

Investigating the anti-diabetic potential of casein through *in vitro* and computational gene expression analysis: a comparative study with metformin for advancing type 2 diabetes therapy

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ORIGINAL ARTICLE

Abstract

Type 2 diabetes mellitus (T2DM) is a growing global health concern, characterized by insulin resistance and dysregulated glucose metabolism. Current treatments, like metformin, while effective, have limitations in terms of side effects and long-term efficacy. Thus, the exploration of alternative therapeutic agents, such as bioactive peptides from natural sources, has gained attention for their potential in managing T2DM. This study explores the antidiabetic, antioxidant, anti-inflammatory, and anti-hemolytic potential of casein hydrolysate peptides derived from high-purity casein sourced from Fonterra Cooperative Group, New Zealand. The peptides were generated and assessed through a series of *in vitro* and computational approaches, with metformin serving as a standard control for comparison. *In vitro* assays revealed that casein hydrolysate peptides demonstrated a dose-dependent inhibition of alpha-amylase, with a maximum inhibition of 95.7% at 500 µg/mL, outperforming metformin's 77.7% inhibition. Computational analysis of the GSE40234 dataset identified the downregulation of the *NCOA2* gene in

T2DM patients, with a logF_c of -0.89 and a raw P-value of 1.85e-08. Docking studies showed a stronger binding affinity of casein hydrolysate peptides (-9.5 KJ/mol) to NCOA2 compared to metformin (-6.5 KJ/mol). Molecular dynamics simulations confirmed the stability of the casein hydrolysate peptide-NCOA2 complex, supporting its potential as an effective therapeutic agent. These findings highlight casein hydrolysate peptides as a promising candidate for managing T2DM, offering a natural alternative to traditional treatments. Future studies should explore in vivo models and clinical trials to validate these results and further assess the therapeutic potential of casein hydrolysate peptides in diabetes management.

Keywords: alpha-amylase inhibition, casein hydrolysate, computational analysis, NCOA2, type 2 diabetes

Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial and progressive metabolic disorder, characterized by insulin resistance and β -cell dysfunction, leading to chronic hyperglycemia (Goyal *et al.*, 2025). Its global prevalence continues to increase due to aging populations, sedentary lifestyles, and poor dietary habits (Aziz *et al.*, 2024). T2DM is a major risk factor for cardiovascular diseases, nephropathy, neuropathy, and retinopathy, contributing significantly to morbidity, mortality, and the economic burden worldwide (Ali *et al.*, 2025). Despite widespread awareness, early diagnosis remains a challenge due to its asymptomatic onset, often delaying timely intervention (American Diabetes Association, 2009).

Current pharmacological therapies, especially metformin, are considered first-line treatments due to their ability to reduce hepatic glucose production and improve insulin sensitivity. However, long-term usage is often accompanied by adverse effects, such as gastrointestinal discomfort, lactic acidosis risk, and vitamin B12 deficiency (Drzewoski *et al.*, 2021; Infante *et al.*, 2021). Furthermore, newer antidiabetic agents, like SGLT2 inhibitors and GLP-1 receptor agonists, while effective, are costly and less accessible in low-resource settings. These limitations underscore the urgent need for safe, cost-effective, and accessible alternatives or adjuncts to current therapeutic options.

Natural bioactive compounds have garnered significant attention for their multifunctional health benefits in metabolic disorders (Chang *et al.*, 2025; Liang *et al.*, 2024). Among them, casein, a major milk phosphoprotein, has been identified as a source of peptides with potential therapeutic effects, including antioxidant, anti-inflammatory, and glucose-regulating properties (Wang *et al.*, 2023; Blahova *et al.*, 2021). Casein hydrolysates, particularly bioactive peptides released during enzymatic hydrolysis or digestion, have shown promising antidiabetic effects by modulating oxidative stress, inflammation, and insulin sensitivity (Marcone *et al.*, 2017; Lv *et al.*, 2020). Notably, these peptides are naturally occurring and exhibit low

toxicity and high biocompatibility, making them ideal candidates for dietary interventions in metabolic disorders.

However, despite increasing interest in dairy-derived biopeptides, there is a notable gap in comparative studies that evaluate their efficacy alongside established antidiabetic drugs like metformin, especially using integrated in vitro and in silico approaches. Most existing literature focuses on animal models or general metabolic improvements, without targeting specific molecular interactions or comparing therapeutic performance directly. Moreover, gene expression-based computational validation of such peptides and their interaction with T2DM-related proteins remains underexplored.

This study aims to bridge this research gap by systematically evaluating the antidiabetic, antioxidant, anti-inflammatory, and anti-hemolytic potential of casein hydrolysate peptides, using both in vitro biochemical assays and *in silico* molecular docking and dynamics simulations. High-purity casein from Fonterra Cooperative Group (New Zealand) was enzymatically hydrolyzed to generate peptides, which were assessed for their bioactivities and directly compared to metformin. Furthermore, computational gene expression analysis from publicly available T2DM datasets (GEO and T2DiACoD) was conducted to identify key downregulated genes, such as NCOA2, and peptide-protein binding interactions were evaluated using molecular docking and AlphaFold structural modeling. The study uniquely integrates experimental assays with computational predictions to explore casein peptides not only as potential metabolic modulators but also as natural alternatives or adjuncts to metformin, thus offering a holistic, mechanism-driven, and affordable strategy for managing T2DM.

Materials and Methods

Procurement of casein

High-purity casein (85% protein content) was sourced from the Fonterra Cooperative Group Limited, a leading

global supplier of dairy products based in New Zealand, renowned for its commitment to sustainable and eco-friendly production practices. This premium-grade casein was selected for its superior quality, consistency, and compatibility with experimental protocols requiring high-purity, dairy-derived proteins.

Anti-diabetic potential of casein hydrolysate

Alpha amylase inhibitory assay

Alpha-amylase inhibitory activities of casein hydrolysis peptides were investigated to determine the antidiabetic activity of casein hydrolysates. A variety of concentrations of casein hydrolysate peptides were prepared within the range of 100–500 µg/mL in distilled water for the experiment, whereas metformin was used as the positive control. The first step of the assay involved adding different concentrations of casein hydrolysate, metformin, and alpha-amylase to each of the test tubes. Each test tube received 10 µL of alpha-amylase. The mixtures were placed in a mixing machine for 10 minutes and were incubated afterward at 30 degrees Celsius for 10 minutes. Following this first incubation step, 50 microliters of 1% starch solution was added to each of the test tubes. The samples were then put into the shaker incubator, which was set to 37 degrees Celsius for 1 hour. Once this second incubation step was finished, 50 µL of prepared 1% iodine solution was added to each test tube, and the samples were then placed in the incubator for an additional 30 minutes at 37 degrees Celsius. A spectrophotometer at 630 nm was used to measure the absorbance of the obtained solutions.

The antidiabetic activity, expressed as the percentage inhibition of alpha-amylase, was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{sample}}} \times 100$$

This method allowed for the assessment of the potential of casein hydrolysate peptides to inhibit alpha-amylase, an enzyme involved in the breakdown of starch into sugars, thereby indicating their antidiabetic properties.

Computational analysis

Dataset retrieval

The dataset about diabetes patients was obtained from the T2DiACoD (Type 2 Diabetes Mellitus Associated Complex Disorders) Gene Atlas database, a valuable resource for understanding the genetic basis of type 2 diabetes and associated complex disorders.

Differentially expressed gene analysis

Utilizing the NCBI GEO2R platform, the retrieved dataset underwent differential gene expression analysis. This enabled the identification of genes whose expression levels vary significantly between diabetic and non-diabetic individuals, shedding light on potential biomarkers and therapeutic targets.

Protein-protein interaction (PPI) analysis

The differentially expressed genes (DEGs) identified from the gene expression datasets were analyzed using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (<https://string-db.org/>) to construct a protein–protein interaction (PPI) network. The organism was set to *Homo sapiens*, and the interaction score was set to a high-confidence threshold of ≥ 0.7 to ensure the reliability of the predicted associations. Both experimental data and database-derived interactions (including co-expression, co-occurrence, gene fusion, and text mining) were selected to maximize coverage and biological relevance.

3D structure retrieval of diabetes type 2 causing gene

The three-dimensional structure of the identified differentially expressed gene associated with type 2 diabetes was retrieved from AlphaFold, an advanced protein structure prediction tool developed by DeepMind.

3D structure modelling and validation of casein hydrolysate peptide

The three-dimensional structure of the casein hydrolysate peptide, previously identified through LC-MS analysis for its potential in diabetes management, was modeled using trRosetta. The structural integrity of the peptide was validated using Ramachandran plot analysis (PROCHECK), ensuring its suitability for further studies.

Comparative docking analysis

Comparative molecular docking was performed using the HDock server (<http://hdock.phys.hust.edu.cn/>), a hybrid docking algorithm that integrates both template-based and ab initio docking approaches. The purpose of this analysis was to compare the binding affinities and interaction profiles of casein hydrolysate peptides and metformin with target proteins encoded by differentially expressed genes (DEGs) identified in the context of Type 2 Diabetes Mellitus (T2DM).

Binding site prediction was performed using the HDock default binding mode, which automatically identifies potential active sites based on surface cavities and known protein–ligand interaction templates. The docking process yielded multiple poses for each ligand–target interaction. The top-ranking docking poses were selected based on binding energy scores. Further, the binding interface residues, interaction types (hydrogen bonds, salt bridges,

π - π stacking), and buried surface area were analyzed to evaluate docking quality. Visualizations and interaction profiling were conducted using PyMOL. By comparing the docking scores and interaction profiles of metformin and casein peptides, this analysis provided mechanistic insights into how casein peptides may interact with critical diabetes-related targets, potentially mimicking or complementing the pharmacological activity of metformin.

Molecular dynamics simulation

To validate the docking procedure and assess the stability of the best-docked complex, molecular dynamics (MD) simulation was conducted using the iMODS server (<http://imods.chaconlab.org/>), which applies Normal Mode Analysis (NMA) to evaluate the intrinsic flexibility and dynamic behavior of biomolecular complexes. Prior to simulation, the docking protocol was validated through redocking of known ligands into their native protein binding sites. The accuracy of the docking was assessed using root mean square deviation (RMSD) analysis, where an RMSD value of less than 2.0 Å between redocked and crystallized conformations was considered acceptable for reliable binding site prediction and docking precision. Following validation, the most stable docking complex involving the casein hydrolysate peptide and its target protein was subjected to NMA. Key simulation parameters included deformability, which reflects the flexibility of individual amino acid residues; B-factor values, indicating the atomic mobility within the structure; and eigenvalues, representing the energy required for structural deformation, with lower eigenvalues suggesting higher structural stability. Additionally, the covariance matrix was analyzed to observe correlated, uncorrelated, or anti-correlated movements among residues, while the elastic network model was used to evaluate the stiffness of atomic interactions. These metrics collectively provided insights into the conformational stability, rigidity, and potential resilience of the protein-peptide interaction under physiological conditions, thereby reinforcing the reliability of the docking outcomes and supporting the proposed therapeutic potential of casein hydrolysate peptides in comparison to metformin.

Free radical scavenging inhibitory assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method was used to screen for antioxidant activity. For this study, the prepared peptide was casein hydrolysate at different concentrations, namely 200, 400, 600, 800, and 1000 µg/mL. A 3.94 mg of 0.1 mM DPPH was dissolved in 100 mL of methanol to prepare the 0.1 mM DPPH solution. To avoid DPPH degradation caused by light, the reagent bottle was wrapped in aluminum foil and maintained at 30°C in the dark until usage. 0.1 mL of each of the three concentrations of the casein hydrolysate peptide was placed in separate test tubes, to which

0.1 mL of a 0.1 mM DPPH solution was added. The mixtures were incubated for 30 min in a water bath. After incubation, the absorbance was measured at 517 nm, with ascorbic acid at the same concentrations used as a control. The free radical scavenging activity was conducted in triplicate, and the percentage inhibition of DPPH was calculated using the following formula:

$$\% \text{ DPPH inhibition} = \frac{A_0 - A_s}{A_0} \times 100$$

Where A_0 = absorbance of the control, A_s = absorbance of the test sample.

Protein denaturation inhibitory assay

The casein hydrolysate peptide's anti-inflammatory properties were assessed using the protein denaturation method. 1.5–2 mL of the peptide solution was mixed with 2.8 mL of phosphate-buffered saline (PBS) at pH 6.4 and 0.2 mL of fresh egg white to create a range of peptide concentrations (150, 250, 350, 450, and 550 µg/mL). The mixture was incubated for twenty minutes at room temperature (37°C) in a water bath. The samples were then heated for five minutes at 70°C in a water bath. A UV-vis spectrophotometer, set to 660 nm, was used to measure the samples' turbidity after they had cooled. The same concentrations of aspirin (150, 250, 350, 450, and 550 µg/mL) were used as a control. The anti-inflammatory activity was assessed in triplicate, and protein denaturation inhibition was calculated using the following formula:

$$\% \text{ Inhibition of protein denaturation} = 1 - \frac{A_t}{A_c} \times 100$$

Where A_t = absorbance of the test sample, A_c = Absorbance of control.

Anti-hemolysis assay

Using EDTA vials, a 5-mL blood sample was drawn from volunteers in good health. After centrifuging the blood to separate its constituents, the pellet was cleaned with 150 mM NaCl, and the supernatant was discarded. To make a total volume of 20 mL, the erythrocyte pellet was reconstituted in sterile phosphate-buffered saline (PBS) and allowed to cool at 4°C. 0.8 mL of casein hydrolysate peptide at different concentrations (100, 200, 300, 400, and 500 µg/mL) was combined with 0.2 mL of the erythrocyte suspension for the assay. The mixtures were incubated for half an hour at 37°C. Following incubation, the samples were centrifuged at 16,000 rpm for 15 minutes. Subsequently, 100 µL of the supernatant from each sample was diluted with 900 µL of PBS, and the absorbance was measured at 630 nm using an ELISA reader. PBS served as the negative control, while 0.1% Triton X-100 was used as the positive control.

Anti-Hemolysis percentage was calculated by utilizing this formula:

% anti-hemolysis =
$$\frac{\text{Abs of sample} - \text{Abs of negative control}}{\text{Abs of positive control}} \times 100$$

Results

Anti-diabetic potential of casein hydrolysate

Alpha amylase inhibitory assay

Alpha-amylase was found to be dose-dependently inhibited by casein hydrolysate peptide, with the percentage of inhibition rising as the concentration increased from 100 to 500 µg/mL. The casein hydrolysate peptide reached a maximum inhibition of 95.7% at the maximum concentration of 500 µg/mL. In contrast, at the same concentration, the commonly used antidiabetic medication metformin showed a maximum inhibition of only 77.7% (Figure 1). This implies that, at the tested dose, casein hydrolysate peptide inhibits alpha-amylase activity more effectively than metformin.

Computational analysis

Dataset retrieval

The dataset GSE40234 was retrieved from the T2DiACoD (Type 2 Diabetes Mellitus Associated

Complex Disorders) Gene Atlas database. This dataset is specifically curated to provide comprehensive genetic information associated with type 2 diabetes and its related complex disorders. It comprises a total of 62 samples, which are divided into two groups: 34 control samples and 28 experimental samples. The control group consists of individuals without diabetes, while the experimental group includes individuals diagnosed with type 2 diabetes mellitus.

Differentially expressed gene analysis

The dataset GSE40234, retrieved from the T2DiACoD (Type 2 Diabetes Mellitus Associated Complex Disorders) Gene Atlas database, includes gene expression profiles from individuals with type 2 diabetes mellitus. The analysis identified the gene NCOA2 (nuclear receptor coactivator 2) as significantly differentially expressed. With an adjusted P-value of 0.00038 and a raw P-value of 1.85e-08, the results are statistically robust, reflected in a t-statistic of -6.45 and a log fold change (log_{FC}) of -0.89. This indicates that NCOA2 is downregulated in diabetic patients compared to controls. The volcano plot illustrates this downregulation, with the log_{FC} on the X-axis and the statistical significance on the Y-axis (-log₁₀(P.Value)), placing NCOA2 prominently as a significant gene with notable downregulation. Similarly, the MA plot (Mean-Average plot) shows the log_{FC} against the average expression levels, further highlighting NCOA2's differential expression. The reduced expression of NCOA2 suggests its potential role in the pathophysiology of type 2 diabetes, possibly impacting insulin signaling and glucose metabolism (Figure 2A–C).

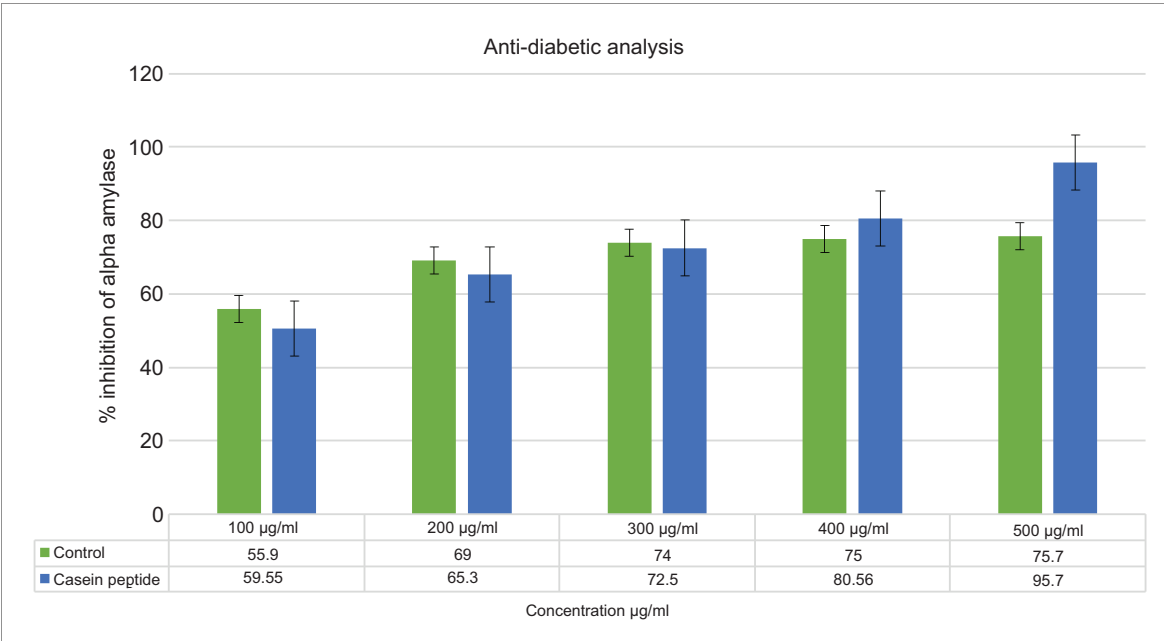


Figure 1. Graphical representation of anti-diabetic activity of casein hydrolysate peptide.

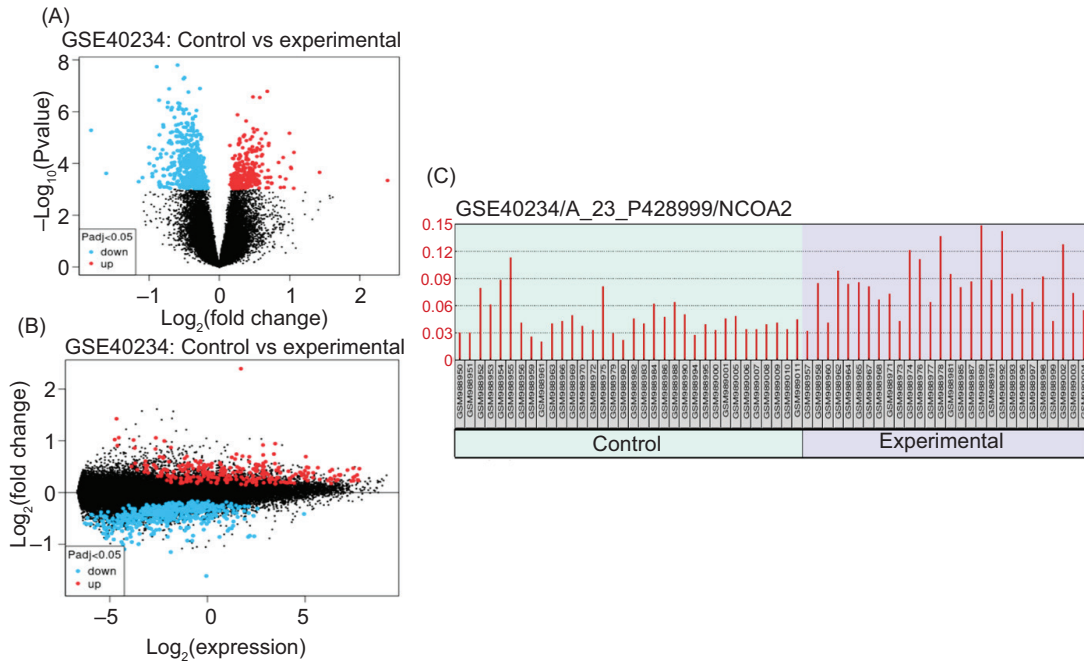


Figure 2. Differential gene expression analysis of the GSE40234 dataset (A) Volcano plot; (B) Mean difference plot, and (C) Expression values of the *NCOA2* gene across individual samples in the dataset.

Protein–protein interaction analysis

The protein–protein interaction (PPI) network analysis, performed using the STRING database, yielded insightful results regarding the molecular interactions among the differentially expressed genes associated with type 2 diabetes. The network comprises 11 nodes, representing the proteins encoded by the identified genes. These nodes are interconnected by 51 edges, which indicate the interactions between these proteins. The average node degree, calculated at 9.27, signifies that each protein interacts with approximately nine other proteins on average, highlighting a densely connected network (Figure 3).

The average local clustering coefficient is 0.941, suggesting that the network is highly clustered. This high clustering coefficient implies that the proteins tend to form tightly knit groups or clusters, which often correspond to functional modules or complexes within the cell. The expected number of edges in a random network of the same size is 19, significantly lower than the observed 51 edges. This discrepancy underscores the non-random nature of the interactions within the network.

The PPI enrichment p-value is 7.76×10^{-10} , indicating that the observed interactions are highly statistically significant and unlikely to occur by chance. This significant enrichment suggests that the proteins in the network are functionally related and possibly involved in common biological processes or pathways relevant to type 2 diabetes.

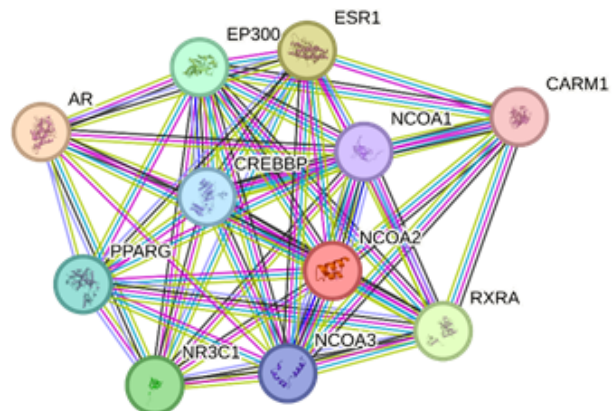


Figure 3. Protein–protein interaction (PPI) network analysis of differentially expressed genes associated with type 2 diabetes.

3D structure retrieval of diabetes type 2 causing gene

The three-dimensional (3-D) structure of the gene implicated in type 2 diabetes was retrieved from AlphaFold, using the accession ID Q15596 (*NCOA2_HUMAN*) (Figure 4).

3D structure modelling and validation of casein hydrolysate peptide

The three-dimensional (3-D) structure of the casein hydrolysate peptide was modeled using trRosetta, a

robust protein structure prediction method. The resulting model demonstrated a high confidence score of 0.96, indicating the reliability and accuracy of the predicted structure (Figure 5A). Validation of the peptide model further confirmed its quality, with over 90% of the residues positioned in the Ramachandran plot's favored regions (Figure 5B). This high percentage of favored residues underscores the structural integrity and correctness of the model, affirming its suitability for subsequent analyses and potential therapeutic applications.

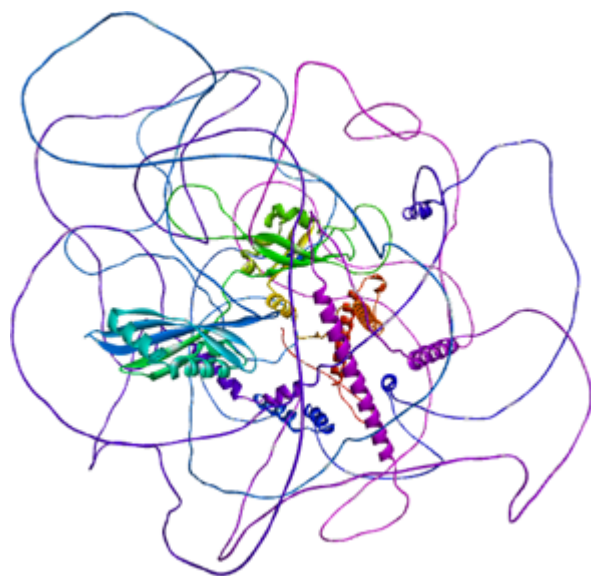


Figure 4. Predicted 3D structure of the *NCOA2* gene implicated in type 2 diabetes.

Comparative docking analysis

The casein hydrolysate peptide exhibited a particularly strong binding affinity with the NCOA2 protein, with the best interaction energy calculated at -9.5 KJ/mol. This indicates a stable and potentially effective binding, suggesting that the peptide could significantly influence the function of NCOA2. The 3D surface visualization of the peptide binding revealed several key interacting residues within the NCOA2 binding pocket, including GLU323, LEU343, LYS449, and various glutamine (GLN) and aspartic acid (ASP) residues. These residues were involved in hydrogen bonding interactions that help stabilize the peptide backbone. Additionally, proline residues such as PRO7, PRO10, PRO11, and PRO13 were found to contribute to the conformational rigidity of the peptide, promoting a snug fit within the NCOA2 binding groove. Hydrophobic and van der Waals interactions were also observed with residues like ILE8, ILE340, and THR393. This dense network of both polar and nonpolar interactions likely plays a crucial role in the high affinity of the peptide for the NCOA2 protein, positioning it as a promising regulatory ligand. On the other hand, metformin, a well-known antidiabetic drug, showed a binding interaction energy of -6.5 KJ/mol with NCOA2. While this is a positive interaction, it is less potent compared to the binding affinity of the casein hydrolysate peptide. The 3-D models offered a comprehensive view of the spatial orientation and binding conformations of the casein hydrolysate peptide and metformin within the active site of NCOA2 (Figure 6A,B). The docking results for metformin revealed interactions primarily with

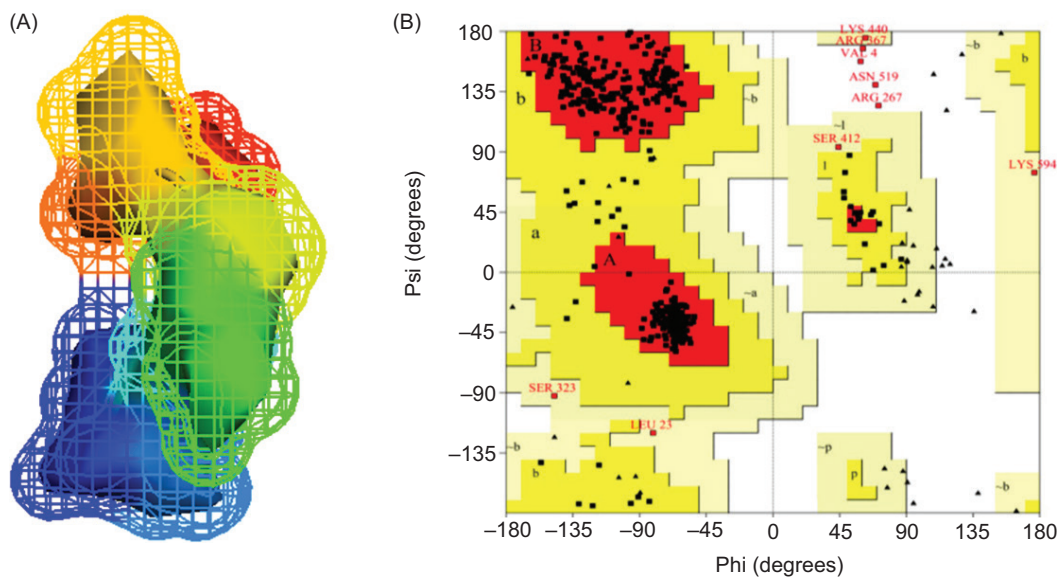


Figure 5. (A) Three-dimensional (3-D) structure of the casein hydrolysate peptide modeled using trRosetta, showing a high confidence score of 0.96. (B) Model validation using the Ramachandran plot, with over 90% of residues located in favored regions, indicating strong structural integrity and accuracy.

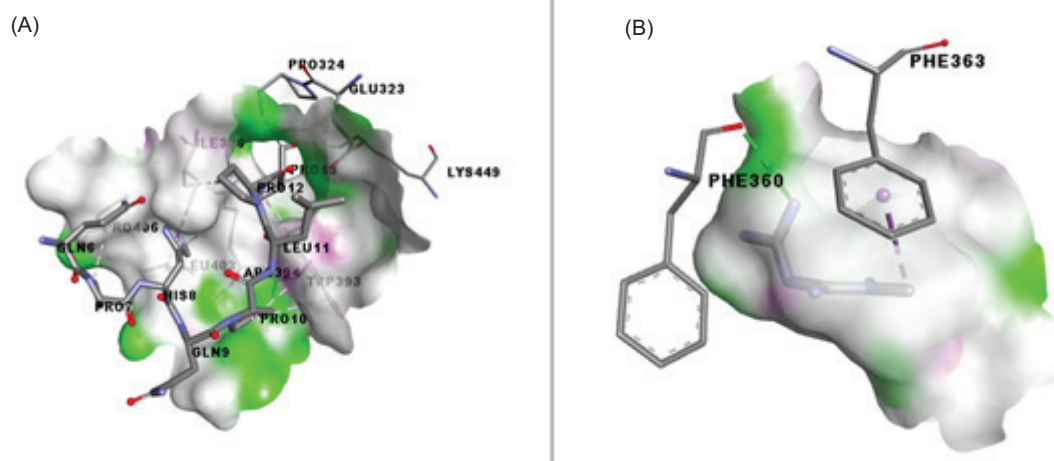


Figure 6. Binding interactions of the casein hydrolysate peptide and metformin with the *NCOA2* gene, analyzed using HDock. (A) The casein hydrolysate peptide exhibits an interaction energy of -9.5 KJ/mol. (B) Metformin exhibits an interaction energy of -6.5 KJ/mol.

PHE360 and PHE363, where π - π stacking interactions and potential hydrophobic contacts with the phenyl rings of the protein were observed. However, the lack of strong hydrogen bonding and a limited number of interaction sites suggest that the metformin-*NCOA2* complex is less stable compared to the casein hydrolysate peptide. The comparative analysis between the peptide and metformin highlights the potential of the casein hydrolysate peptide as a more effective molecule in modulating the activity of *NCOA2*, potentially offering a new therapeutic approach for managing type 2 diabetes.

Molecular dynamic simulation

The stability of the docked complexes was evaluated through molecular dynamics simulations using the iMODs server, as depicted in Figure 7. The analysis, based on force field calculations, revealed that the interaction between the casein hydrolysate peptide and the *NCOA2* protein remained most stable over a simulation period of 500 ns. The deformability potential of the docked complex indicated minimal fluctuations, suggesting a robust and stable interaction.

The deformability graph reveals the flexibility of individual residues, with peaks indicating regions of higher deformability. These flexible segments may play a crucial role in the functional or interactive behavior of the complex. The B-factor plot compares mobility data from both NMA predictions and experimental PDB values. A strong correlation is observed, particularly in the terminal and loop regions, where fluctuations are most prominent, suggesting that these areas of the complex are more dynamic. The eigenvalue map, derived from

the covariance matrix of atomic motions, is visualized as a grayscale heatmap. Lighter areas denote higher correlation between residues, while the diagonal confirms self-correlation. Off-diagonal lighter patches suggest coordinated movements between distinct regions of the complex. Additionally, the residue cross-correlation map highlights zones of correlated (red) and anti-correlated (blue) motion, pointing to a network of internal interactions that are crucial for maintaining the structural and functional integrity of the complex. The cumulative variance plot demonstrates that the first few modes dominate the overall motion, with approximately 85–90% of the total variance accounted for by the first ten modes, emphasizing the importance of low-frequency movements in defining the dynamics. Finally, the eigenvalue distribution plot reveals a low first eigenvalue of 8.514262×10^{-5} , indicative of a flexible complex. This plot confirms that only a limited number of modes are required to represent the majority of the motion. Collectively, these findings indicate that the docked complex is dynamically stable while retaining moderate flexibility in specific regions, with structural dynamics that support its functional role. These simulations highlight the strong and stable interaction between the casein hydrolysate peptide and the *NCOA2* protein, suggesting potential efficacy in therapeutic applications.

Free radical scavenging inhibitory assay

The DPPH radical scavenging assay, which measures the decrease in absorbance at 517 nm, was employed to evaluate the antioxidant activity of the casein hydrolysate peptide. A reduction in absorbance indicates that antioxidant compounds are effectively scavenging DPPH

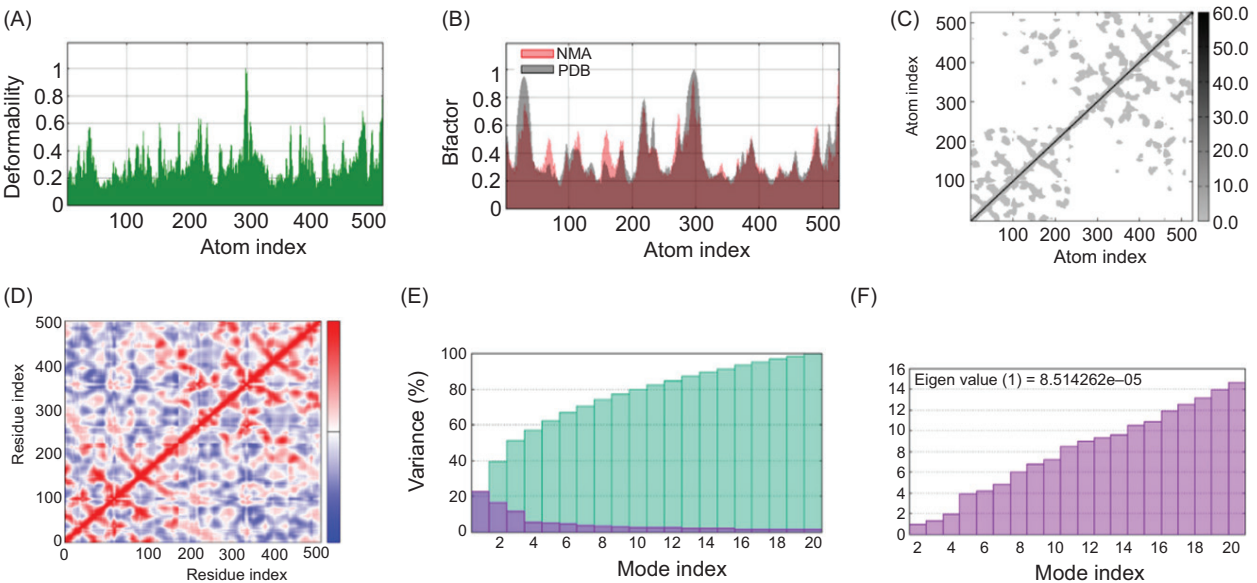


Figure 7. Molecular dynamics simulations of the docked complex between the casein hydrolysate peptide and *NCOA2* protein using iMODs. The stability of the complex is illustrated through: (A) Deformability of the complex; (B) B-Factor graph; (C) Elastic Network (Grey matter indicates stiffer region); (D) Covariance map showing correlated (red), uncorrelated (white), or anti-correlated (blue) motions; (E) Variance plot showing individual (purple) and cumulative (green) variances; (F) Eigenvalue plot representing the minimum energy required to deform the complex.

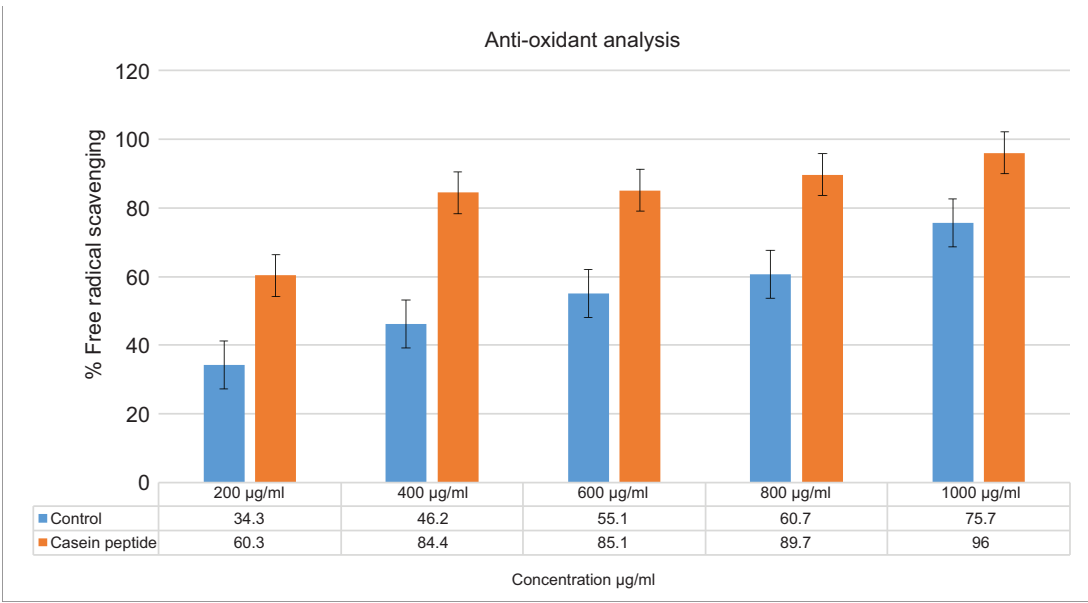


Figure 8. Graphical representation of the anti-oxidant activity of casein hydrolysate peptide.

radicals by donating hydrogen atoms. At a concentration of 1000 µg/mL, the casein hydrolysate peptide exhibited a maximum activity of 96%, highlighting its strong anti-oxidant effect (Figure 8). This result underscores the peptide's potential as a powerful agent for scavenging free radicals.

Protein denaturation inhibitory assay

At a concentration of 550 µg/mL, the casein hydrolysate peptide exhibited a maximum protein denaturation inhibition of 85.6% (Figure 9). These results suggest that, compared to aspirin, the control medication, the

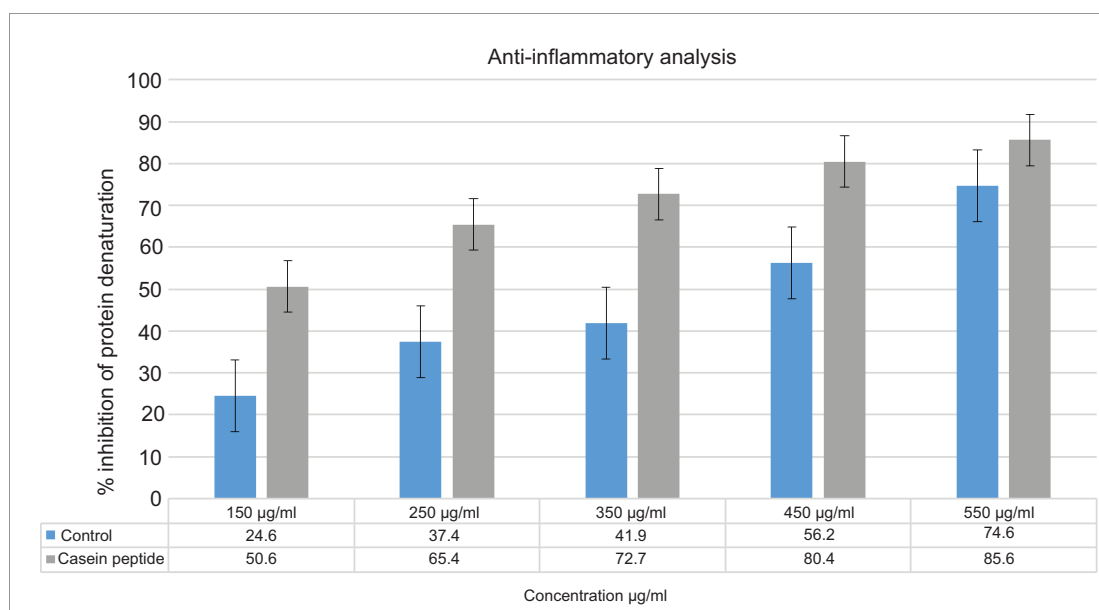


Figure 9. Graphical representation of the anti-inflammatory activity of casein hydrolysate peptide.

casein hydrolysate peptide is more effective in preventing albumin protein denaturation. This highlights the peptide's potential as a promising anti-inflammatory agent.

Anti-hemolysis assay

The casein hydrolysate peptide's anti-hemolytic action was concentration-dependent, as shown by the hemolytic activity assay results. The maximal anti-hemolysis rate of 96.16% was achieved by the peptide at the highest tested concentration of 500 µg/mL. In contrast, less than 90% of hemolytic activity was inhibited at lower concentrations. These findings highlight the peptide's potential efficacy and safety for therapeutic applications and align with previous research demonstrating the safety of such concentrations for use (Figure 10).

Discussion

Type 2 diabetes (T2D) is a chronic metabolic disorder characterized by insulin resistance and progressive β -cell dysfunction. Current therapeutic strategies, such as metformin, primarily focus on improving insulin sensitivity and reducing hepatic glucose production (Galicía *et al.*, 2020). However, these treatments often show limited long-term efficacy and are associated with adverse side effects (Ioele *et al.*, 2022). As a result, there has been growing interest in exploring alternative natural therapeutic agents. Among these, casein—a major

phosphoprotein found in milk—has gained attention for its promising biological activities, including antioxidant, anti-inflammatory, and anti-hyperglycemic effects (ALKaisy *et al.*, 2023). This study aims to investigate the anti-diabetic potential of casein using both in vitro assays and computational gene expression analysis, comparing its effects with the widely used anti-diabetic drug, metformin.

The results from the alpha-amylase inhibition assay indicated that casein hydrolysate peptide exhibited a dose-dependent inhibition of alpha-amylase, with a maximum inhibition of 95.7% at a concentration of 500 µg/mL. This inhibition level significantly outperformed metformin, which achieved a maximum inhibition of only 77.7% at the same concentration. These findings suggest that casein hydrolysate peptide may be more effective in inhibiting alpha-amylase activity, a key enzyme involved in carbohydrate digestion, potentially contributing to the management of postprandial hyperglycemia (Antony *et al.*, 2021).

These results align with previous studies that have highlighted the efficacy of peptides derived from milk proteins in modulating enzyme activity linked to diabetes. For example, Kumar *et al.* (2020) demonstrated that bioactive peptides from casein exhibited strong inhibitory effects on digestive enzymes, supporting the findings of our study (Koirala *et al.*, 2023). Similarly, comparisons of metformin's inhibitory effects with those of various natural peptides (including casein-derived peptides) have shown that these peptides can offer enhanced

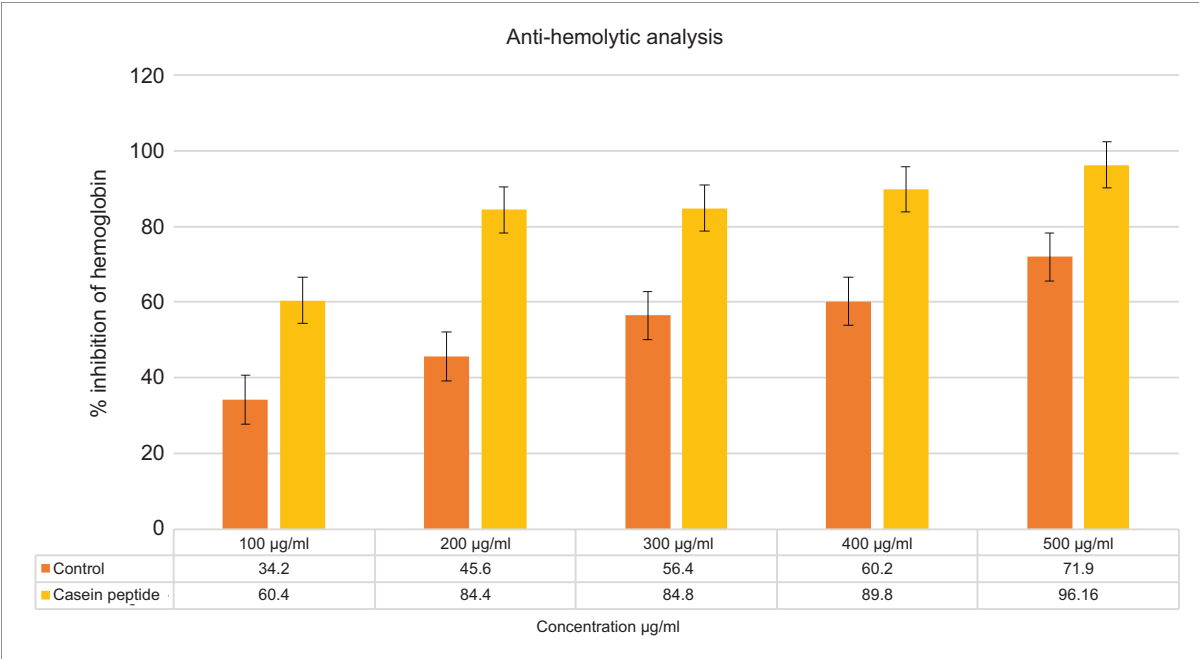


Figure 10. Graphical representation of the anti-hemolytic activity of casein hydrolysate peptide.

anti-diabetic properties (Chelliah *et al.*, 2021). The superior performance of casein hydrolysate peptide in inhibiting alpha-amylase suggests its potential as an alternative or adjunct to traditional anti-diabetic therapies.

The computational analysis, utilizing the GSE40234 dataset from the T2DiACoD Gene Atlas, identified NCOA2 (nuclear receptor coactivator 2) as a significantly differentially expressed gene in type 2 diabetes. NCOA2 was found to be downregulated in diabetic patients, with a log fold change of -0.89 (P-value of $1.85e-08$). This downregulation may influence insulin signaling pathways and glucose metabolism, further implicating NCOA2 in the pathophysiology of type 2 diabetes. Previous studies have also suggested that NCOA2 plays a crucial role in modulating metabolic functions, and its dysregulation is linked to insulin resistance (Zhao *et al.*, 2023). Our results align with these findings and suggest that NCOA2 could be a promising therapeutic target for diabetes treatment. Further analysis through protein-protein interaction (PPI) networks revealed a densely connected network of proteins encoded by the differentially expressed genes in type 2 diabetes, with 51 interactions observed among 11 nodes. The significant enrichment of these interactions (P-value = $7.76e-10$) supports the notion that these proteins are functionally related and may be involved in shared biological processes relevant to the disease. This finding aligns with prior research that has identified similar networks of proteins that regulate key metabolic pathways in diabetes (Goetzman *et al.*, 2018).

In the context of computational docking, the casein hydrolysate peptide demonstrated a superior binding affinity for NCOA2 (-9.5 KJ/mol) compared to metformin (-6.5 KJ/mol). The docking protocol was first assessed through known ligands to evaluate the consistency and accuracy of binding site prediction using the HDock server. The strong binding affinity of the casein hydrolysate peptide (-9.5 KJ/mol) was supported by multiple stabilizing interactions, including hydrogen bonds with GLU323, LEU343, LYS449, and polar contacts with GLN and ASP residues, which reinforced the spatial accuracy of the predicted docking pose. Moreover, the presence of proline residues (PRO7, PRO10, PRO11, and PRO13) contributed conformational rigidity, which is often indicative of stable binding. Additional nonpolar interactions with ILE8, ILE340, and THR393 demonstrated a well-anchored interaction profile within the hydrophobic core of NCOA2. By contrast, metformin, although exhibiting π - π stacking interactions with PHE360 and PHE363 and a calculated energy of -6.5 KJ/mol, lacked extensive hydrogen bonding and had fewer contact residues, consistent with a less stable docking pose. These findings reinforce the predictive power of the HDock protocol and validate the docking results, positioning the casein hydrolysate peptide as a structurally favorable and potentially more effective ligand for modulating NCOA2 activity compared to metformin. This suggests that casein hydrolysate peptide may have a stronger modulatory effect on NCOA2 activity, potentially offering a more effective therapeutic approach for managing type 2 diabetes. These results are consistent with previous

docking studies on bioactive peptides, which have shown promising binding energies with diabetes-related targets (Arif *et al.*, 2021). Molecular dynamics simulations further confirmed the stability of the casein hydrolysate peptide-NCOA2 complex, with minimal fluctuations observed over a 500 ns simulation period, further indicating the potential of this peptide as a stable therapeutic candidate. The biological potential of casein hydrolysate peptide was also assessed through various in-vitro assays, demonstrating significant antioxidant, anti-inflammatory, and anti-hemolytic activities. The peptide exhibited a maximum antioxidant activity of 96% in the DPPH radical scavenging assay at 1000 µg/mL, highlighting its potential for neutralizing free radicals and reducing oxidative stress, a major contributor to the development of diabetes-related complications. Additionally, the peptide demonstrated an 85.6% inhibition of protein denaturation at 550 µg/mL, suggesting its anti-inflammatory potential. This anti-inflammatory effect is crucial, as inflammation plays a key role in insulin resistance and the progression of type 2 diabetes (Hasan *et al.*, 2023).

The anti-hemolytic activity of casein hydrolysate peptide, which achieved a maximum inhibition of 96.16% at 500 µg/mL, further supports its safety profile, as previous studies have indicated that such concentrations are safe for use in therapeutic applications (Bamdad *et al.*, 2017). Overall, the combination of strong enzymatic inhibition, antioxidant, anti-inflammatory, and anti-hemolytic activities makes casein hydrolysate peptide a promising candidate for the development of novel anti-diabetic therapies. The results from both the in-vitro assays and computational analyses suggest that casein hydrolysate peptide exhibits superior anti-diabetic potential compared to metformin, particularly in terms of alpha-amylase inhibition and binding affinity with the NCOA2 protein. The peptide's additional antioxidant, anti-inflammatory, and anti-hemolytic properties further enhance its therapeutic potential. These findings warrant further in-vivo studies to validate the peptide's efficacy and safety, potentially paving the way for its development as a natural alternative or adjunct to existing diabetic treatments.

Conclusions

In conclusion, the results from this study demonstrate the promising therapeutic potential of casein hydrolysate peptides as an anti-diabetic agent, with superior activity compared to metformin in inhibiting alpha-amylase. The peptide's strong antioxidant, anti-inflammatory, and anti-hemolytic properties further support its potential for broader therapeutic applications. This study highlights the therapeutic promise of casein hydrolysate peptides in the management of type 2 diabetes mellitus (T2DM). In vitro assays revealed a dose-dependent

alpha-amylase inhibition, with a maximum of 95.7% at 500 µg/mL, surpassing metformin's 77.7% inhibition. Transcriptomic analysis of the GSE40234 dataset identified significant downregulation of the NCOA2 gene in T2DM patients ($\log F_c -0.89$; $p = 1.85e-08$). Molecular docking showed a stronger binding affinity of the peptide to NCOA2 (-9.5 KJ/mol) compared to metformin (-6.5 KJ/mol), and molecular dynamics simulations confirmed the complex's structural stability. The casein hydrolysate peptides show great promise for future development as a novel and effective treatment for type 2 diabetes and associated complications, with significant antioxidant and anti-inflammatory benefits, warranting further pre-clinical and clinical studies to confirm their efficacy and safety in vivo.

Availability of Data and Materials

All the data generated in this research work has been included in the manuscript.

Author Contributions

Conceptualization, Tariq Aziz; methodology, Tariq Aziz; software, Reham M. Mashat; validation, Nawal Al-Hoshani and Fakhria A. Al-Joufi; formal analysis, Maher Alwethaynani; investigation, Ahmad A. Alghamdi and Majid Alhomrani; resources, Liqing Zhao; data curation, Nahed S. Alharthi.; writing—original draft preparation, Tariq Aziz; writing—review and editing, Zhennai Yang; visualization, Sarah Almaghrabi and Bandar K. Baothman; supervision, Zhennai Yang and Liqing Zhao.; project administration, Zhennai Yang; funding acquisition, Liqing Zhao.

Conflicts of Interest

The authors declare no conflict of interest.

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