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In Silico-Based Screening of Vannamei Shrimp Myosin Protein as a Potential Source of Antihypertensive and Antidiabetic Bioactive Peptides

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ABSTRACT

Vannamei shrimp (*Penaeus vannamei*) is an important aquaculture commodity and a potential source of bioactive peptides with diverse health benefits. However, its myosin protein remains underexplored as a source of the peptides. This study aimed to predict antihypertensive and antidiabetic activities of the peptides derived from the shrimp's myosin using an *in silico* approach. The myosin sequence was retrieved from the National Center for Biotechnology Information (NCBI) database (GenBank accession number: ROT81-948.1). Basic Local Alignment Search Tool (BLAST) analysis revealed high similarity to myosin heavy chains from several shrimp species, with 93-100% query coverage and 87.67 and 91.43% identity values. Enzymatic hydrolysis was simulated using Bioactive Peptides Database (BIOPEP-UWM) to predict bioactive peptide release. The ficin-bromelain combination released 30 unique bioactive dipeptides, compared with 23 sequences from ficin-papain combination, and produced more dipeptides associated with ACE- and DPP-IV-inhibitory activities. Five dipeptides (EK, DR, EG, IR, and NY) were predicted to exhibit dual ACE- and DPP-IV-inhibitory effects. EA and DL emerged as the most frequent ACE-inhibitory peptides, whereas AL and QL were the most frequent DPP-IV-inhibitory peptides. All identified dipeptides had average molecular weights of <320 Da, consistent with typical bioactive dipeptides. These findings highlight the potential of shrimp myosin hydrolyzed with food-grade proteases as a promising source of bioactive peptides for functional food development.

Keywords: Bioactive peptides, BIOPEP-UWM, *in silico*, myosin, vannamei shrimp

INTRODUCTION

Bioactive peptides are specific protein fragments that exert beneficial effects on physiological functions and may contribute to human health (Kadam *et al.,* 2015). These peptides consist of two or more amino acid residues linked by covalent peptide bonds and are generally composed of 2–20 amino acids. Bioactive peptides can be released from precursor proteins or polypeptides through enzymatic hydrolysis and may exhibit a wide range of biological activities, including antimicrobial, antithrombotic, antihypertensive, immunomodulatory, antioxidant, and antidiabetic effects (Sánchez and Vázquez, 2017). The biological activity of these peptides is influenced by their amino acid sequence, molecular size, hydrophobicity, and structural characteristics. Enzymatic hydrolysis is therefore an important approach for generating peptides with specific health-promoting properties. Bioactive peptides have been identified from various animal and plant protein sources, including milk, eggs, meat, soybeans, and wheat, as well as marine resources such as fish, algae, shellfish, and sea cucumbers (Bhat *et al.,* 2015; Cunha and Pintado, 2022).

Among the reported biological activities, anti-hypertensive activity has received considerable attention because bioactive peptides may modulate blood pressure through inhibition of angiotensin-converting enzyme (ACE), a key enzyme in the renin-angiotensin system involved in blood pressure regulation (Pearman *et al.,* 2025; Ramlal *et al.,* 2022; Zhang *et al.,* 2025). Previous studies have suggested that short-chain peptides, particularly those with molecular weights below 3 kDa and hydrophobic amino acid residues, may exhibit relatively strong ACE-inhibitory activity (Ichim *et al.,* 2024). Marine organisms have also been recognized as promising sources of antihypertensive peptides. For example, GFPGLP from sea cucumber and AEYLCEAC from oyster have been reported as peptide sequences with ACE-inhibitory potential (Chen *et al.,* 2022; Liang *et*

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al., 2025). These findings indicate that marine-derived proteins may serve as valuable precursors for generating peptides with potential applications in blood pressure management.

Beyond antihypertensive effects, marine-derived peptides have shown potential antidiabetic activity, particularly by inhibiting DPP-IV. Takahashi *et al.* (2021) reported that peptides derived from salmon milt exhibited DPP-IV-inhibitory activity and reduced postprandial blood glucose levels in an *in vivo* model. Similarly, CunhaNeves *et al*. (2022) demonstrated that protein hydrolysates from blue mussel (*Mytilus edulis*) exhibited antidiabetic potential through DPP-IV inhibition. Hossain *et al.* (2020) further reported that protein hydrolysates from Atlantic red sea cucumber (*Cucumaria frondosa*) contained bioactive peptides with ACE- and DPP-IV-inhibitory activities, together with antioxidant properties. These multi-functional activities are particularly relevant because oxidative stress and disturbances in blood pressure and glucose regulation frequently coexist in metabolic disorders. In another study, Neves *et al.* (2017) identified peptides from salmon gelatin hydrolysates that exhibited ACE-inhibitory, DPP-IV-inhibitory, and antioxidant activities. Collectively, these findings support the potential of marine proteins as sources of multifunctional bioactive peptides for the development of functional foods and nutraceutical ingredients.

Indonesia possesses abundant marine biological resources that remain underexplored for developing bioactive peptide-based functional ingredients. Among Indonesia's major aquaculture commodities, including in regions like East Java, whiteleg shrimp (*Penaeus vannamei*) is particularly relevant due to its high protein content and substantial production. However, research on the bioactive potential of specific structural proteins from *P. vannamei*, particularly myosin heavy chain type A (MHC-A), remains limited. MHC-A is one of the major structural proteins in shrimp muscle and may serve as a precursor for bioactive peptides after enzymatic hydrolysis. Identification of potential bioactive sequences from MHC-A could therefore provide an alternative strategy for valorizing shrimp protein resources and supporting the development of marine-derived functional ingredients.

In silico approaches provide a rapid and cost-effective preliminary strategy to screen protein sequences for potential bioactive peptides prior to experimental validation. Computational analysis can facilitate the prediction of peptide sequences that may exhibit specific biological activities and thereby reduce the number of candidates requiring subsequent laboratory testing (Iwaniak *et al.,* 2024; Minkiewicz *et al.,* 2019; Minkiewicz *et al.,* 2022). Nevertheless*, in silico* predictions represent theoretical potential rather than confirmed biological activity and should therefore be validated using appropriate in vitro and, subsequently, *in vivo* assays.

Therefore, this study aimed to predict potential bioactive peptides generated from the hydrolysis of *P. vannamei* MHC-A using an *in silico* approach, with particular emphasis on ACE- and DPP-IV-inhibitory activities as indicators of potential antihypertensive and antidiabetic effects. The findings are expected to provide a preliminary basis for the subsequent experimental validation and development of *P. vannamei* MHC-A as a potential source of bioactive peptides for functional food or nutraceutical applications.

MATERIALS AND METHOD

Protein sequence and bioinformatics resources

The protein sequence analyzed in this study was the MHC-A of *P. vannamei*, retrieved from the National Center for Biotechnology Information (NCBI) Protein database. The sequence was obtained in FASTA format under GenBank accession number ROT81948.1 and consisted of 1,299 amino acid residues. This protein was selected as a potential precursor of bioactive peptides because myosin is one of the major structural proteins involved in muscle contraction. The bioinformatics databases and tools used in this study included NCBI (https://www.ncbi.nlm.nih.gov) for protein sequence retrieval and sequence similarity analysis using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), BIOPEP-UWM (https://biochemia.uwm.edu.pl/biopep/start_biopep.php), for *in silico* enzymatic digestion and prediction of potential biological activities, and ToxinPred for preliminary toxicity prediction. All analyses were performed using a computer with internet access. Three food-grade proteases, namely ficin (EC 3.4.22.3), bromelain (EC 3.4.22.32), and papain (EC 3.4.22.2), were selected for the *in silico* hydrolysis simulation. Ficin is obtained from fig (*Ficus carica*), bromelain from pineapple (*Ananas comosus*), and papain mainly from papaya latex (*Carica papaya*). These plant-derived cysteine proteases have been investigated for protein hydrolysis and bioactive peptide production (Morellon-Sterling *et al.,* 2020; Tacias-Pascacio *et al.,* 2023). Despite sharing similar catalytic mechanisms, they differ in substrate specificity. Bromelain exhibits broad proteolytic activity, while ficin and papain show preferences for peptide bonds involving hydrophobic residues, including phenylalanine, depending on the substrate (Venetikidou *et al.,* 2025). These differences may generate distinct peptide profiles and influence the predicted occurrence of ACE-inhibitory (antihypertensive) and DPP-IV-inhibitory (antidiabetic) peptides.

Study workflow

The study consisted of six main stages: (1) retrieval of the MHC-A protein sequence from the NCBI database; (2) sequence similarity analysis using BLASTp; (3) *in silico* protein hydrolysis using BIOPEP-UWM; (4) identification of potential biological activities of the resulting peptides; (5) toxicity screening and selection of potential non-toxic bioactive peptides; and (6) descriptive analysis and interpretation of the results.

Retrieval and sequence confirmation of MHC-A

The MHC-A sequence was identified by searching the NCBI Protein database using the keyword "*Penaeus vannamei* myosin heavy chain type A." The retrieved sequences were screened based on the organism and protein annotation. The sequence with GenBank accession number ROT81948.1 was selected and downloaded in FASTA format for subsequent analysis. The selected sequence consisted of 1,299 amino acid residues. Sequence identity and similarity were subsequently evaluated using the BLASTp algorithm against the NCBI protein database. The analysis was performed to confirm the similarity of the selected sequence to myosin proteins from other species. The main parameters considered were query coverage and sequence identity. Query coverage represents the percentage of the query sequence aligned with the subject sequence, whereas sequence identity represents the percentage of identical amino acid residues within the aligned region. Higher query coverage and sequence identity values indicate greater sequence similarity.

***In silico* protein hydrolysis**

In silico protein hydrolysis was performed using the *in silico* digestion function of the BIOPEP-UWM database according to previously described procedures (Iwaniak *et al.,* 2024; Minkiewicz *et al.,* 2019). Simulated enzymatic hydrolysis was performed using the "Enzyme(s) action" tool in the BIOPEP-UWM database. The simulation was based on enzyme-specific recognition and cutting sequences, where the recognition sequence represents the amino acid sequence recognized by an enzyme, and the cutting sequence identifies the residue associated with the peptide bond susceptible to hydrolysis. Complete hydrolysis was applied, cleaving all eligible cleavage sites according to the enzyme-specific cleavage rules implemented in BIOPEP-UWM. Individual and combined enzyme treatments were simulated according to the cleavage specificities encoded in BIOPEP-UWM. The resulting peptide fragments and their positions within the parent protein sequence were recorded for subsequent bioactivity screening. Because BIOPEP-UWM uses a theoretical model of proteolysis, the predicted fragments represent potential hydrolysis products rather than experimentally confirmed peptides. Because this was a computational simulation, experimental hydrolysis conditions, including pH, temperature, enzyme-to-substrate ratio, and hydrolysis time, were not specified.

Prediction of potential biological activities

The peptide fragments generated by the simulated hydrolysis were analyzed using BIOPEP-UWM to identify potential biological activities based on sequence matches with bioactive peptides deposited in the database (Minkiewicz *et al.,* 2022). The activities assessed included angiotensin-converting enzyme (ACE)-inhibitory, antioxidative, renin-inhibitory, calcium/calmodulin-dependent phosphodiesterase (CaMPDE)-inhibitory, dipeptidyl peptidase IV (DPP-IV)-inhibitory, dipeptidyl peptidase III (DPP-III)-inhibitory, α-glucosidase-inhibitory, glutamate carboxypeptidase-inhibitory, glutamate carboxypeptidase II-inhibitory, citrate lyase deacetylase-inhibitory, thymidylate synthase-inhibitory, and neprilysin-inhibitory activities. Dipeptide sequences are represented using the standard one-letter codes for amino acids, according to the IUPAC-IUB nomenclature. The official list of amino acid codes and their corresponding names is available at https://iupac.qmul.ac.uk/AminoAcid/AA212.html. The BIOPEP-UWM parameters used for comparison were the frequency of occurrence (AE) and the theoretical number of bioactive peptides (d). The frequency of occurrence is expressed as the ratio between the sum of bioactive peptide occurrences and the total number of amino acid residues in the precursor protein, according to the Equation 1.

$$AE = \frac{d}{N} \quad \text{..... (1)}$$

AE represents the frequency of occurrence of a given bioactive peptide activity, *d* represents the theoretical number of bioactive peptides associated with that activity, and *N* represents the total number of amino acid residues in the precursor protein. In the present study, the MHC-A precursor protein consisted of 1,299 amino acid residues. For comparative analysis, d was used as the primary parameter to describe the predicted abundance of peptide fragments associated with each biological activity after different enzyme treatments. The AE values were considered as normalized frequency parameters that account for the length of the precursor protein.

Toxicity prediction and selection of potential peptides

The toxicity of the peptides generated through the simulated hydrolysis was evaluated using Toxin-

Pred (https://webs.iiitd.edu.in/raghava/toxinpred/) (Gupta *et al.,* 2013), employing the SVM (Swiss-Prot)-based model with the default SVM threshold of 0.0. Peptides were classified as toxic or non-toxic according to their SVM scores relative to the selected threshold. Peptide sequences were entered in FASTA format and analyzed using the ToxinPred prediction models based on Support Vector Machine (SVM) algorithms.

The available prediction features included amino acid composition, dipeptide composition, binary profiles, and motif-based profiles. Peptides exhibiting the targeted biological activities (particularly ACE- and DPP-IV-inhibitory activities) and predicted to be non-toxic were selected as potential candidates for further consideration. Toxicity prediction was used only as a preliminary computational screening step and does not replace experimental safety assessment.

Physicochemical characteristics, including hydrophobicity, hydrophilicity, and net charge, were also evaluated using the available ToxinPred tools. Hydrophobicity and hydrophilicity values were determined based on the physicochemical characteristics of the amino acid residues comprising each peptide sequence. Net charge was determined from the contribution of charged amino acid residues within the peptide sequence.

The results were used as an initial screening criterion to identify peptides predicted to be non-toxic according to the tool's prediction criteria. These predictions do not establish the actual safety of the peptides, as the assessment was based on a single computational tool. Experimental toxicity testing is therefore necessary to confirm their safety before further application.

Data analysis

The results of the *in silico* hydrolysis and bioactivity prediction were analyzed descriptively. Data obtained from BIOPEP-UWM and ToxinPred were tabulated according to enzyme combination, peptide sequence, predicted biological activity, peptide position, theoretical peptide occurrence (d), molecular weight (MW), physicochemical characteristics (hydrophobicity, hydrophilicity, and net charge), and predicted toxicity status.

The results were presented as tables and graphs to facilitate comparison of the predicted peptide profiles generated by the different enzyme combinations. Microsoft Excel was used for data organization, processing, and the preparation of tables and graphs to present the results of the analysis. Peptides predicted to exhibit non-toxicity alongside ACE- and DPP-IV-inhibitory activities were prioritized as potential candidates for subsequent experimental validation.

RESULTS AND DISCUSSION

Sequence similarity analysis

Table 1 presents the results of the protein sequence similarity analysis of the target MHC-A using BLASTp. The analysis was performed to confirm the sequence similarity of the selected *P. vannamei* MHC-A protein with myosin proteins from other shrimp species. Protein sequences from closely related Penaeus species were included as comparators due to their taxonomic relationship and the conserved nature of muscle myosin proteins. Myosin is one of the major structural proteins in shrimp muscle and plays an important role in muscle contraction (Koyama *et al.,* 2012).

The BLASTp analysis showed query coverage values ranging from 93 to 100% and sequence identity values ranging from 87.67% to 91.43% among the selected homologous proteins (Table 1). Query coverage represents the proportion of the target sequence aligned with the subject sequence, whereas sequence identity represents the percentage of identical amino acid residues within the aligned region. The highest query coverage was observed for the *P. vannamei* myosin heavy chain type 1 protein (ROT83632.1), with 100% query coverage and 89.92% sequence identity. The high sequence similarity observed among the identified myosin proteins confirms that the selected ROT81948.1 sequence is highly similar to annotated myosin heavy chain proteins in related shrimp sequences. The biological implication of this sequence conservation is that the selected MHC-A sequence represents a relevant candidate for investigating potential bioactive peptide precursors.

Table 1. BLAST analysis results of myosin heavy chain protein sequence in NCBI

Organism/species	Protein Description	Accession Number	Query Cover (%)	Identity (%)
Penaeus vannamei	Myosin heavy chain type 1	ROT83632.1	100	89.92
Penaeus chinensis	Myosin heavy chain, muscle-like	XP_047497312.1	98	91.43
Penaeus japonicus	Myosin heavy chain, muscle-like	XP_042884531.1	98	87.67
Penaeus vannamei	Myosin heavy chain type A	ROT81945.1	93	87.67

Note: BLAST analysis was performed using the NCBI protein database. Query cover indicates the percentage of the query sequence aligned with the subject sequence, while identity indicates the percentage of identical amino acid residues in the aligned region. Accession numbers refer are unique identifiers for the corresponding protein sequences in the NCBI database

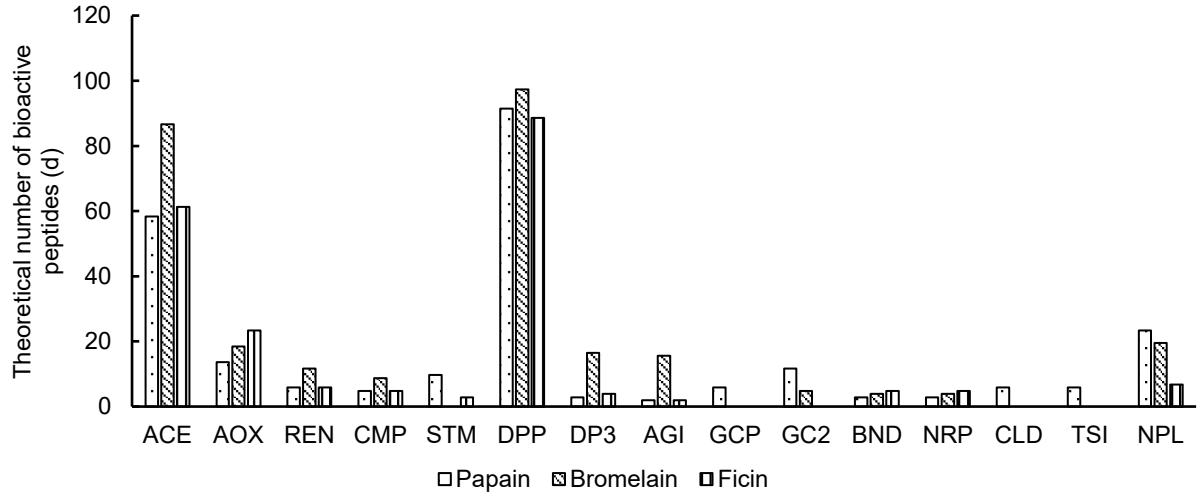
However, sequence similarity primarily supports the identification of homologous proteins and does not directly demonstrate their capacity to generate bioactive peptides. The potential bioactivity of peptides derived from MHC-A depends on their amino acid sequences, the cleavage specificity of the selected enzymes, and the release of relevant peptide sequences during proteolysis. Myosin heavy chain comprises the N-terminal motor domain, neck, and C-terminal tail, which support muscle contraction and filament assembly (Heissler & Sellers, 2016). These regions have been characterized in penaeid shrimp (Koyama *et al.,* 2012), but their roles in bioactive peptide generation depend on peptide sequences and enzymatic cleavage. This study analyzed full-length MHC-A; therefore, the contribution of individual regions remains to be investigated.

The selected MHC-A sequence was subsequently subjected to *in silico* enzymatic hydrolysis using BIOPEP-UWM to identify potential bioactive peptide sequences (Rivero-Pino *et al.,* 2023). The predicted peptides were then evaluated based on their potential bioactivities. These computational predictions provide an initial assessment of MHC-A as a potential source of bioactive peptides, although experimental validation is required to confirm peptide release and biological activity.

In silico hydrolysis of MHC-A using BIOPEP-UWM

Figure 1 shows the predicted bioactive peptide profiles obtained from *in silico* hydrolysis of the MHC-A sequence using papain, bromelain, and ficin. The simulation was performed to assess the potential occurrence of bioactive peptide sequences based on the cleavage patterns of each protease. Differences in the predicted bioactivity profiles were observed among the enzymes, indicating that the cleavage specificity of each protease influenced the bioactive sequences that could potentially be released from the MHC-A precursor.

Among the individual enzyme treatments, bromelain showed the highest theoretical number of DPP-IV-inhibitory peptides (d= 97.30), followed by papain (91.45) and ficin (88.59). Similarly, for ACE-inhibitory peptides, bromelain exhibited the highest theoretical number of bioactive peptides (d= 86.64), followed by ficin (61.31) and papain (58.33). In addition to ACE inhibition, the potential of vannamei shrimp myosin to generate peptides targeting other enzymes associated with blood pressure regulation was also evaluated, particularly neprilysin (NPL). Among the individual enzyme treatments, papain exhibited the highest theoretical number of NPL-inhibitory peptides (d= 23.38), followed by bromelain (19.49) and ficin (6.75). This result suggests that papain-mediated hydrolysis may favor the generation of peptide sequences with potential NPL-inhibitory activity



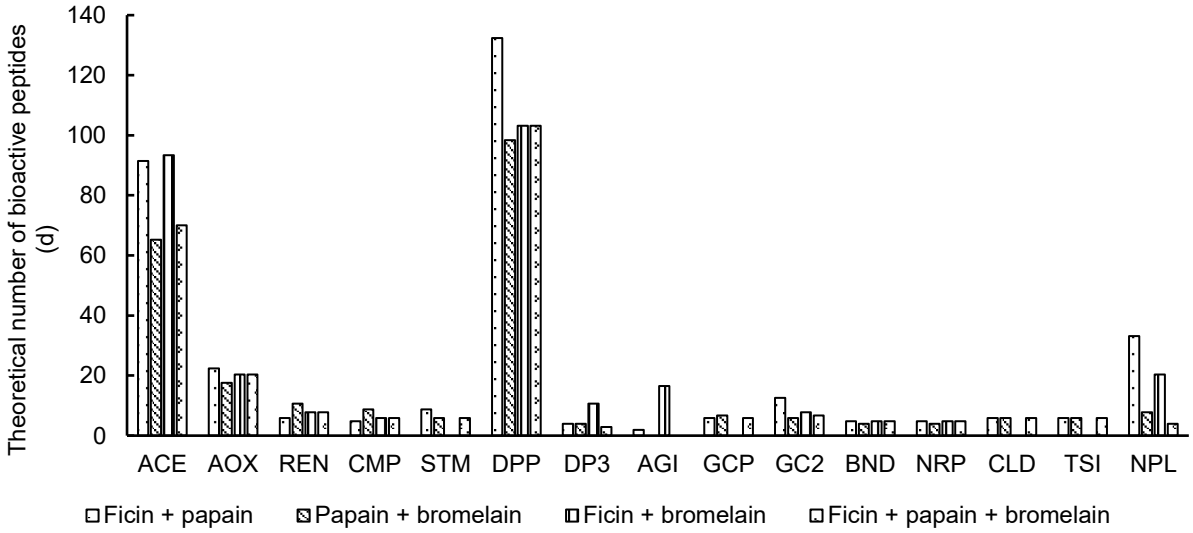
Note: ACE= angiotensin-converting enzyme inhibitor; AOX= antioxidant; REN= renin inhibitor; CMP= calcium/calmodulin-dependent phosphodiesterase (CaMPDE) inhibitor; DPP= dipeptidyl peptidase IV inhibitor; DP3= dipeptidyl peptidase III inhibitor; AGI= alpha-glucosidase inhibitor; GCP= glutamate carboxypeptidase inhibitor; GC2= glutamate carboxypeptidase II inhibitor; BND= binding; NRP= neuropeptide; CLD= citrate lyase deacetylase inhibitor; TSI= thymidylate synthase inhibitor; NPL= neprilysin inhibitor

Figure 1. Theoretical number of bioactive peptides generated from *in silico* protein hydrolysis of MHC-A of vannamei shrimp using papain, stem bromelain, and ficin enzymes. The x-axis represents the predicted biological activities of the generated peptides, while the y-axis represents the theoretical number of bioactive peptides (d), calculated as d= AE×N (equivalent to AE= d/N), where AE is the frequency of occurrence and N is the number of amino acid residues in MHC-A (N= 1299). The value of d is a theoretical estimate and has no physical unit

The differences in the theoretical number of bioactive peptides among the enzyme treatments may be attributed to the distinct substrate specificities and cleavage preferences of each protease. In the present study, bromelain exhibited the highest theoretical number of DPP-IV and ACE-inhibitory peptides, whereas papain showed the highest value for NPL-inhibitory peptides. These findings suggest that bromelain-mediated hydrolysis of MHC-A may favor the release of peptide sequences associated with antidiabetic and ACE-inhibitory activities, while papain may be more favorable for generating sequences associated with NPL inhibition. Such differences are likely related to the recognition of specific amino acid residues surrounding proteolytic cleavage sites, which determines the peptide profiles generated during enzymatic hydrolysis. Previous studies have demonstrated that papain and bromelain exhibit distinct substrate preferences and can consequently generate different peptide profiles (Choe et al., 2006; Arámburo-Gálvez et al., 2024).

The enzyme combinations were evaluated to investigate whether the complementary cleavage specificities of different proteases could enhance the release of peptide sequences associated with various bioactivities from MHC-A. Figure 2 shows that ACE- and DPP-IV-inhibitory peptides were predicted across all enzyme combinations, indicating the presence of sequence motifs associated with these activities in MHC-A. The highest theoretical number of ACE-inhibitory peptides was observed for the ficin-bromelain combination (93.40), followed by ficin-papain (91.45). For DPP-IV-inhibitory peptides, ficin-papain exhibited the highest value (132.36), suggesting that this combination may favor the release of peptide sequences associated with antidiabetic activity. NPL-inhibitory peptides were also predicted, with the ficin-papain combination showing the highest theoretical number (33.12). This finding suggests that combining ficin and papain may also favor the generation of peptide sequences associated with potential neprilysin-inhibitory activity.

One possible explanation is that sequential or combined cleavage may alter peptide boundaries and consequently reduce the occurrence of intact sequences matching the bioactive peptide entries in the BIOPEP-UWM database. However, this inter-pretation cannot be directly attributed to excessive hydrolysis because the present analysis does not experimentally determine the degree of hydrolysis, peptide concentration, or peptide degradation. Further experimental analysis is therefore required to determine whether extensive proteolysis reduces the abundance or activity of specific bioactive peptides.



Note: ACE= angiotensin-converting enzyme inhibitor; AOX= antioxidant; REN= renin inhibitor; CMP= calcium/calmodulin-dependent phosphodiesterase (CaMPDE) inhibitor; DPP= dipeptidyl peptidase IV inhibitor; DP3= dipeptidyl peptidase III inhibitor; AGI= alpha-glucosidase inhibitor; GCP= glutamate carboxypeptidase inhibitor; GC2= glutamate carboxypeptidase II inhibitor; BND= binding; NRP= neuropeptide; CLD= citrate lyase deacetylase inhibitor; TSI= thymidylate synthase inhibitor; NPL= neprilysin inhibitor

Figure 2. Theoretical number of bioactive peptides generated from *in silico* protein hydrolysis of MHC-A of vannamei shrimp using combined papain, stem bromelain, and ficin enzymes. The x-axis represents the predicted biological activities of the generated peptides, while the y-axis represents the theoretical number of bioactive peptides (d), calculated as d= AE×N (equivalent to AE= d/N), where AE is the frequency of occurrence and N is the number of amino acid residues in MHC-A (N= 1299). The value of d is a theoretical estimate and has no physical unit

The biological potential of the generated peptides is also influenced by their sequence and physicochemical characteristics. Peptide length, molecular weight, hydrophobicity, net charge, and amino acid composition may affect their interaction with target enzymes and their stability during gastrointestinal digestion (Ahmed *et al.,* 2022). Although short peptides containing approximately 2–20 amino acid residues are frequently reported among bioactive peptides (Zaky *et al.,* 2022), peptide length alone does not determine biological activity. Therefore, the predicted bioactive sequences identified in the present study require further evaluation using gastrointestinal digestion simulations and experimental ACE- and DPP-IV-inhibitory assays to determine their stability and biological activity.

Characterization of bioactive peptides identified from *in silico* hydrolysis

Table 2 presents the dipeptide sequences identified following *in silico* hydrolysis of the MHC-A protein using the ficin-papain combination, with particular emphasis on ACE- and DPP-IV-inhibitory activities. These two activities were selected because they correspond to the antihypertensive and anti-diabetic potential targeted in this study and showed relatively high predicted values in the preceding BIOPEP-UWM analysis.

A total of 23 bioactive dipeptide sequences were identified, including peptides associated with either single or dual biological activities. Several sequences, including EK (Glu-Lys), DR (Asp-Arg), EG (Glu-Gly), IR (Ile-Arg), AY (Ala-Tyr), NY (Asn-Tyr), VK (Val-Lys), and EI (Glu-Ile), were identified in the BIOPEP-UWM database as being associated with both ACE- and DPP-IV-inhibitory activities. The occurrence of a peptide sequence in both activity categories indicates that the same sequence has previously been associated with more than one biological activity in the database. Such multi-functionality may be related to the physicochemical properties and amino acid composition of the peptide sequence, including the identity and position of individual residues.

Table 2. Identification and characterization of bioactive dipeptides acting as ACE and DPP-IV inhibitors generated by protein hydrolysis using ficin and papain of vannamei shrimp

Sequence	Inhibitory activity	Position range	Frequency (n)	MW average (Da)
EK	ACE, DPP-IV	147–955	5	275.3007
DR	ACE, DPP-IV	392–1289	5	289.2876
EG	ACE, DPP-IV	108–1156	5	204.1801
IR	ACE, DPP-IV	188–1246	5	287.3576
PG	ACE, DPP-IV	676–677	1	172.1813
MF	ACE, DPP-IV	90–91	1	296.3855
AY	ACE, DPP-IV	177–904	2	252.2658
VY	ACE, DPP-IV	940–941	1	280.3188
EA	ACE	97–1250	17	218.206
DL	ACE	14–1248	15	246.2587
AL	DPP-IV	98–1319	17	202.2501
AS	DPP-IV	293–1251	9	176.17
AT	DPP-IV	55–895	6	190.1965
QL	DPP-IV	18–981	11	259.3013
NL	DPP-IV	63–1321	9	245.2748
NY	ACE, DPP-IV	166–893	3	295.2905
VK	ACE, DPP-IV	32–1078	3	245.3177
EI	ACE, DPP-IV	320–1047	2	260.2861
EH	DPP-IV	65–897	4	284.2679
MK	DPP-IV	121–806	3	277.3838
MR	DPP-IV	151–1207	3	305.3972
QY	DPP-IV	366–1093	3	309.317

Note: Dipeptide sequences are represented using the standard one-letter amino acid codes, for example, EK= Glu-Lys, DR= Asp-Arg, EG= Glu-Gly, and IR= Ile-Arg. The position range indicates the amino acid residue positions where the corresponding dipeptide sequences occur within the MHC-A protein sequence and is expressed as residue numbers (no unit). MW (molecular weight) represents the average molecular weight of the dipeptides, expressed in Daltons (Da). Predicted bioactive peptide sequences and their associated biological activities were identified using the BIOPEP-UWM database (Minkiewicz *et al.*, 2019)

For ACE inhibition, residues at or near the C-terminal region are frequently considered important for interaction with the ACE active site, whereas DPP-IV recognition may be influenced by amino acid residues at the N-terminal region as well as other sequence characteristics. Consequently, differences in amino acid composition and residue position may contribute to differences in the predicted interaction of a peptide with each target enzyme. Hydrophobic residues, such as alanine (A) and isoleucine (I), may contribute to hydrophobic interactions with enzyme binding sites; however, inhibitory activity is determined by the overall sequence context rather than by the presence of a single residue (Carrera-Alvarado *et al.,* 2022). Among the ACE-inhibitory dipeptides, EA (Glu-Ala) and DL (Asp-Leu) showed the highest frequencies of occurrence, with *n*= 17 and *n*= 15, respectively. Their relatively frequent occurrence indicates that the corresponding EA and DL sequences are present at multiple positions within the MHC-A sequence that are compatible with the simulated cleavage patterns of ficin and papain. The ACE-inhibitory potential of EA has also been reported experimentally, with an ACE-inhibition value of 47.11% in a previous study (Moreno-Mariscal *et al.,* 2025). However, the frequency observed in the present study reflects the occurrence of the sequence within the precursor protein and should not be interpreted as a measure of its inhibitory potency. For DPP-IV-inhibitory activity, AL (Ala-Leu), QL (Gln-Leu), NL (Asn-Leu), and AS (Ala-Ser) were among the more frequently occurring sequences, with frequencies of up to *n*= 17. The presence of these sequences is consistent with previous reports describing the contribution of specific amino acid residues and sequence characteristics to DPP-IV-inhibitory activity (Zhang *et al.,* 2023). The broad distribution of the identified dipeptides across the MHC-A sequence suggests that the predicted bioactive sequences are not restricted to a single region of the precursor protein.

All identified dipeptides had molecular weights below approximately 320 Da, as expected for peptides consisting of two amino acid residues. Their small molecular size may be favorable for intestinal transport; however, molecular weight alone does not establish peptide absorption or bioavailability. Therefore, the potential bioavailability of these dipeptides requires further assessment using gastrointestinal digestion, intestinal transport, and experimental bioavailability studies (Sun *et al.,* 2020).

Table 3 presents the bioactive dipeptides identified following *in silico* hydrolysis using the ficin-bromelain combination. This treatment generated a diverse profile of dipeptides associated with ACE- and DPP-IV-inhibitory activities, including sequences associated with both activities. Several dipeptides, including PA (Pro-Ala), IA (Ile-Ala), EK (Glu-Lys), DR (Asp-Arg), EG (Glu-Gly), IR (Ile-Arg), EV (Glu-Val), and PG (Pro-Gly), were identified as having both ACE- and DPP-IV-inhibitory activities according to the BIOPEP-UWM database. Among these sequences, EK showed a relatively high frequency of occurrence (*n*= 7).

EA and DL were again among the most frequently occurring ACE-inhibitory dipeptides, with *n*= 17 and *n*= 15, respectively. Their repeated occurrence under different enzyme treatments suggests that these sequences are distributed at multiple locations within the MHC-A sequence and can be generated under the cleavage rules of both enzyme combinations. However, the recurrence of a sequence does not necessarily indicate greater ACE-inhibitory potency because peptide activity depends on the specific sequence and its interaction with the target enzyme.

The DPP-IV-inhibitory group included several moderately to highly occurring sequences, such as NA (Asn-Ala) and NL (Asn-Leu) (*n*= 10 each), ES (Glu-Ser) (*n*= 8), and QA (Gln-Ala) and QL (Gln-Leu) (*n*= 5 each). These sequences correspond to peptide entries associated with DPP-IV inhibition in the BIOPEP-UWM database and may therefore represent potential candidates for further investigation. Nevertheless, their biological activity should be confirmed experimentally because database-based sequence matching does not establish actual inhibitory activity in the generated hydrolysate. A total of identified 15 common dipeptide sequences: DL, DR, EA, EG, EH, EK, IR, MF, MK, MR, NL, NY, PG, QL, and QY. These shared sequences suggest overlapping cleavage outcomes between the enzyme treatments; however, their predicted bioactivities depend on the individual peptide sequences and require experimental validation.

The ficin-bromelain treatment generated 30 bioactive dipeptide sequences, compared with 23 sequences identified following ficin-papain hydrolysis. Thus, the two enzyme combinations produced distinct predicted peptide profiles in terms of sequence diversity and occurrence. Both treatments generated sequences associated with ACE- and DPP-IV-inhibitory activities, including several sequences associated with both activities. These differences highlight the influence of protease cleavage specificity on the predicted bioactive peptide profile of MHC-A and support the use of different enzyme combinations as a preliminary strategy for screening potential bioactive peptide sequences.

Toxicity prediction and physicochemical characteristics of ACE- and DPP-IV-inhibitory dipeptides

The toxicity prediction results showed that all dipeptides evaluated in this study were predicted to be non-toxic by ToxinPred (Table 4). This finding

indicates that none of the identified ACE- and DPP-IV-inhibitory dipeptides was classified as toxic by the computational prediction model under the applied conditions. Similar computational studies have used ToxinPred as an initial screening tool for assessing the potential toxicity of bioactive peptide candidates (Gupta *et al.,* 2013). However, the predicted non-toxic status should be interpreted as a preliminary safety indication rather than evidence of biological safety. Experimental evaluation, including appropriate in vitro cytotoxicity assays and subsequent *in vivo* studies, would be required to establish the safety profile of the candidate peptides.

The amino acid composition of the identified dipeptides resulted in differences in their predicted hydrophobicity and hydrophilicity. Dipeptides containing non-polar or aromatic residues, such as leucine, valine, phenylalanine, and tyrosine, tended to exhibit greater hydrophobic characteristics. Hydrophobicity is an important physicochemical property of many bioactive peptides and may contribute to their interactions with target enzymes, including ACE and DPP-IV (Acquah *et al.,* 2018). However, hydrophobicity alone does not determine peptide bioactivity or toxicity. Excessive hydrophobicity may, under certain conditions, increase interactions with biological membranes, whereas peptide charge, sequence composition, length, and structural characteristics may also influence membrane interactions and potential cytotoxicity (Du *et al.,* 2026; Patil *et al.,* 2022). The predicted dipeptides also exhibited differences in net charge, reflecting variation in the occurrence of charged amino acid residues within their sequences.

Table 3. Identification and characterization of bioactive dipeptides acting as ACE and DPP-IV inhibitors generated by simulated protein hydrolysis using ficin and bromelain of vannamei shrimp

Sequence	Inhibitory Activity	Position Range	Frequency (n)	MW Average (Da)
PA	ACE, DPP-IV	9–10	1	186.2078
IA	ACE, DPP-IV	369–1096	2	202.2501
EK	ACE, DPP-IV	147–955	7	275.3007
DR	ACE, DPP-IV	392–1289	5	289.2876
EG	ACE, DPP-IV	108–1156	5	204.1801
IR	ACE, DPP-IV	188–1246	5	287.3576
MF	ACE	90–91	1	296.3855
IY	ACE	340–1067	2	295.2895
DA	ACE	404–1262	8	204.1806
EA	ACE	97–1250	17	218.206
QK	ACE	161–1264	9	259.3013
NY	ACE	166–893	3	295.2905
NK	ACE	327–1054	2	260.3081
EY	ACE	362–1089	2	310.3018
EV	ACE, DPP-IV	296–1212	4	246.2596
PH	ACE	662–663	1	252.2691
DY	ACE	709–710	1	296.2766
EF	ACE	882–883	1	294.2773
ER	ACE	371–1271	4	287.316
DL	ACE	14–1248	15	246.2587
EH	DPP-IV	65–897	4	284.2679
ES	DPP-IV	23–1040	8	234.206
ET	DPP-IV	399–1126	2	248.2325
MK	DPP-IV	121–806	3	277.3838
ML	DPP-IV	457–1184	3	262.3692
MR	DPP-IV	151–1207	3	305.3972
NA	DPP-IV	12–1143	10	203.1953
NH	DPP-IV	560–1287	2	269.2566
NL	DPP-IV	63–1321	10	245.2748
PG	ACE, DPP-IV	676–677	1	172.1813
QA	DPP-IV	233–1163	5	217.2218
QL	DPP-IV	18–981	5	259.3013
QV	DPP-IV	25–986	4	245.2748
QY	DPP-IV	692–693	1	309.317

Note: Dipeptide sequences are represented using the standard one-letter amino acid codes, for example, EK= Glu-Lys, DR= Asp-Arg, EG= Glu-Gly, and IR= Ile-Arg. Position range indicates the amino acid residue positions where the corresponding dipeptide sequences occur within the MHC-A protein sequence and is expressed as residue numbers (no units). MW (molecular weight) represents the average molecular weight of the dipeptides, expressed in Daltons (Da). Predicted bioactive peptide sequences and their associated biological activities were identified using the BIOPEP-UWM database (Minkiewicz *et al.*, 2019)

Table 4. Physicochemical characteristics and toxicity prediction of dipeptides

Sequence	Prediction	Hydrophobicity	Hydrophilicity	Charge
PA	Non-toxic	0.09	−0.25	0.00
IA	Non-toxic	0.49	−1.15	0.00
EK	Non-toxic	−0.86	3.00	0.00
DR	Non-toxic	−1.24	3.00	0.00
EG	Non-toxic	−0.23	1.50	−1.00
IR	Non-toxic	−0.52	0.60	1.00
MF	Non-toxic	0.43	−1.90	0.00
IY	Non-toxic	0.38	−2.05	0.00
DA	Non-toxic	−0.23	1.25	−1.00
EA	Non-toxic	−0.18	1.25	−1.00
QK	Non-toxic	−0.90	1.60	1.00
NY	Non-toxic	−0.31	−1.05	0.00
NK	Non-toxic	−0.87	1.60	1.00
EY	Non-toxic	−0.30	0.35	−1.00
EV	Non-toxic	−0.04	0.75	−1.00
PH	Non-toxic	−0.24	−0.25	0.50
DY	Non-toxic	−0.35	0.35	−1.00
EF	Non-toxic	−0.01	0.25	−1.00
ER	Non-toxic	−1.19	3.00	0.00
DL	Non-toxic	−0.09	0.60	−1.00
EH	Non-toxic	−0.51	1.25	−0.50
ES	Non-toxic	−0.44	1.65	−1.00
ET	Non-toxic	−0.40	1.30	−1.00
MK	Non-toxic	−0.42	0.85	1.00
ML	Non-toxic	0.40	−1.55	0.00
MR	Non-toxic	−0.75	0.85	1.00
NA	Non-toxic	−0.20	−0.15	0.00
NH	Non-toxic	−0.52	−0.15	0.50
NL	Non-toxic	−0.05	−0.8	0.00
PG	Non-toxic	0.04	0.00	0.00
QA	Non-toxic	−0.22	−0.15	0.00
QL	Non-toxic	−0.08	−0.80	0.00
QV	Non-toxic	−0.07	−0.65	0.00
QY	Non-toxic	−0.33	−1.05	0.00
AT	Non-toxic	0.04	−0.45	0.00
AY	Non-toxic	0.14	−1.40	0.00
VY	Non-toxic	0.28	−1.90	0.00
AL	Non-toxic	0.39	−1.15	0.00
AS	Non-toxic	−0.01	−0.10	0.00
VK	Non-toxic	−0.28	0.75	1.00
EI	Non-toxic	0.05	0.60	−1.00

Note: Hydrophobicity, hydrophilicity, and charge were calculated based on the amino acid sequences using ToxinPred. Positive and negative values indicate the corresponding hydrophobic or hydrophilic tendency according to the tool's scale. Toxicity was predicted using ToxinPred , and "Non-toxic" indicates that the peptide was predicted to be non-toxic

Differences in peptide charge may influence electrostatic interactions with biological targets and, together with hydrophobicity and amino acid sequence, contribute to peptide-target interactions (Agoni *et al.,* 2025). For example, the presence of charged residues such as lysine (K), arginine (R), glutamate (E), and aspartate (D) can alter the net charge of a peptide and potentially influence its interaction with the active site or binding regions of target enzymes.

Taken together, the predicted non-toxic profile, combined with the observed variation in hydrophobicity, hydrophilicity, and charge, provides useful preliminary information for prioritizing candidate peptides for further investigation. Nevertheless, these physicochemical characteristics should not be used independently to infer safety or biological efficacy. Experimental validation is required to determine whether the predicted ACE- and DPP-IV-inhibitory activities are retained under physiological conditions and whether the selected peptides exhibit acceptable safety profiles.

CONCLUSION

The MHC-A protein of *Penaeus vannamei* was identified as a potential precursor of bioactive peptides based on *in silico* hydrolysis analysis. Simulated hydrolysis using food-grade protease

combinations identified dipeptide sequences associated with ACE- and DPP-IV-inhibitory activities, including several sequences associated with both biological activities. The identified dipeptides consisted of two amino acid residues, with molecular weights below approximately 320 Da, and were distributed across different regions of the MHC-A sequence. The ficin-papain treatment yielded 23 bioactive dipeptide sequences, whereas the ficin-bromelain treatment yielded 30 dipeptide sequences with diverse predicted activities. Several sequences, including EK, DR, EG, IR, and NY, were associated with both ACE- and DPP-IV-inhibitory activities in the BIOPEP-UWM database. All evaluated candidate dipeptides were predicted to be non-toxic by ToxinPred, providing preliminary information for their prioritization as potential bioactive peptide candidates. Overall, the findings support the potential of *P. vannamei* MHC-A as a source of candidate ACE- and DPP-IV-inhibitory peptides. However, the present findings are based on computational prediction and sequence-based database matching. Therefore, experimental validation encompassing peptide identification, ACE- and DPP-IV-inhibitory assays, gastrointestinal stability assessments, and safety evaluations is required to confirm the biological activity and applicability of these predicted peptides.

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