 derived peptides such as PAGNFLPPVAAAPVM, MLPLMLPFTMGY, and
339 LRFPL demonstrated inhibitory potential against PL through molecular
340 docking analysis (Baba et al., 2021). These peptides interacted with PL
341 primarily via hydrogen bonds, salt bridges, and hydrophobic interactions,
342 highlighting their potential to destabilize the open-lid conformation of the
343 enzyme.

344 Validated peptides like VPPR, LADR, LSPR, and TVGPR further
345 exhibited uncompetitive inhibition of PL, emphasizing the role of structural
346 motifs and interaction types for enzyme regulation. Other studies identified
347 peptides such as MSNFYF (camel casein) and MMML (cow casein) that bind
348 at the active site of PL, confirming their regulatory activity (Mudgil et al.,
349 2022). Additionally, plant-derived peptides from sources like cocoa beans,
350 yellow field peas, and pinto beans revealed the importance of non-polar
351 residues in forming stable complexes with PL (Mudgil et al., 2018; Coronado-
352 Cáceres et al., 2020; Awosika & Aluko, 2019; Ngoh et al., 2017). These findings
353 collectively demonstrate the potential of bioactive peptides as natural
354 pancreatic lipase inhibitors, offering promising strategies for managing
355 obesity and associated metabolic disorders.

356 Molecular docking analyses of α-amylase revealed significant
357 interactions between bioactive peptides and the enzyme's key active sites.
358 α-Amylase consists of three subunits—A, B, and C—with the primary active
359 sites Asp197, Glu233, and Asp300 located in subunit A, along with calcium-
360 binding (Asp100, Arg158, Asp167, His201) and chloride-binding (Arg195,
361 Asn298, Arg337) domains, which are critical for enzymatic activity (Esfandi
362 et al., 2022).

363 In our study, binding energies of peptides with α-amylase ranged
364 from 71 to −8.4 kcal/mol, indicating diverse affinities **(Fig. 3.4. d).**
365 Notably, peptides AAPFIWL and WQDGSWQF exhibited the strongest
366 binding energies of −8.4 and −8.1 kcal/mol, respectively **(Fig. 3.4. d)**.
367 AAPFIWL formed 5 hydrogen bonds and 7 pi bonds, contributing to
368 stability within the enzyme's binding site. Similarly, WQDGSWQF

369 established 8 hydrogen bonds and 8 pi bonds, highlighting its robust
370 interactions with α-amylase **(Fig. 3.4. e & f)**. These diverse modes of
371 interaction demonstrate the peptides' potential as α-amylase inhibitors.

372 On of the study, Mudgil, (2024) identified peptides
373 IMEQQQTEDEQQDK from camel milk and DQHQKAMKPWTQPK from
374 goat milk, which showed strong binding affinity towards α-amylase.
375 Another study by Su, (2024) identified QEPVPDPVRGL from camel casein
376 protein, which exhibited potent antidiabetic activity by effectively
377 occupying the enzyme's active cavities through hydrogen bonds, salt
378 bridges, and pi-alkyl interactions. Furthermore, Singh, (2024)
379 demonstrated the enzymatic hydrolysis of whey protein from the
380 Himalayan goat breed "Gaddi" using alcalase, flavourzyme, and their
381 combination. This process yielded bioactive peptides TPEVDKEALEK (−5.7
382 kcal/mol), DDSPDLPK (−6.5 kcal/mol), and EMPFPK (−7.0 kcal/mol).
383 These peptides exhibited strong and efficient interactions with α-amylase,
384 further highlighting their potential as bioactive molecules for therapeutic
385 applications.

386 Collectively, these results emphasize the critical role of bioactive
387 peptides in modulating α-amylase activity, underscoring their potential in
388 developing functional foods and therapeutic agents for managing metabolic
389 disorders.

390 In summary, these findings underscore the potential of select
391 bioactive peptides sourced from whey protein hydrolysates to interact with
392 and potentially influence the activities of pancreatic lipase and alpha-
393 amylase. Insight into the molecular mechanisms governing these
394 interactions is essential for the identification of innovative therapeutic agents
395 or functional food constituents targeting lipid and starch metabolism.

396 3.5. Peptide sequence logos for pancreatic lipase and α-amylase binding 397 peptide

398 The predicted binding energies of peptides interacting with the active sites of
399 pancreatic lipase and α-amylase ranged from -3.1 to -8.4. Sequence logo
400 analysis of peptides binding to pancreatic lipase indicated tryptophan,

methionine, phenylalanine and proline as the most dominant hydrophobic and aromatic residues. In contrast, sequence logo analysis of peptides binding to α-amylase identified tryptophan, leucine and proline as the predominant aromatic and hydrophobic residues **(Fig. 3.5a & b).**

Conclusion

This study successfully identified 2,883 peptides in whey protein hydrolysates using HR-LC/MS guided library preparation, with 28 peptides predicted as bioactive based on a high probability score (0.9–1) from PeptideRanker. These peptides, predominantly 8 to 13 amino acids in length, demonstrated significant inhibitory potential against pancreatic lipase and alpha-amylase through molecular docking analysis. For pancreatic lipase, binding energies ranged from 121.9 to −6.5 kcal/mol, with WPGIMR and WQDGSWQF showing the strongest affinities, forming multiple hydrogen and pi bonds that underscored their robust interactions with the enzyme. Similarly, alpha-amylase docking revealed binding energies between −8.4 and −7.1 kcal/mol, with AAPFIWL and WQDGSWQF exhibiting the highest affinities, demonstrating diverse interaction modes and structural stability. The findings highlight the potential of whey protein-derived peptides as inhibitors of key metabolic enzymes involved in lipid and carbohydrate digestion. These results contribute valuable insights into the molecular mechanisms of enzyme inhibition, paving the way for their application as functional food components or therapeutic agents for managing metabolic disorders such as obesity and diabetes. Future studies should address limitations by exploring toxicity, pharmacokinetics, and conducting *in vitro* and *in-vivo* trials to validate the safety and efficacy of these bioactive peptides, enabling their practical application in dietary and pharmaceutical interventions.

AUTHOR CONTRIBUTIONS

Manish Singh Sansi: Conceptualization, Data curation, Methodology, Visualization, writing– original draft | **Daraksha Iram:** Data curation, Visualization, writing – review | **Sudarshan Kumar:** Writing – review and editing | **Suman Kapila:** Provide instruments, writing – review and editing | **Kamal Gandhi:** Provide instruments, writing – review and editing | **Sunita Meena:** Conceptualization, Project administration, writing – review and editing

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

Data availability statement

The data and materials supporting our findings are available from the corresponding author upon reasonable request.

References

1. Ali, S. A., Kaur, G., Kaushik, J. K., Malakar, D., Mohanty, A. K., & Kumar, S. (2017). Examination of pathways involved in leukemia inhibitory factor (LIF)-induced cell growth arrest using label-free proteomics approach. *Journal of proteomics*, *168*, 37-52.
2. UniProt, C. (2019). UniProt: a worldwide hub of protein knowledge Nucleic Acids Res 47. *D506-D515*, *10*.
3. Mooney, C., Haslam, N. J., Pollastri, G., Shields, D. C., & Kurgan, L. (2012). Towards the improved discovery and design of functional peptides: Common features of diverse classes permit generalized prediction of bioactivity. *PLoS One*, 7(10), e45012.
4. Coscueta, E. R., Batista, P., Gomes, J. E. G., da Silva, R., & Pintado, M. M. (2022). Screening of novel bioactive peptides from goat casein: In silico to in vitro validation. *International Journal of Molecular Sciences*, *23*(5), 2439.
5. Xiao, C., Toldrá, F., Zhao, M., Zhou, F., Luo, D., Jia, R., & Mora, L. (2022). In vitro and in silico analysis of potential antioxidant peptides obtained from chicken hydrolysate produced using Alcalase. *Food Research International*, *157*, 111253.
6. Romay, C. H., Gonzalez, R., Ledon, N., Remirez, D., & Rimbau, V. (2003). C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and

469 neuroprotective effects. *Current Protein and Peptide Science*, 4(3), 207-216.

470 7. Hermoso, J., Pignol, D., Kerfelec, B., Crenon, I., Chapus, C., & Fontecilla-
471 Camps, J. C. (1996). Lipase activation by nonionic detergents: the crystal
472 structure of the porcine lipase-colipase-tetraethylene glycol monooctyl ether
473 complex. *Journal of Biological Chemistry*, *271*(30), 18007-18016.

474 8. Baba, W. N., Mudgil, P., Baby, B., Vijayan, R., Gan, C. Y., & Maqsood, S.
475 (2021). New insights into the cholesterol esterase-and lipase-inhibiting
476 potential of bioactive peptides from camel whey hydrolysates: Identification,
477 characterization, and molecular interaction. *Journal of Dairy Science*, *104*(7),
478 7393-7405.

479 9. Mudgil, P., Baba, W. N., Kamal, H., FitzGerald, R. J., Hassan, H. M., Ayoub,
480 M. A., & Maqsood, S. (2022). A comparative investigation into novel
481 cholesterol esterase and pancreatic lipase inhibitory peptides from cow and
482 camel casein hydrolysates generated upon enzymatic hydrolysis and in-vitro
483 digestion. *Food chemistry*, *367*, 130661.

484 10. Esfandi, R., Seidu, I., Willmore, W., & Tsopmo, A. (2022). Antioxidant,
485 pancreatic lipase, and α amylase inhibitory properties of oat bran hydrolyzed
486 proteins and peptides. *Journal of Food Biochemistry*, *46*(4), e13762.

487 11. Mudgil, P., Al Dhaheri, M. K. O., Alsubousi, M. S. M., Khan, H., Redha, A. A.,
488 Yap, P. G., & Maqsood, S. (2023). Molecular docking studies on α-amylase
489 inhibitory peptides from milk of different farm animals. *Journal of Dairy*
490 *Science*.

491 12. Su, N., Yi, L., He, J., Ming, L., Jambal, T., Mijiddorj, B., & Ji, R. (2024).
492 Identification and molecular docking of a novel antidiabetic peptide from
493 protamex-camel milk protein hydrolysates against α-amylase and DPP-
494 IV. *International Dairy Journal*, *152*, 105884.

495 13. Klein, S., Gastaldelli, A., Yki-Järvinen, H., & Scherer, P. E. (2022). Why does
496 obesity cause diabetes? *Cell metabolism*, *34* (1), 11-20.

497 14. Hiyoshi, T., Fujiwara, M., & Yao, Z. (2017). Postprandial hyperglycemia and
498 postprandial hypertriglyceridemia in type 2 diabetes. *Journal of biomedical*
499 *research*, *33*(1), 1.

500 15. Kaur, N., Kumar, V., Nayak, S. K., Wadhwa, P., Kaur, P., & Sahu, S. K. (2021).
501 Alpha amylase as molecular target for treatment of diabetes mellitus: A
502 comprehensive review. *Chemical biology & drug design*, *98*(4), 539-560.

16. Aguiar, A. J. F. C., de Medeiros, W. F., da Silva-Maia, J. K., Bezerra, I. W. L., Piuvezam, G., & Morais, A. H. D. A. (2024). Peptides evaluated in silico, in vitro, and in vivo as therapeutic tools for obesity: A systematic review. *International journal of molecular sciences*, *25*(17), 9646.
17. Patil, M., Patil, R., Bhadane, B., Mohammad, S., & Maheshwari, V. (2017). Pancreatic lipase inhibitory activity of phenolic inhibitor from endophytic Diaporthe arengae. *Biocatalysis and agricultural biotechnology*, *10*, 234-238.
18. Bello, M., Basilio-Antonio, L., Fragoso-Vázquez, J., Avalos-Soriano, A., & Correa-Basurto, J. (2017). Molecular recognition between pancreatic lipase and natural and synthetic inhibitors. *International journal of biological macromolecules*, *98*, 855-868.
19. Buysschaert, B., Aydin, S., Morelle, J., Hermans, M. P., Jadoul, M., & Demoulin, N. (2016). Weight loss at a high cost: Orlistat-induced late-onset severe kidney disease. *Diabetes & metabolism*, *42*(1), 62-64.
20. Birari, R. B., & Bhutani, K. K. (2007). Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug discovery today*, *12*(19-20), 879-889.
21. Ramasubbu, N., Ragunath, C., & Mishra, P. J. (2003). Probing the role of a mobile loop in substrate binding and enzyme activity of human salivary amylase. *Journal of molecular biology*, *325*(5), 1061-1076.
22. Butterworth, P. J., Warren, F. J., & Ellis, P. R. (2011). Human α□amylase and starch digestion: An interesting marriage. *Starch□Stärke*, *63*(7), 395-405.
23. Labes, A., Karlsson, E. N., Fridjonsson, O. H., Turner, P., Hreggvidson, G. O., Kristjansson, J. K., & Scho□nheit, P. (2008). Novel members of glycoside hydrolase family 13 derived from environmental DNA. *Applied and environmental microbiology*, *74*(6), 1914-1921.
24. Dhital, S., Warren, F. J., Butterworth, P. J., Ellis, P. R., & Gidley, M. J. (2017). Mechanisms of starch digestion by α-amylase—Structural basis for kinetic properties. *Critical reviews in food science and nutrition*, *57*(5), 875-892.
25. Kato, C. G., Gonçalves, G. D. A., Peralta, R. A., Seixas, F. A. V., de Sá-Nakanishi, A. B., Bracht, L., & Peralta, R. M. (2017). Inhibition of α□amylases by condensed and hydrolysable tannins: focus on kinetics and hypoglycemic actions. *Enzyme Research*, *2017*(1), 5724902.
26. Jayaraj, S., Suresh, S., & Kadeppagari, R. K. (2013). Amylase inhibitors and their biomedical applications. *Starch□Stärke*, *65*(7□8), 535-542.

537 27. Dinika, I., Verma, D. K., Balia, R., Utama, G. L., & Patel, A. R. (2020).
538 Potential of cheese whey bioactive proteins and peptides in the development
539 of antimicrobial edible film composite: A review of recent trends. *Trends in*
540 *Food Science & Technology*, *103*, 57-67.
541 28. Verma, S. K., Iram, D., Sansi, M. S., Pandey, K. K., Vij, S., & Sood, S. K.
542 (2023). Sustainable utilization of dairy waste paneer whey by pediococcus
543 pentosaceus NCDC 273 for lactic acid production. *Biocatalysis and*
544 *Agricultural Biotechnology*, *47*, 102588.
545 29. Fabre, B., Combier, J. P., & Plaza, S. (2021). Recent advances in mass
546 spectrometry–based peptidomics workflows to identify short-open-reading-
547 frame-encoded peptides and explore their functions. *Current opinion in*
548 *chemical biology*, *60*, 122-130.
549 30. Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and
550 functionality. *International dairy journal*, *16*(9), 945-960.
551 31. Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011).
552 Antihypertensive peptides: Production, bioavailability and incorporation into
553 foods. *Advances in colloid and interface science*, *165*(1), 23-35.
554 32. Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., & Yadav, H.
555 (2011). Bioactive peptides derived from milk proteins and their health
556 beneficial potentials: an update. *Food & function*, *2*(1), 18-27.
557 33. Ahmed, A. S., El-Bassiony, T., Elmalt, L. M., & Ibrahim, H. R. (2015).
558 Identification of potent antioxidant bioactive peptides from goat milk
559 proteins. *Food Research International*, *74*, 80-88.
560 34. Park, Y. W. (2009). Bioactive components in goat milk. *Bioactive components*
561 *in milk and dairy products*, *4381*.
562 35. Muro Urista, C., Álvarez Fernández, R., Riera Rodriguez, F., Arana Cuenca, A.,
563 & Tellez Jurado, A. (2011). Production and functionality of active peptides
564 from milk. *Food Science and Technology International*, *17*(4), 293-317.
565 36. Hirose, Y., Kurimoto, M., Yuda, N., & Tanaka, M. (2024). Identification of
566 Whey Protein□Derived Anti□Obesity Peptides Through 3T3□L1 Adipocyte
567 Differentiation Assay. *Food Science & Nutrition*.
568 37. Baba, W. N., Mudgil, P., Baby, B., Vijayan, R., Gan, C. Y., & Maqsood, S.
569 (2021). New insights into the cholesterol esterase-and lipase-inhibiting
570 potential of bioactive peptides from camel whey hydrolysates: Identification,

characterization, and molecular interaction. *Journal of Dairy Science*, *104*(7), 7393-7405.

38. Mudgil, P., Kamal, H., Yuen, G. C., & Maqsood, S. (2018). Characterization and identification of novel antidiabetic and anti-obesity peptides from camel milk protein hydrolysates. *Food chemistry*, *259*, 46-54.
39. Coronado-Cáceres, L. J., Rabadán-Chávez, G., Mojica, L., Hernández-Ledesma, B., Quevedo-Corona, L., & Lugo Cervantes, E. (2020). Cocoa (Theobroma cacao L.) seed proteins' anti-obesity potential through lipase inhibition using in silico, in vitro and in vivo models. *Foods*, *9*(10), 1359.
40. Ngoh, Y. Y., Choi, S. B., & Gan, C. Y. (2017). The potential roles of Pinto bean (Phaseolus vulgaris cv. Pinto) bioactive peptides in regulating physiological functions: Protease activating, lipase inhibiting and bile acid binding activities. *Journal of Functional Foods*, *33*, 67-75.
41. Awosika, T. O., & Aluko, R. E. (2019). Inhibition of the in vitro activities of α□amylase, α□glucosidase and pancreatic lipase by yellow field pea (Pisum sativum L.) protein hydrolysates. *International Journal of Food Science & Technology*, *54*(6), 2021-2034.
42. Ngoh, Y. Y., Choi, S. B., & Gan, C. Y. (2017). The potential roles of Pinto bean (Phaseolus vulgaris cv. Pinto) bioactive peptides in regulating physiological functions: Protease activating, lipase inhibiting and bile acid binding activities. *Journal of Functional Foods*, *33*, 67-75.
43. Mudgil, P., Al Dhaheri, M. K. O., Alsubousi, M. S. M., Khan, H., Redha, A. A., Yap, P. G., & Maqsood, S. (2024). Molecular docking studies on α-amylase inhibitory peptides from milk of different farm animals. *Journal of Dairy Science*, *107*(5), 2633-2652.
44. Su, N., Yi, L., He, J., Ming, L., Jambal, T., Mijiddorj, B., & Ji, R. (2024). Identification and molecular docking of a novel antidiabetic peptide from protamex-camel milk protein hydrolysates against α-amylase and DPP-IV. *International Dairy Journal*, *152*, 105884.
45. Singh, B. P., Paul, S., & Goel, G. (2024). Shotgun proteomics and molecular simulations on multifunctional bioactive peptides derived from the whey of unexplored "Gaddi" goat of Himalayas. *Food Chemistry*, *430*, 137075.

Legends and contents

605 **Figure 3.0. Workflow of profiling and identification of bioactive**
606 **peptides from WP-PTC hydrolysate.**

607 **Figure 3.1. (a) Nano-HPLC chromatogram and (b) total ion spectra**
608 **of the whey protein peptides.**

609 **Figure 3.2 a. Numbers of peptides amino acid residues.**

610 **Figure 3.2 b. Bioactivity score of identified peptides from whey**
611 **protein hydrolysate.**

612 **Figure 3.2 c. Peptide sequence logos were generated for the**
613 **sequences predicted with bioactivity scores ranging from 0.90 to 1.**
614 **The peptide sequence logo for these sequences displayed cysteine,**
615 **proline, tryptophan, phenylalanine, methionine, and glycine as the**
616 **dominant residues.**

617 **Figure 3.2 d. Toxic and nontoxic peptide predicted with bioactivity**
618 **scores ranging from 0.90 to 1.**

619 **Figure 3.3. Molecular weight of identified peptides.**

620 **Figure 3.4. (a) Binding energy of the interacted bioactive peptides**
621 **with pancreatic lipase.**

622 **Figure 3.4. b. 2-D binding analysis of the bioactive peptide**
623 **WPGIMR with pancreatic lipase.**

624 **Figure 3.4. c. 2-D Binding analysis of the bioactive peptide**
625 **WQDGSWQF, with pancreatic lipase.**

626 **Figure 3.4. d. Binding energy of the interacted bioactive peptides**
627 **with α-amylase.**

628 **Figure 3.4. e. 2-D Binding analysis of the bioactive peptide**
629 **AAPFIWL with Alpha amylase.**

630 **Figure 3.4. f. 2-D binding analysis of the bioactive peptide**
631 **WQDGSWQF, with Alpha amylase.**

632 **Figure 3.5. The binding energy, ranging from -3.1 to -8.4, of peptide**
633 **sequence logos was generated for the sequences predicted to bind**
634 **with the pancreatic lipase and α-amylase pockets. (a) The peptide**

sequence logo for the pancreatic lipase binding peptide sequences displayed tryptophan, methionine, phenylalanine and proline as the dominant residues. (b) The peptide sequence logo for the predicted α-amylase binding peptide sequences highlighted tryptophan, leucine and proline as the predominant the dominant residues.

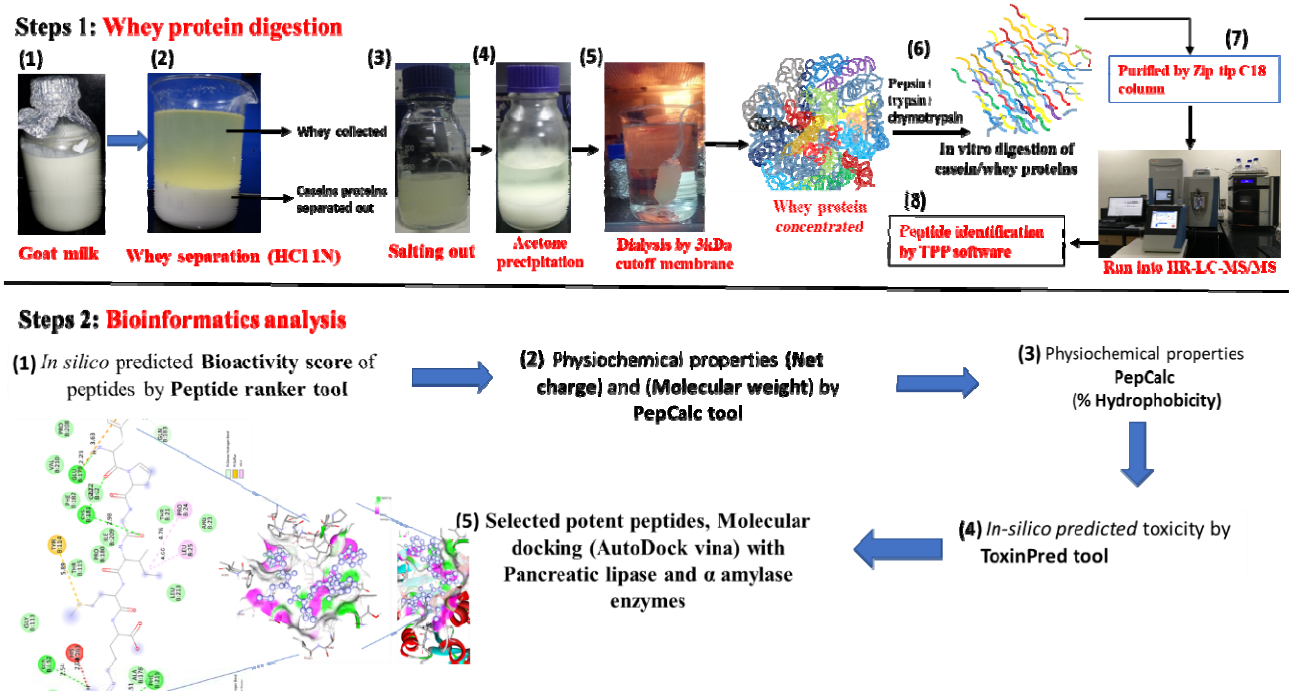
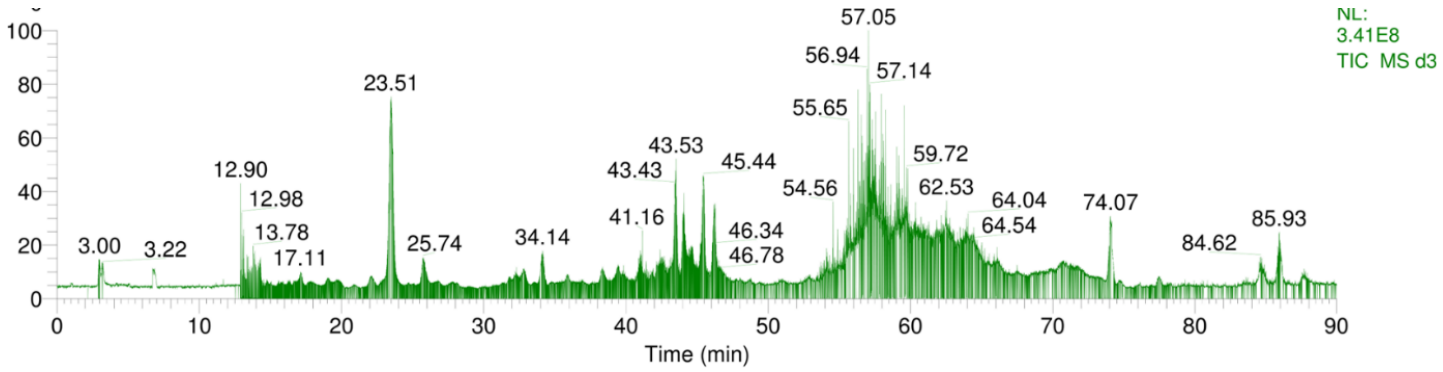

Steps 1: Whey protein digestion
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
Whey collected
Caseins proteins separated out
Goat milk
Whey separation (HCl 1N)
Salting out
Acetone precipitation
Dialysis by 3kDa cutoff membrane
Whey protein concentrated
Pepsin ; trypsin ; chymotrypsin
In vitro digestion of casein/whey proteins
Purified by Zip tip C18 column
Peptide identification by TPP software
Run into HR-LC-MS/MS
Steps 2: Bioinformatics analysis
(1) In silico predicted Bioactivity score of peptides by Peptide ranker tool
(2) Physiochemical properties (Net charge) and (Molecular weight) by PepCalc tool
(3) Physiochemical properties PepCalc (% Hydrophobicity)
(4) In-silico predicted toxicity by ToxinPred tool
(5) Selected potent peptides, Molecular docking (AutoDock vina) with Pancreatic lipase and α amylase enzymes

Figure 3.0.

(a)


NL:
3.41E8
TIC MS d3
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Time (min)
3.00
3.22
12.90
12.98
13.78
17.11
23.51
25.74
34.14
41.16
43.43
43.53
45.44
46.34
46.78
54.56
55.65
56.94
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57.14
59.72
62.53
64.04
64.54
74.07
84.62
85.93

(b)

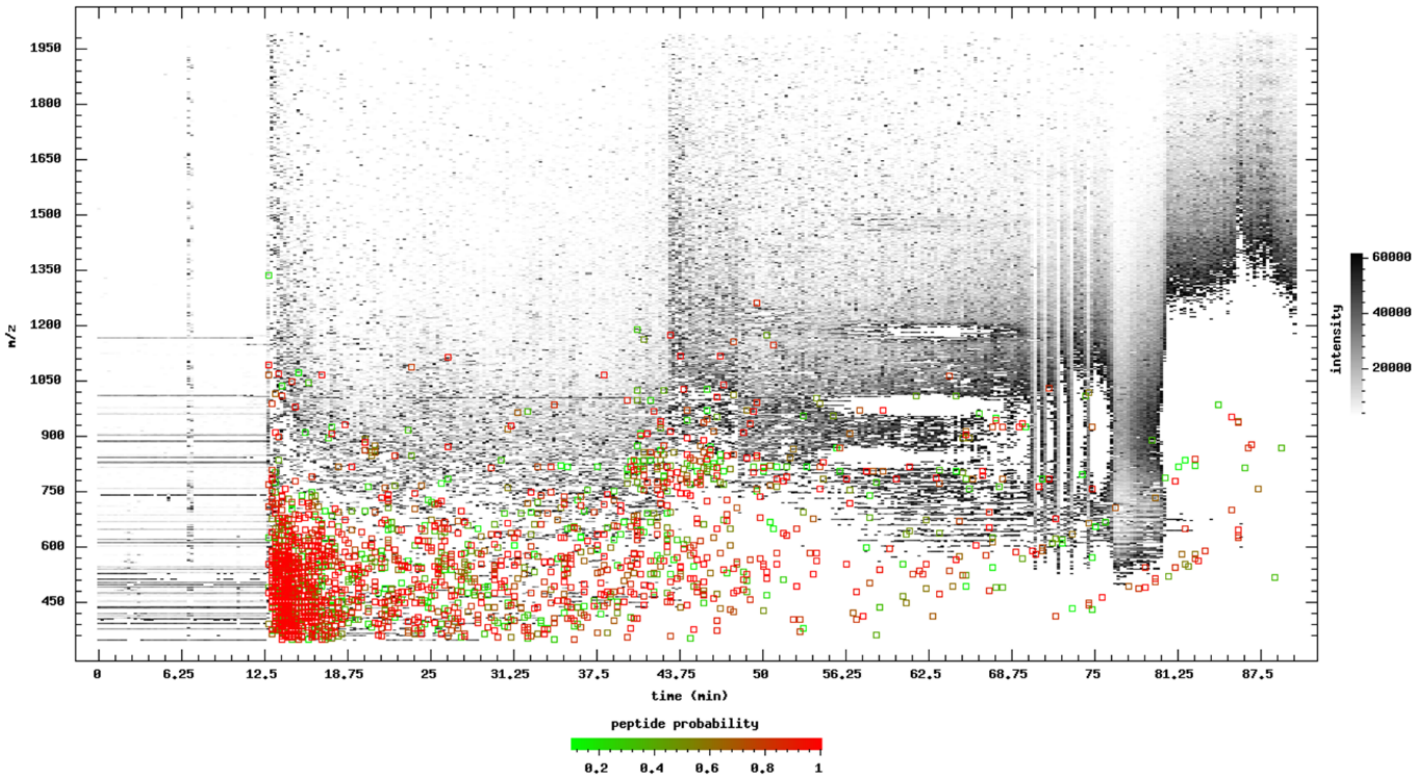


Figure 3.1. a & b

(a)

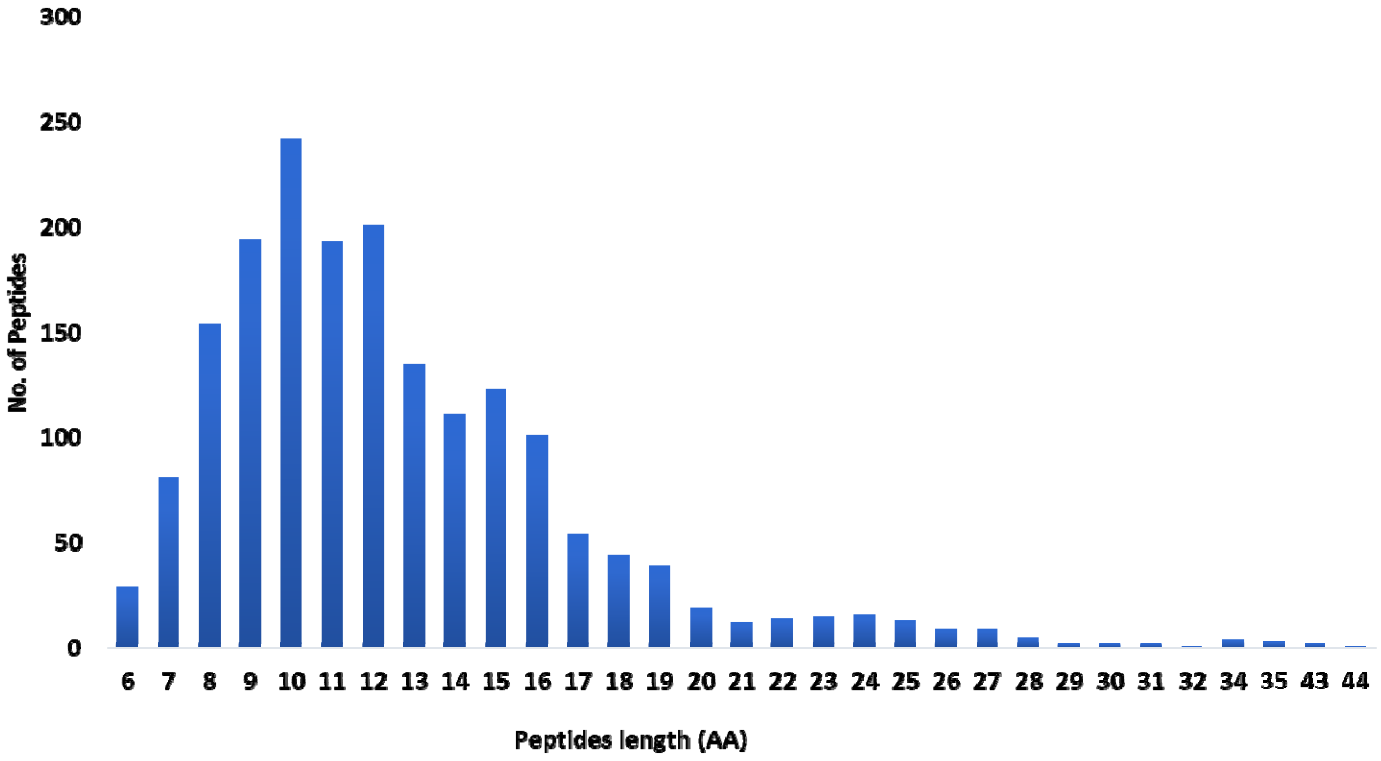
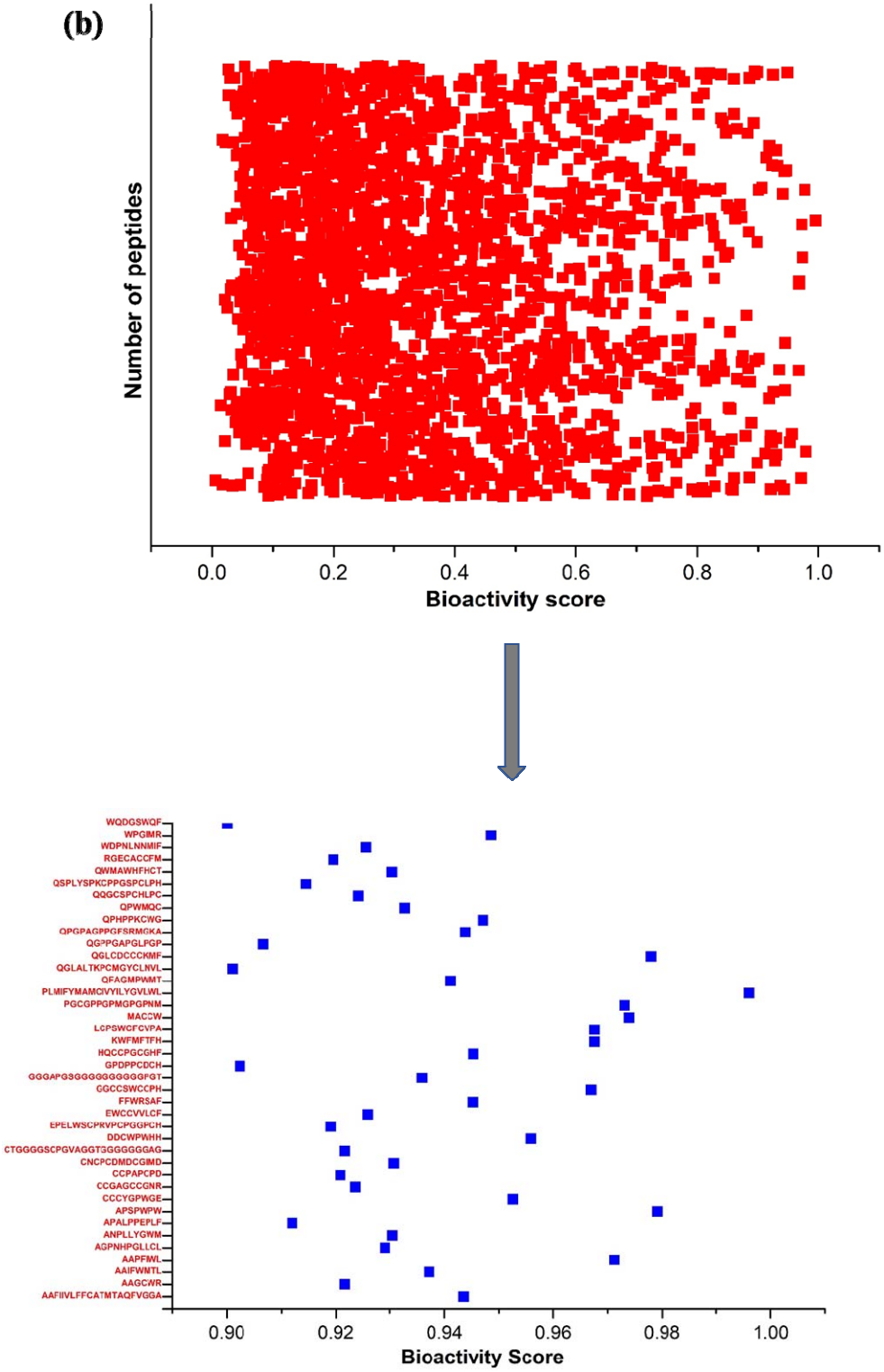

300
250
200
150
100
50
0
No. of Peptides
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 34 35 43 44
Peptides length (AA)

Figure 3.2 a.


(b)
Number of peptides
0.0
0.2
0.4
0.6
0.8
1.0
Bioactivity score
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WPGMR
WDPNLNNMIF
RGECACCFM
QWMAWHFHCT
QSPLYSPKCPPGSPCLPH
QQGCSPCHLPC
QPWMQC
QPHPPKCWG
QPGPAGPPGFSRMGKA
QGPPGAPGLFGP
QGLCDCCCKMF
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QFAGMPWMT
PLMIFYMAMCIVYILYGVLWL
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MACCW
LCPSWCFCVPA
KWFMFTFH
HQCCPGCGHF
GPDPPCDCH
GGGAPGSGGGGGGGGGGFGT
GGCCSWCCPH
FFWRSAF
EWCCVVLCF
EPELWSCPKVPCPGGPCH
DDCWPWHH
CTGGGGSCPGVAGGTGGGGGGGAG
CNCPCDMDCGIMD
CCPAPCPD
CCGAGCCGNR
CCCYGPWGE
APSPWPW
APALPPEPLF
ANPLLYGWM
AGPNHPGLLCL
AAPFWL
AAIFWMTL
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0.90
0.92
0.94
0.96
0.98
1.00
Bioactivity Score

Figure 3.2 b.

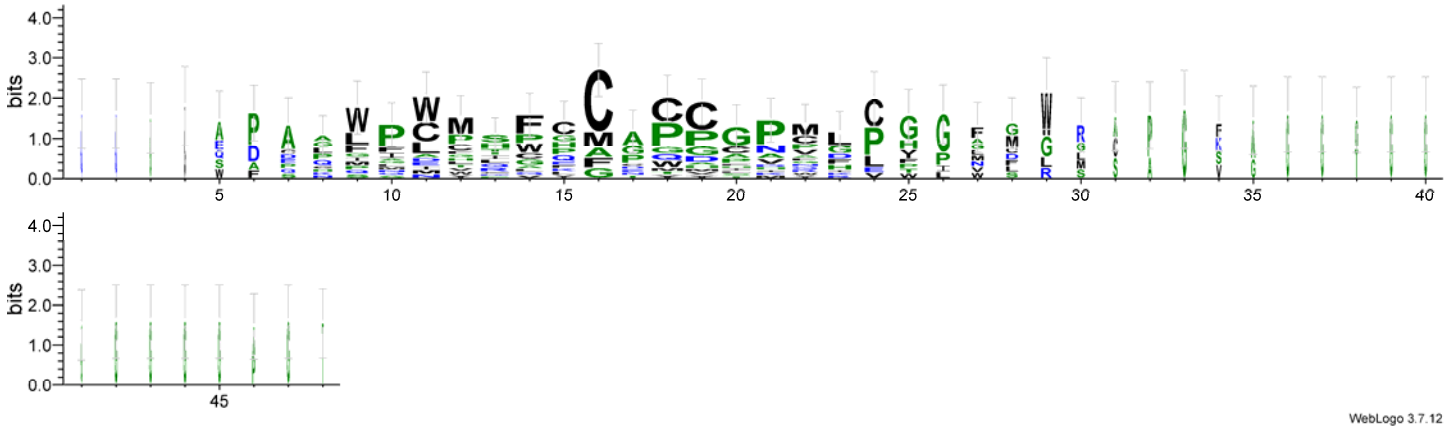


Figure 3.2 c:

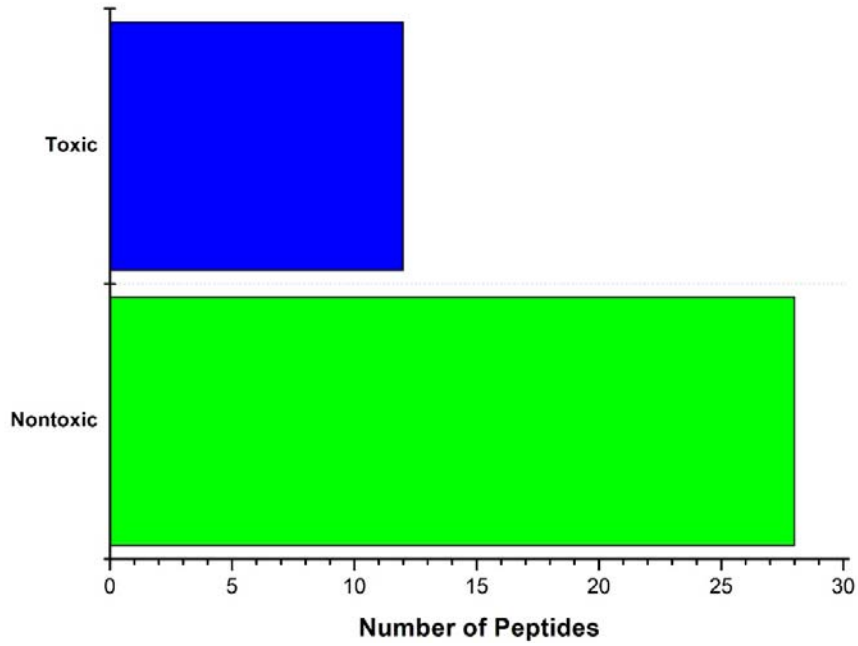
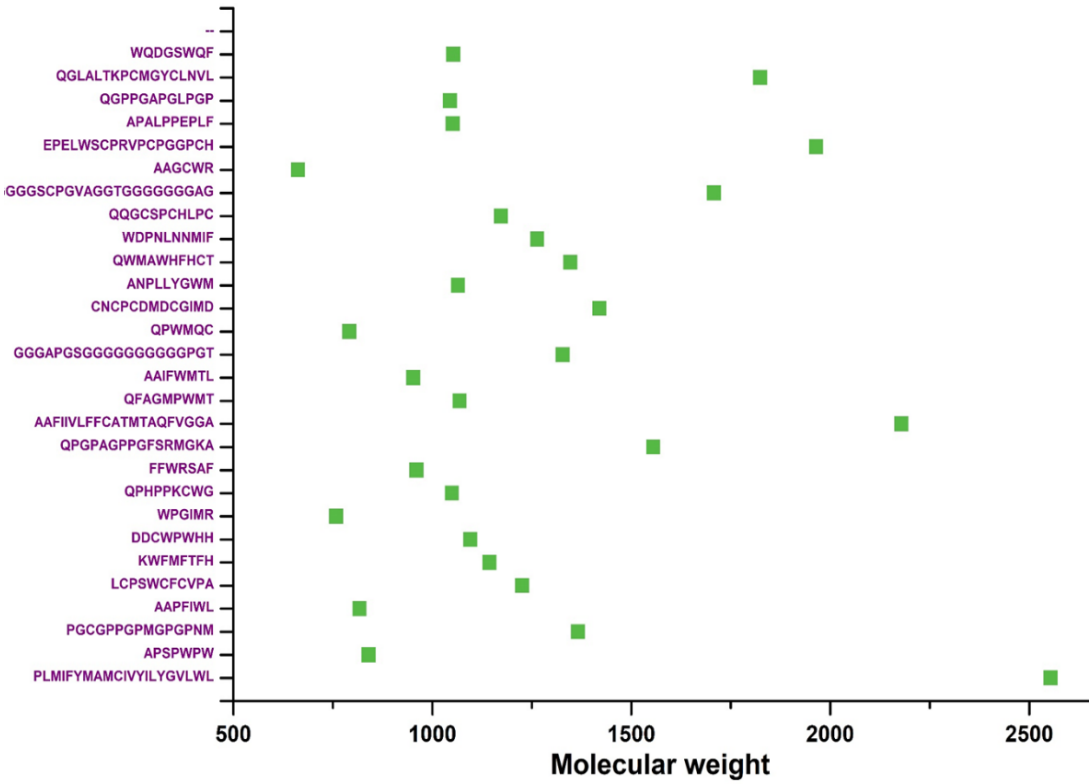


Figure 3.2 d: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ANPLLYGWM
CNCPCDMDCGIMD
QPWMQC
GGGAPGSGGGGGGGGGGPGT
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AAFIIVLFFCATMTAQFVGGA
QPGPAGPPGFSRMGKA
FFWRSAF
QPHPPKCWG
WPGIMR
DDCWPWHH
KWFMFTFH
LCPSWCFCVPA
AAPFIWL
PGCGPPGPMGPGPNM
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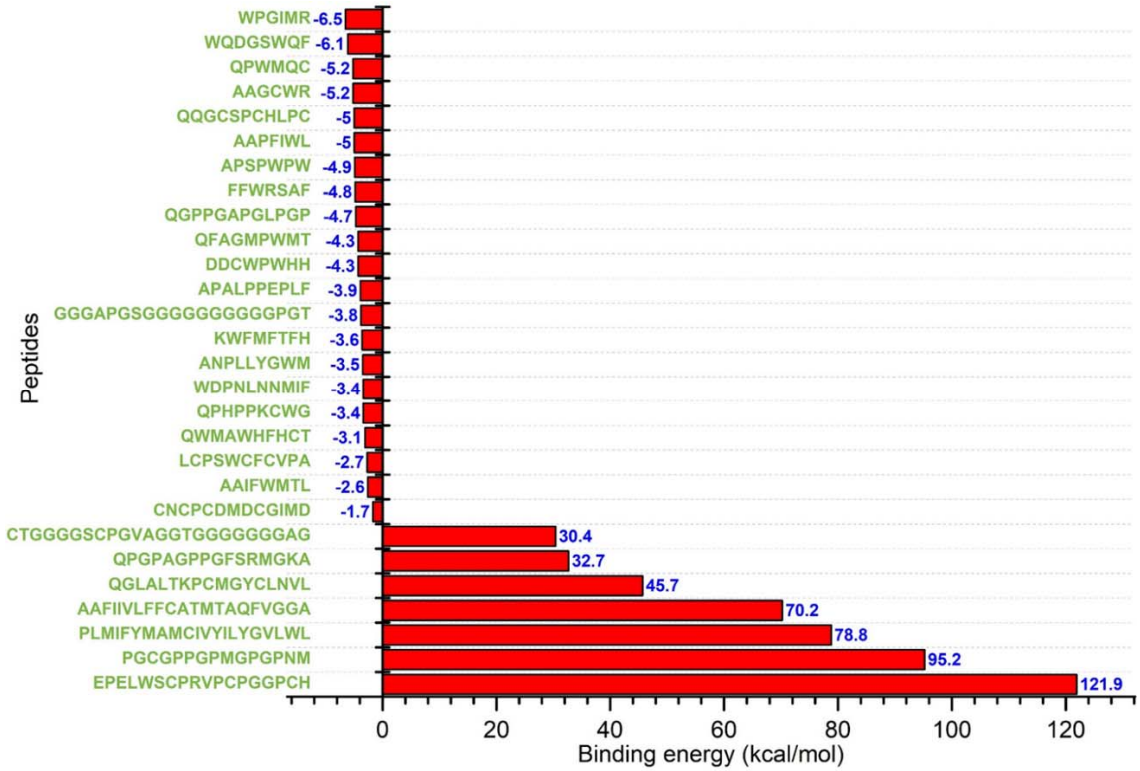
PLMIFYMAMCIVYILYGVLWL
500
1000
1500
2000
2500
Molecular weight

Figure 3.3.

(a)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WQDGSWQF -6.1
QPWMQC -5.2
AAGCWR -5.2
QQGCSPCHLPC -5
AAPFIWL -5
APSPWPW -4.9
FFWRSAF -4.8
QGPPGAPGLPGP -4.7
QFAGMPWMT -4.3
DDCWPWHH -4.3
APALPPEPLF -3.9
GGGAPGSGGGGGGGGGGPGT -3.8
KWFMFTFH -3.6
ANPLLYGWM -3.5
WDPNLNNMIF -3.4
QPHPPKCWG -3.4
QWMAWHFHCT -3.1
LCPSWCFCVPA -2.7
AAIFWMTL -2.6
CNCPCDMDCGIMD -1.7
CTGGGGSCPGVAGGTGGGGGGGAG 30.4
QPGPAGPPGFSRMGKA 32.7
QGLALTKPCMGYCLNVL 45.7
AAFIIVLFFCATMTAQFVGGA 70.2
PLMIFYMAMCIVYILYGVLWL 78.8
PGCGPPGPMGPGPNM 95.2
EPELWSCPRVPCPGGPCH 121.9
Peptides
0
20
40
60
80
100
120
Binding energy (kcal/mol)

Figure 3.4. (a)

(b)

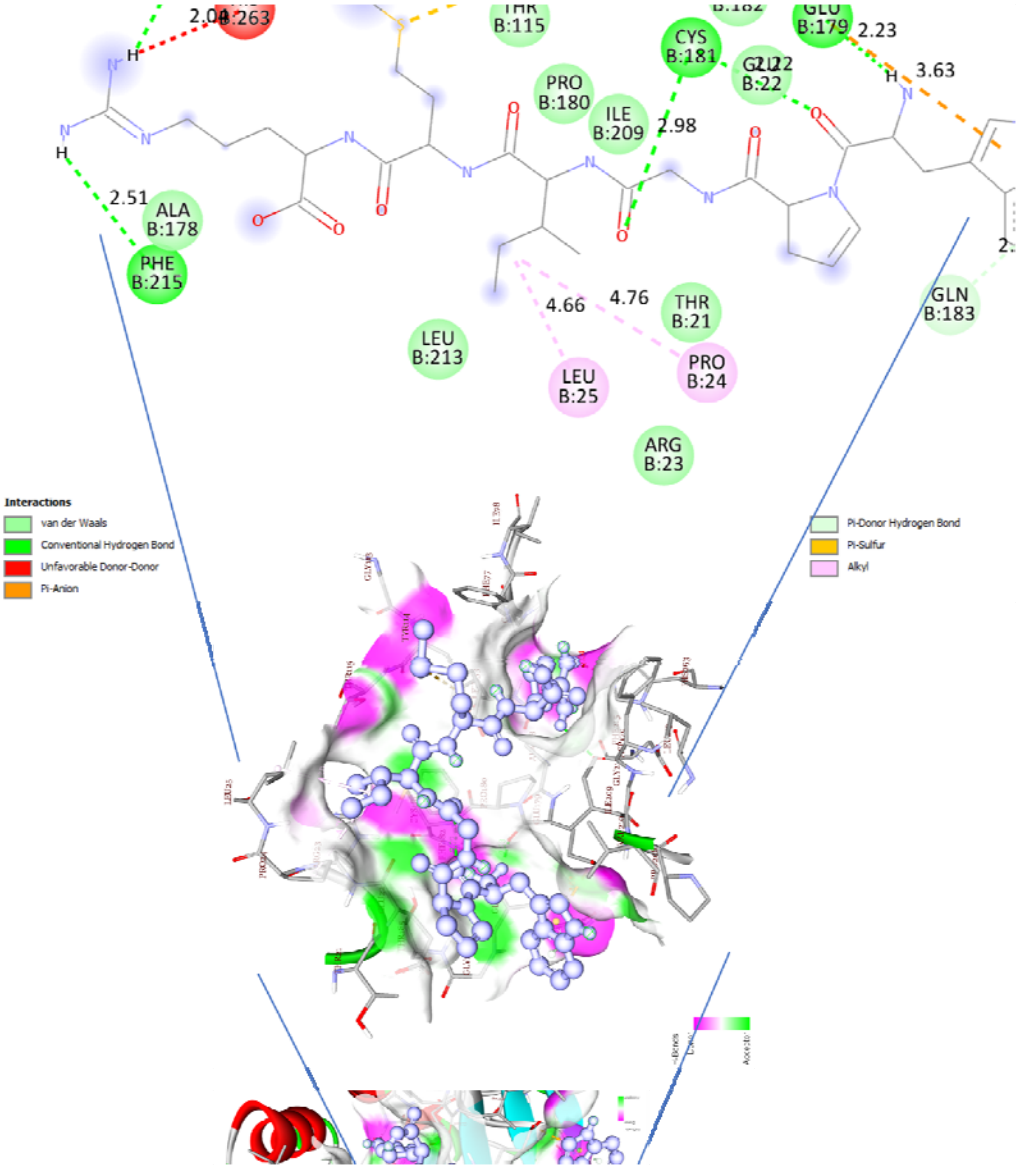
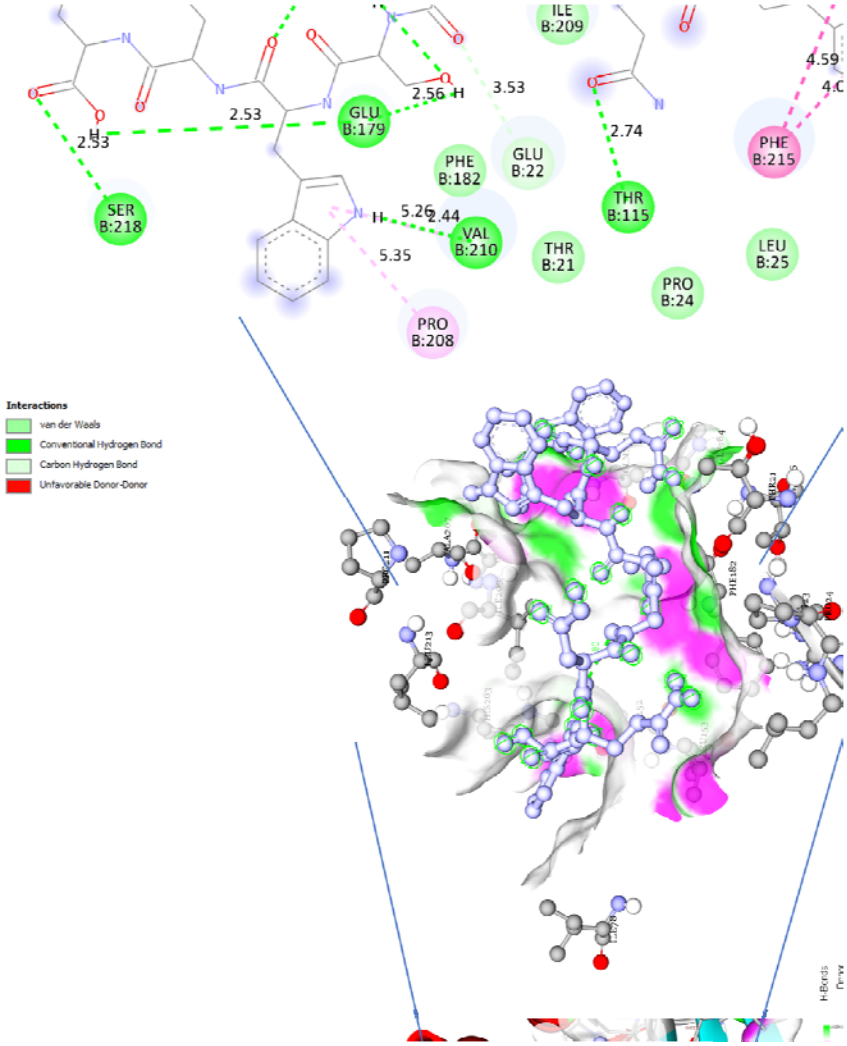


Figure 3.4. b

(c)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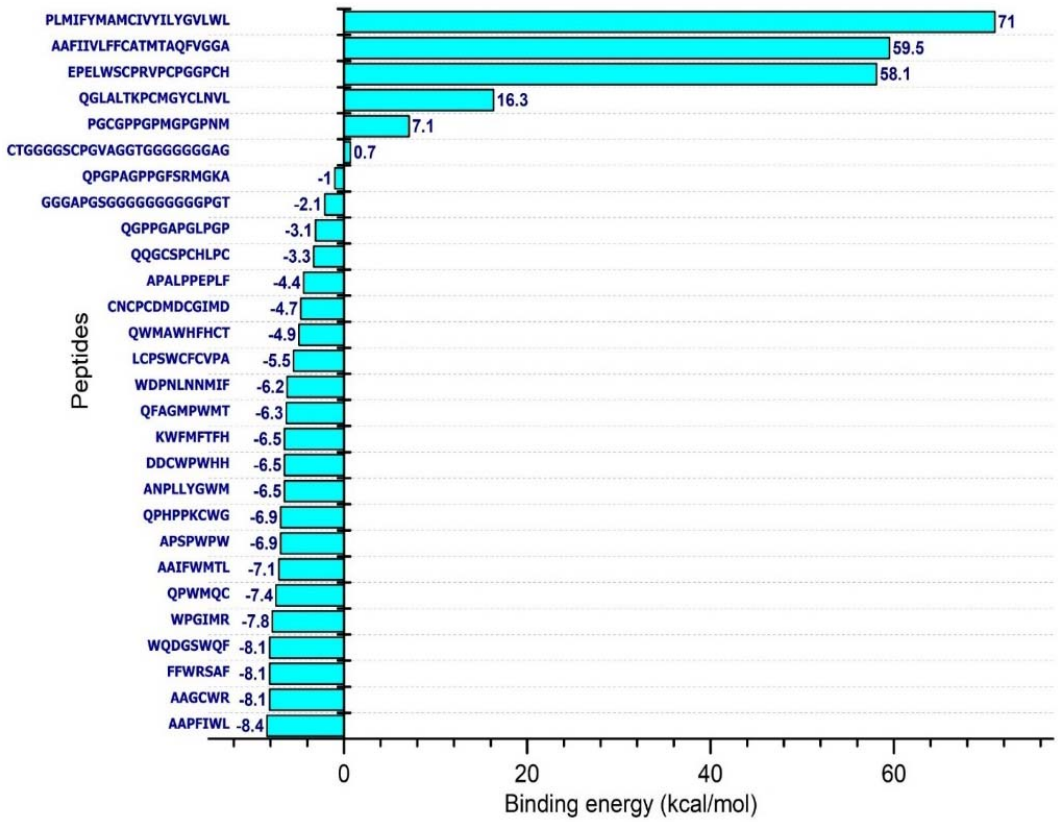
3.53
2.56
2.53
2.53
GLU
B:179
2.74
PHE
B:215
PHE
B:182
GLU
B:22
THR
B:115
SER
B:218
5.26
2.44
VAL
B:210
THR
B:21
LEU
B:25
5.35
PRO
B:24
PRO
B:208
Interactions
van der Waals
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Unfavorable Donor-Donor

Figure 3.4. c.

(d.)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DDCWPWHH
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QPHPPKCWG
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QPWMQC
WPGIMR
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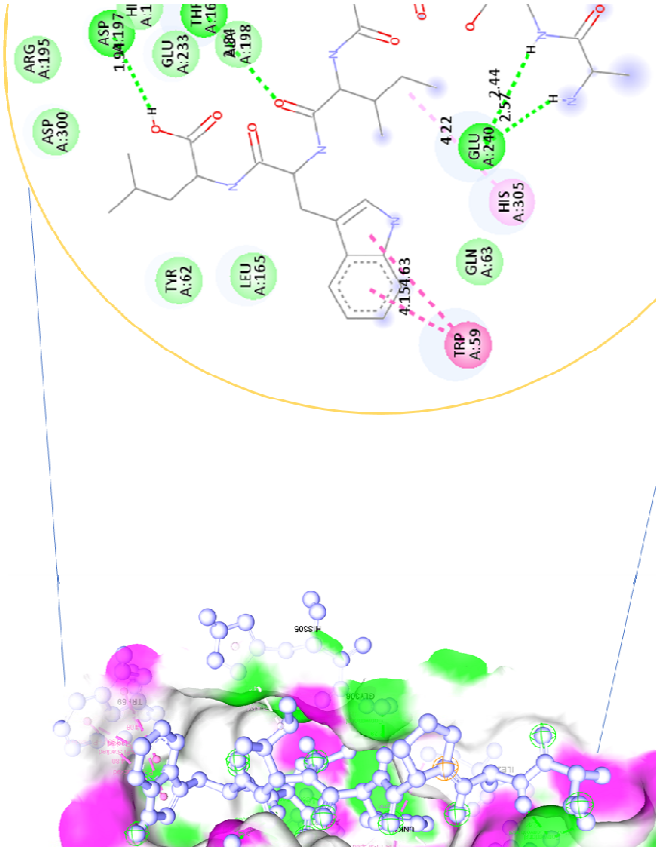
FFWRSAF
AAGCWR
AAPFIWL
71
59.5
58.1
16.3
7.1
0.7
-1
-2.1
-3.1
-3.3
-4.4
-4.7
-4.9
-5.5
-6.2
-6.3
-6.5
-6.5
-6.5
-6.9
-6.9
-7.1
-7.4
-7.8
-8.1
-8.1
-8.1
-8.4
Peptides
0
20
40
60
Binding energy (kcal/mol)

Figure 3.4. d.

(e)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GLN
A:63
TYR
A:62
LEU
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TRP
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2.44
2.57
4.154
4.63

Figure 3.4. e.

(f)

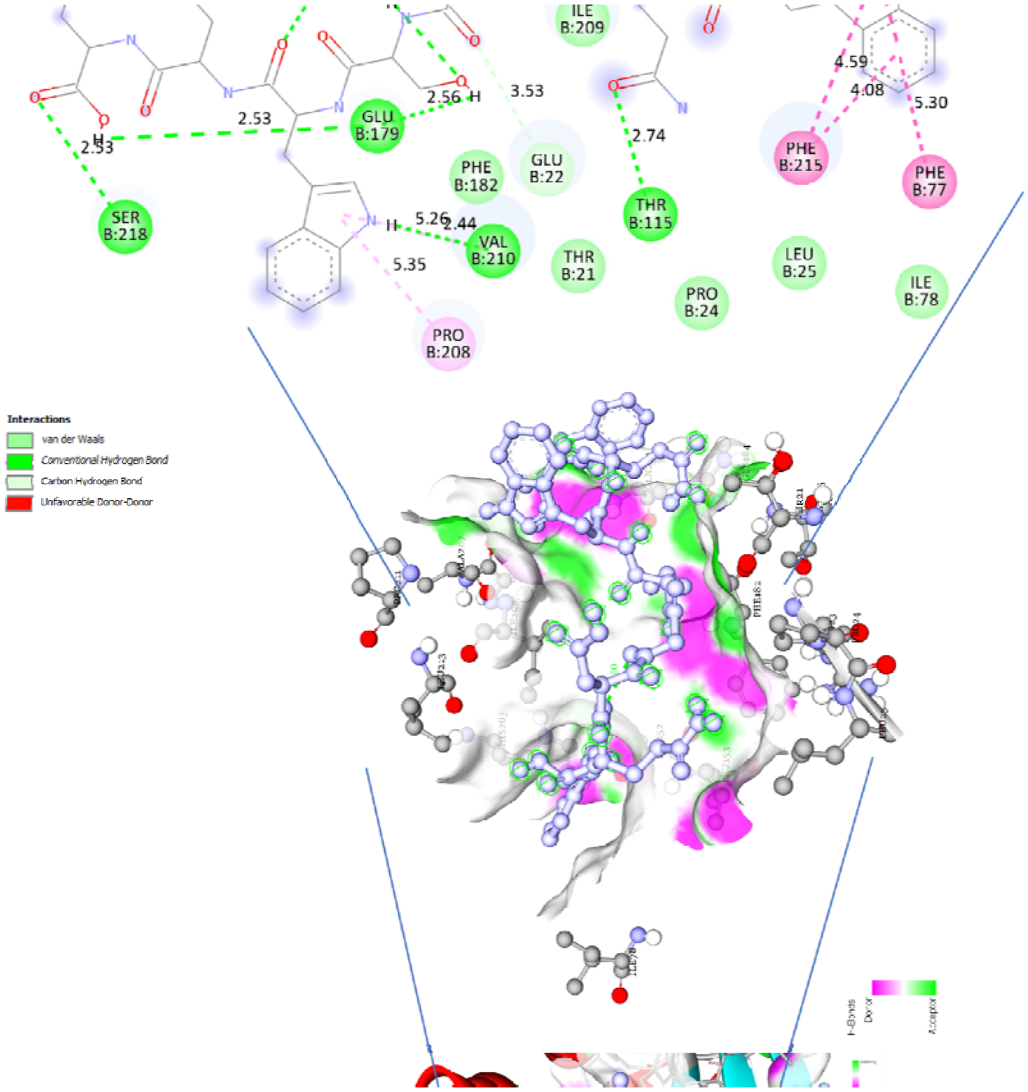
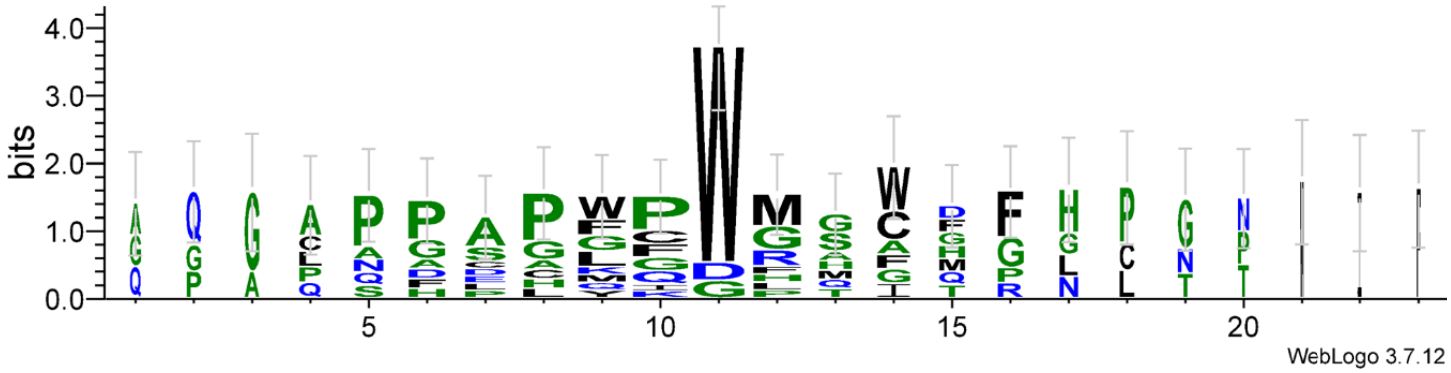


Figure 3.4. f. 2-D

(a)



(b)

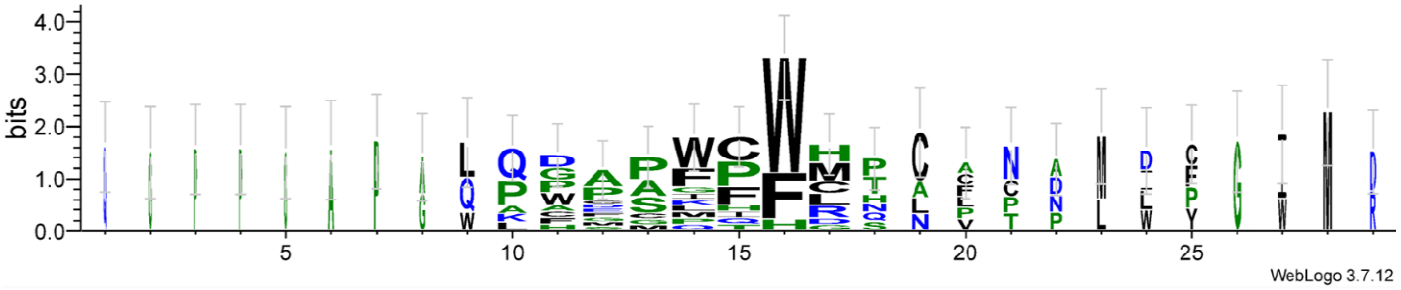


Figure 3.5.