Antihyperuricemic activity and inhibition mechanism of xanthine oxidase inhibitory peptides derived from whey protein by virtual screening

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ABSTRACT

Xanthine oxidase (XO), a rate-limiting enzyme in uric acid production, is the pivotal therapeutic target for gout and hyperuricemia. In this study, 57 peptides from α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) were obtained via virtual enzymatic hydrolysis, and 10 XO inhibitory peptides were virtually screened using molecular docking. Then toxicity, allergenicity, solubility, and iso-electric point of the obtained 10 novel peptides were evaluated by in silico tools. The XO activity of these synthetic peptides was tested using an in vitro assay by high-performance liquid chromatography. Their inhibitory mechanism was further explored by molecular docking. The results showed that 4 peptides GL, PM, AL, and AM exhibited higher inhibitory activity, and their IC50 in vitro was 10.20 ± 0.89, 23.82 ± 0.94, 34.49 ± 0.89, and 40.45 ± 0.92 mM, respectively. The peptides fitted well with XO through hydrogen bond, hydrophobic interaction, and van der Waals forces, and amino acid residues Glu802, Leu873, Arg880, and Pro1076 played an important role in this process. Overall, this study indicated 4 novel peptides GL, PM, AL, and AM from whey protein exhibited XO inhibitory activity, and they might be useful and safe XO inhibitors for hyperuricemia prevention and treatment.

Keywords: XO inhibitor, whey protein, peptide sequence, molecular docking

INTRODUCTION

Hyperuricemia (HUA) as a chronic disease is becoming a serious public health problem according to large epidemiological studies (Zhang and Qiu, 2018). The prevalence of HUA among adults in the United States is 20.1%, according to a study by American College of Rheumatology (ACR). The prevalence of HUA in China reached 14.0% in 2018–2019, with a higher rate in males than in females (Zhang et al., 2022). HUA is a risk factor for gout, uric acid nephrolithiasis, hypertension, diabetes, and other metabolic diseases (Jalal et al., 2013, Otaki et al., 2020). Generally, HUA is characterized by impaired purine metabolism or decreased uric acid excretion (Dalbeth et al., 2021). Thus, to prevent and alleviate HUA, improvement of purine metabolism and increased uric acid excretion are of great importance.

HUA is characterized by a high level of uric acid (above 6.8 mg/dL) (Dalbeth et al., 2017), and treatment can tailor to this feature. Xanthine oxidase (XO) is the key non-specific hydroxylase and catalyzes the oxidation of hypoxanthine to xanthine and further oxidation to uric acid. XO has become an important target for the development of uric acid-lowering drugs in the study and application (White, 2018). Allopurinol and febuxostat are recognized effective XO inhibitor (Terkeltaub et al., 2006), but they could cause severe side effects such as hypersensitivity, aggravate renal toxicity. Therefore, there is an urgent need to produce effective and safe compounds as XO inhibitors for antihyperuricemic.

Bioactive peptides have been attracting much attention for their potential antihyperuricemic activities (Murota et al., 2014). Many studies have shown that short peptides (5–10 amino acid residues) were more likely to be absorbed by the human body and had a stronger XO inhibitory activity. The conventional method for obtaining peptides with biological activity includes enzymolysis, separation, purification and mass spectrometry identification. Molecular docking is often used to illustrate the active mechanism of XO inhibitory peptides. Peptides WPPKN (XO inhibitory activity is around 18% at concentration of 20 mM) and ADIYTE (XO inhibitory activity is around 42% at the same concentration of 20 mM) from dephenolized walnut meal hydrolysates inhibited XO in different ways, including entering the hydrophobic channel and occupying the entrance of hydrophobic channel of XO (Liu...
et al., 2020). Thirteen dipeptides and tripeptides were identified from tuna flesh hydrolysates and FH (IC$_{50}$ = 25.7 mM) exerted the strongest XO inhibitory effects. Molecular docking studies indicated that there were 2 hydrogen bonds and one π-π stacking interaction with residue Phe 914 which plays a key role in binding with XO (He et al., 2019). These studies have demonstrated the presence of various antihyperuricemic peptides derived from various sources, including plants and seafood. As for dairy, many clinical and epidemiological studies have shown that drinking milk and dairy products could reduce the content of uric acid in human serum. However, the studies of antihyperuricemia peptide inhibitors are still in their infancy (Gao et al., 2018, Nongonierma et al., 2013, Whey proteins and peptides Special Summer Double Issue). It was reported that tryptophan-containing milk-protein-derived dipeptides have XO inhibitory activity in vitro (Nongonierma and FitzGerald, 2012). Whey protein, an important source of milk-derived peptides, is an effective functional component in decreasing serum uric acid levels in Potassium oxonate-induced hyperuricemia in rats (Qi et al., 2021). However, the available antihyperuricemic peptide sequences derived from whey protein are still quite limited and mechanisms have been much less studied.

According to the rationale outlined above, the aim of this study was to discover novel XO inhibitory peptides from whey protein and investigate their mechanisms of action. The 2 main components of the whey proteins, α-LA (123 amino acids) and β-LB (162 amino acids) were hydrolyzed by alcalase to produce peptides. Ten peptides that were predicted to bind with the XO enzyme were filtered via virtual screening and then synthesized of the identified peptides were predicted to bind with the XO enzyme and enzyme were probed by molecular docking.

**Virtual enzymolysis of whey protein**

The amino acid sequence of α-LA and β-LB was obtained from Universal Protein (https://www.uniprot.org/). The proteolysis simulation process of α-LA and β-LB was performed using NOVOPRO protease digestion tool (https://novopro.cn/tools/protease-digestion-tool.html). Alcalase the common commercial protease with low specificity, was used to virtually enzymatic hydrolysis of α-lactalbumin and β-lactoglobulin. Subsequently, unknown peptides were selected for the following virtual screening by molecular docking.

**Molecular docking**

The molecular docking simulation was conducted using the method outlined by (Dai et al., 2017) with minor adjustments. The structure of peptides obtained by virtual hydrolysis was constructed using Hyperchem 8.0 (Hypercube Inc.). The 3D crystal structure of XO with ligands (PDB ID: 1FIQ) was obtained from the Universal Protein (https://www.uniprot.org/). Discovery Studio 2019 (Neotrident Technology Ltd., Beijing, China) was used to perform molecular docking. The XO crystal unit had 2 flavin molecules, 2 Mo atoms and 8 Fe atoms. Before docking, the XO without ligands macromolecular was removed water and added hydrogen. The peptides obtained by virtual enzymatic hydrolysis of whey protein were energy minimized by the charm force field. XO inhibitory drug (allopurinol) was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov). The binding sites were created at X: 28.3837, Y: 11.3587, Z: 110.692 with a radius of 18. The pose cluster radius was 0.5 and the other parameters were left at default values. The docking program was conducted with the partial flexibility program CDOCKER protocol. Evaluation of molecular docking was performed according to (−) CDOCKER energy, (−) CDOCKER interaction energy.

**In silico analysis of peptides**

Allergenicity, toxicity, solubility, and iso-electric point of the identified peptides were predicted by the tool including Allergen FP v.1.0. ToxinPred, Innovagen and ProtParam, respectively. The water-solubility and iso-electric point of the peptides were analyzed using peptide calculator (https://pepcalc.com/) (Zhao et al., 2020). Subsequently, the online tool (http://web.expasy.org/protparam/) was utilized to evaluate the stability of the peptides (Mohd Salim and Gan, 2020).

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**MATERIALS AND METHODS**

**Materials and reagents**

Xanthine oxidase (X1875–5UN, derived from bovine milk), xanthine (≥99%) and uric acid (≥99%) were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). Methanol and Ammonium phosphate monobasic were purchased from Macklin and were of chromatographic grade. All other reagents and chemicals were of analytical grade.

The peptides (GGV, SL, CM, AM, AL, PM, GL, GI, SF, and QI) were produced by RoyoBiotech Co., Ltd. (Shanghai, China).
In vitro evaluation of XO inhibitory activity

The XO inhibitory activity in vitro was determined using HPLC following the method with a slight change (He et al., 2019). All solutions were dissolved in 50 mM phosphate buffer (pH 7.4). The reaction mixtures consisted of 250 μL sample solution or buffer solution and 250 μL of XO (0.15 U/mL) were added together at 37°C for 10 min. Subsequently, 750 μL of 0.7 mM xanthine was added to start the reaction at 37°C for 15 min. The reaction was terminated by adding 400 μL of 1 M HCl. After that, a 10 μL reaction solution was measured by an Agilent ZORBAX SB-C18 column (4.6 × 250mm, 5 μm), and a mobile phase consisting of 85% aqueous solution (10 mM Ammonium phosphate monobasic) and 15% methanol at a flow rate of 1 mL/min were utilized. The amount of xanthine in final mixture was determined from the absorbance at 290 nm. The percentage of inhibition based on the HPLC method was calculated using the following equation:

\[
P(XO) = \frac{C_{\text{control}} - C_{\text{sample}}}{C_{\text{control}}} \times 100\%,
\]

where \( C_{\text{Sample}} \) and \( C_{\text{Control}} \) represent the concentration of uric acid generated by the reaction of the sample and control, respectively.

Statistical analysis

All experiments and analyses were conducted at least in triplicate, and the results are expressed as ‘mean ± standard deviation’. Significant differences were evaluated by one-way ANOVA followed by Duncan’s multiple-range test using SPSS software (version 22.0, IBM Corp., USA). The statistical significance was set at \( P < 0.05 \).

RESULTS AND DISCUSSIONS

Virtual enzymolysis of α-LA and β-LB

To obtain biological active peptides massively and efficiently, the α-LA and β-LB were subjected to virtual enzymatic hydrolysis by the low specificity Alcalase. The whey protein hydrolysates have been demonstrated to exert the inhibitory activity of XO (Qi et al., 2021), especially the dipeptides or tripeptides with phenylalanine. In addition, long-chain peptides are less capable of binding to the active site of XO. Considering the bioavailability and inhibitory effect, polypeptides with less than 6 amino acids were selected for further analysis. Finally, 17 dipeptides, nineteen tripeptides, 9 tetrapeptides, and 12 pentapeptides were obtained as the results of virtual enzymolysis of both proteins (shown in Table 1).

Virtual screening for XO inhibitory peptides by molecular docking

To screen the potential XO inhibitory peptides, allopurinol and 57 peptides were docked to XO using the CDOCKER program from the Discovery Studio 2019 software. Allopurinol, a XO inhibitor, as the positive control was bound to the enzyme. According to the results, it showed allopurinol had a negative CDOCKER energy and a negative CDOCKER interaction energy in agreement with literature reports and the active sites of the XO were correct. The negative CDOCKER energy value indicated that ligands could dock into the protein macromolecule. The lower the energy, the more likely it is for the peptides to dock into XO and achieve favorable conformation. Table 2 showed the rank of CDOCKER energy and CDOCKER interaction energy of the 10 peptides docked into XO successfully. The CDOCKER energy value of the allopurinol was −6.48 kcal/mol and the CDOCKER interaction energy was −28.61 kcal/mol, which are higher than the other 10 peptides. Collectively, these data indicated that the 10 peptides could associate with XO receptor spontane-

<table>
<thead>
<tr>
<th>Length</th>
<th>Source</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>dipeptide</td>
<td>α-LA</td>
<td>AL, CM, NY, PM, QI, SF, SL</td>
</tr>
<tr>
<td></td>
<td>β-LG</td>
<td>AL, AM, CV, DI, EI, GI, HI, KI, RL, RV, SP</td>
</tr>
<tr>
<td>tripeptide</td>
<td>α-LA</td>
<td>CNI, DKV, DQW, GGV, KDL, KGY, KKI, PEW, REL</td>
</tr>
<tr>
<td></td>
<td>β-LG</td>
<td>DAL, EEL, EKF, KAL, KCL, KGL, KKY, PAV, QKV, QKW</td>
</tr>
<tr>
<td>tetrapeptide</td>
<td>α-LA</td>
<td>CEKL, CTTF, DDDL, NNKI, TDDI</td>
</tr>
<tr>
<td></td>
<td>β-LG</td>
<td>AGTW, DKL, DTDY, TQTM</td>
</tr>
<tr>
<td>pentapeptide</td>
<td>α-LA</td>
<td>AHKAL, CSEKL, DTQAI, CSEKL, DTQAI, HTSGY, SCDFK, TKCEV,</td>
</tr>
<tr>
<td></td>
<td>β-LG</td>
<td>AASDI, ACQCL, DDEAL, DDEAL, NENKV, NPTQL, RTPEV</td>
</tr>
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</table>
ously and were more able to stably bind to the active site than allopurinol.

**Prediction of allergenicity, toxicity, water solubility and iso-electric point of screened peptides in silico**

The allergenicity, toxicity, water solubility and iso-electric point of dipeptides and tripeptides were predicted. The results showed that all of them were poor water solubility and non-toxic. Subsequently, these peptides didn’t exhibit allergenicity but GGV peptides (shown in Table 3). Food allergy is a public health problem (van Hengel, 2007) so we further studied the results of allergenicity prediction by comparing with identified major allergenic epitopes. The cow milk protein allergen demonstrated 5 IgE-binding sites including f1–15, f6–20, f46–60, f71–85, and f101–115 in α-lactalbumin amino acid sequences. Moreover, 4 IgG binding epitopes were identified at f6–20, f21–35, f36–50, and f86–100 (Golkar et al., 2019, Yanjun et al., 2016). Peptide GGV is at f19–21 which does not totally belong to the aforementioned major allergenic epitopes. Thus, 10 peptides (1 tripeptide and 9 dipeptides) with non-toxin were evaluated the XO inhibitory activity in vitro.

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### Table 2. The CDOCKER energy and CDOCKER interaction energy of 10 peptides that were docked with xanthine oxidase

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Ligands</th>
<th>Pose</th>
<th>-CDOCKER energy (kcal/mol)</th>
<th>-CDOCKER interaction energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopurinol</td>
<td>6</td>
<td>10</td>
<td>6.48</td>
<td>28.61</td>
</tr>
<tr>
<td>AL</td>
<td>2</td>
<td>10</td>
<td>39.44</td>
<td>43.40</td>
</tr>
<tr>
<td>AM</td>
<td>2</td>
<td>20</td>
<td>36.71</td>
<td>41.58</td>
</tr>
<tr>
<td>EI</td>
<td>2</td>
<td>20</td>
<td>37.46</td>
<td>41.27</td>
</tr>
<tr>
<td>GI</td>
<td>2</td>
<td>20</td>
<td>36.48</td>
<td>41.08</td>
</tr>
<tr>
<td>PM</td>
<td>2</td>
<td>10</td>
<td>34.46</td>
<td>34.65</td>
</tr>
<tr>
<td>QI</td>
<td>2</td>
<td>10</td>
<td>29.12</td>
<td>33.82</td>
</tr>
<tr>
<td>SF</td>
<td>2</td>
<td>10</td>
<td>27.27</td>
<td>32.47</td>
</tr>
<tr>
<td>SL</td>
<td>2</td>
<td>1</td>
<td>23.84</td>
<td>30.20</td>
</tr>
<tr>
<td>GGV</td>
<td>2</td>
<td>10</td>
<td>56.38</td>
<td>52.81</td>
</tr>
</tbody>
</table>

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### Table 3. Allergenicity, toxicity, solubility and iso-electric point of 10 peptides obtained by virtual screening. No, non-toxic

<table>
<thead>
<tr>
<th>Number</th>
<th>Sequence</th>
<th>Source</th>
<th>Allergenicity</th>
<th>Toxicity</th>
<th>Solubility</th>
<th>Iso-electric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGV</td>
<td>α-LA</td>
<td>Probable allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.65</td>
</tr>
<tr>
<td>2</td>
<td>SL</td>
<td>α-LA, β-LG</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.37</td>
</tr>
<tr>
<td>3</td>
<td>CM</td>
<td>β-LG</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>2.99</td>
</tr>
<tr>
<td>4</td>
<td>AM</td>
<td>β-LG</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.77</td>
</tr>
<tr>
<td>5</td>
<td>AL</td>
<td>β-LG</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.70</td>
</tr>
<tr>
<td>6</td>
<td>PM</td>
<td>β-LG</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>4.16</td>
</tr>
<tr>
<td>7</td>
<td>GL</td>
<td>α-LA</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.63</td>
</tr>
<tr>
<td>8</td>
<td>GI</td>
<td>α-LA</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.66</td>
</tr>
<tr>
<td>9</td>
<td>SF</td>
<td>α-LA, β-LG</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.43</td>
</tr>
<tr>
<td>10</td>
<td>QI</td>
<td>α-LA</td>
<td>Probable non-allergen</td>
<td>No</td>
<td>Poor</td>
<td>3.37</td>
</tr>
</tbody>
</table>

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**In vitro XO inhibitory activity of synthetic 10 screened peptides**

The XO inhibitory activity of synthetic 10 peptides was determined using HPLC. As shown in Figure 1, the inhibition rate of these peptides (at 100 μg/mL) could inhibit the XO and the inhibitory activity of GL, PM, AL and AM on XO was higher than 40%. Thus, these 4 peptides were further evaluated and the IC50 values were calculated, which is the concentration of peptide required to observe 50% XO inhibition. As shown in Figure 2, the IC50 values of GL, PM, AL, and AM were 10.20 ± 0.89, 23.82 ± 0.94, 34.49 ± 0.89, and 40.45 ± 0.92 mM, respectively. Smaller IC50 indicates the better activity of XO inhibition, so XO inhibition effectiveness of the peptides was ranked-ordered as GL, PM, AL, and AM. The variation in inhibition rates can be attributed to various factors. The peptide length and origin of these peptides were possible reasons. At present, XO inhibitory peptides from aquatic product or plant protein have high inhibitory activity. In contrast to the previous identified XO inhibitory peptides, such as pentapeptide ACECD (IC50 value of 13.40 mM) (Zhong et al., 2021), PGACSN (XO inhibitory activity is around 18% at concentration of 20 mM), WML (XO inhibitory activity is around 42% at the same concentration of 20 mM) from bonito protein (Li et al., 2018) and dipeptide...
FH (IC\textsubscript{50} value of 25.7 mM) from tuna protein (He et al., 2019). Besides, the in vitro XO properties of 2 pure synthesized peptides, WPPKN (97.95 ± 0.39\%) and ADIYTE (73.21 ± 0.62\%) from walnut protein, were tested at the same concentration of 20 μg/mL (Li et al., 2018). As for peptides derived from whey protein, ALPM (IC\textsubscript{50} value of 7.23 ± 0.22 mM) and LWM (IC\textsubscript{50} value of 5.01 ± 0.31 mM) were identified from whey protein isolate hydrolysates (Qi et al., 2022). PEW and LLW exhibited the high XO inhibitory activity, with IC\textsubscript{50} values of 3.46 ± 0.22 mM and 3.02 ± 0.17 mM, respectively (Qi et al., 2022). Eight potential XO inhibitory peptides, including VYPFPGPI, GPVRRGPFPPIIV, VYPFPGPIPN, VPYFPGPIHN, QLKRFSFRSFIWR, LVYPFPGPIHN, AVFPSIVGR, and GFININSLR, exhibited an IC\textsubscript{50} range of 4.67–8.02 mM (Gao et al., 2023). The experiments in vitro demonstrated these 4 peptides gained from virtual screening have promising XO inhibitory activity. Besides, virtual screening might be a feasible approach for discovering XO inhibition peptides and could decrease the consumption of person and time.

**Binding sites and interaction mechanism analysis of peptides with highest inhibition rate**

Molecular docking was employed to further study the binding sites and interactions between peptides (GL, PM, AL, and AM) and XO. From the docking results, the XO inhibitory peptides were well docked into the activity center of XO and bound with amino acid residues of the receptor through different interactions (Figure 3(a-d)). The hydrogen bond, hydrophobic interaction, and \textit{van der Waals} forces play vital roles in the stability between ligands and biomolecules (Zhou et al., 2021). According to the result in Table 4, peptide GL showed hydrogen bond interaction with amino acid residues Ser876, Arg880, Ser1008, Phe1009, Thr1010 and Val1011, hydrophobic contact with Gln802, Ala910, Phe914, Ala1078 and Ala1079 and van der Waals forces with Leu648, Leu873, Leu1014, Pro1076, and Gln1261. Peptide PM also formed the hydrogen bond with amino acids residues (Arg880, Phe914 and Gln1261) on XO, hydrophobic contact with Gln802 and Arg912, and van der Waals forces with Leu648, Gln767, Phe798, Gly799, Leu873, Ser876, Ala910, Phe911, Gly913, Phe1005, Phe1009, Thr1010, Pro1076, Ala1078, Ala1079, Ser1080 and Gln1261. As for AL, there were hydrophobic bonds between peptide and amino acids Gln802 and Arg880 of XO, hydrophobic interaction between peptide and amino acids Gln802, Arg880, Ala910, Phe914 and Ala1078, and van der Waals forces between AL and amino acids Gln767, Phe798, Gly799, Leu873, Ser876, Phe911, Arg912, Gly913, Phe1009, Thr1010, Val1011, Leu1014, Pro1076, Thr1077, Ala1079, Ser1080 and Gln1261. Furthermore, peptide AM exhibited hydrogen bonds with amino acid residues (Glu802, Arg880, and Gln1261) of XO, formed the hydrophobic interactions with the amino acids Gln802 and Arg912 and interacted with Gln767, Phe798, Gly799, Leu873, Phe911, Gly913, Phe914, Phe1009, Thr1010, Val1011, Leu1014, Pro1076, Ala1078, Ala1079 and Ser1080 through \textit{van der Waals} forces. According to the result, we found amino acid Arg880 in XO was most likely to form hydrophobic, Gln802 was likely to form hydrophobic and \textit{van der Waals} forces were established between peptide and amino acid residues Leu873 and Pro1076. However, the same amino acid residue did not always form the same interaction. For example, amino acid residue Gln802 of XO formed hydrogen bond and hydrophobic interaction with AM and AL, but it only formed hydrophobic interaction with PM and GL.

In this analysis of docking, the peptide could dock to the protein through the amino acid residues but not limited to Gln802, Leu873, Arg880 and Pro1076. For the XO inhibitory peptide of results, 20 amino acids, 400 dipeptides and 8000 tripeptides were systematically investigated the potential binding mechanism with XO (Li et al., 2019). The result showed that the Trp interacted with residues Gln802, Leu873, Ser876, Arg880, Phe914, Phe1009, Thr1010, Val1011, Leu1014, Ala1078, Ala1079 and Mos3004 of XO enzyme via different interactions, including hydrogen bonding and hydrophobic interactions (Li et al., 2019). Peptide ACECD displayed \textit{van der Waals} forces, hydrogen bond, π–π stacked and π–π T-shaped interactions with Gln879, Ser876, His875 and Phe649 of XO (Zhong et al., 2021). Peptide FH displayed van der Waals forces, hydrogen bond, π–alkyl interaction, π–π stacked and π–π T-shaped interaction with

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Xanthine oxidase inhibitory activity of 10 synthetic peptides that was found by virtual screening at the concentration of 100 μg/mL. Values were expressed as means ± SEM of 3 independent determinations. Different letters (a-f) indicate significant differences (P < 0.05).
Ser1080, Ser1082, Ala1078, Phe914, Gly799, Gly1260, Arg912, Gln1194, Phe798, Met1038 and Cys150 of XO (He et al., 2019). Peptide WPPKW displayed van der Waals forces, hydrogen bond, π-π stacked and π-π T-shaped interactions with Glu879, Ser876, His875 and Phe649 of XO. Peptide ADYIET displayed salt bridge, hydrogen bond and π-alkyl interactions with Arg871, Glu879, Phe649, Phe1142, Ser1141 and Tyr1140 of XO.

Figure 2. In vitro xanthine oxidase inhibitory activity of synthetic peptides with higher inhibition rate. The half maximal inhibitory concentration (IC_{50}) value of (a) GL, (b) PM, (c) AL, (d) AM and (e). Values were expressed as means ± SEM of 3 independent determinations. Different letters (a-d) indicate significant differences (P < 0.05).
Figure 3. Molecular docking of 4 peptides with higher xanthine oxidase inhibition rate between peptides and enzyme. Three-dimensional and 2-dimensional visual analysis of docked poses and interaction of xanthine oxidase with (a) GL, (b) PM, (c) AL, and (d) AM.
(Li et al., 2018). ALPM and LWM have been proven to exhibit high XO inhibitory activity in vitro and the mechanism of action might interact with residues around the MO structural domain through hydrogen bond and hydrophobic interaction. ALPM interacted with XO amino acid residues Ser876, Glu802, Lys771, Phe914, Phe1009, Phe649, Leu1014, Leu648, Leu873, Val1011, Met770, Pro1076 and Phe1013. LWM interacted with XO amino acid residues Leu648, Met770, Lys771, Ser876, Phe914, Phe1009, Thr1010, Leu1014, Phe1013, Pro1076, Ala1078 and Ala1079 (Qi et al., 2022). In the recent study, the peptides PEW and LLW, which were found to successfully traverse the Caco-2 cell monolayer, could interact with residues in the XO active cavity. Four hydrogen bonds were observed between PEW and Lys771, Asn768, Ser876, and Thr1010. Additionally, 5 hydrogen bonds were formed between LLW and Lys771, Glu802, Ala910, Phe914, Ala1078, Ala1079, and Glu1040. Furthermore, both PEW and LLW demonstrated hydrophobic interactions with residues neighboring the XO active cavity, including π–π stacking with Phe1009 and Phe914 (Qi et al., 2022). Eight potential XO inhibitory peptides, including VYPFPGPLH, VYPFPFGPPIV, VYPFPFGPPIPN, VYPFPFGPPIHN, QLKRFSFRSF1WR, LVYPFPGP1HN, AVFPSIVGR, and GF1INNSLR were purified and identified from alkaline protease hydrolysates using gel filtration, LC-MS/MS, and PeptideRanker. These peptides primarily bind to XO residues (Ser774, Glu879, His879, Ser710, and Lys771) through hydrogen bonds to ensure stable binding between the peptides and XO. Residue Ser774 interacted with VYPFPGP1HN, LVYPFPGP1HN, and GF1INNSLR through a hydrogen bond. Residue Glu879 interacted with GPVRGPPFIIV, AVFPSIVGR, and GF1INNSLR through a hydrogen bond. Residue His875 interacted with VYPFPGP1HN, QLKRFSFRSF1WR, and LVYPFPGP1HN through a hydrogen bond (Gao et al., 2023). It has been reported that Mo domain of XO consisted of Phe649, Asn768, Glu802, Leu873, Arg880 and Phe914, Phe1009, Thr1010, Leu1014 and Glu1261 among which Glu802, Leu873, Arg880 and Pro1076 were confirmed to play a key role in the inhibitory reaction. Above all, amino acids Glu802, Leu873, Arg880 and Pro1076 were found to form the active site of XO and the XO inhibitory peptides were likely to interact with them. The small peptides were pointed into the hydrophobic and associated with XO stably by the active site. Thus, peptide as an inhibitor prevented xanthine from binding on the XO and inhibited XO activity.

**CONCLUSIONS**

In this study, XO inhibitory peptides could be successfully screened out from whey protein using multiple in silico techniques including virtual enzymatic hydrolysis, in silico properties analysis, and molecular docking. Based on the results of virtual screening, the inhibitory activity of synthetic peptides was verified in vitro, and 4 peptides GL, PM, AL, and AM exhibited the best XO inhibitory effect. In the investigation mechanisms, inhibitory peptides were found able to interact with several crucial residues in the XO active sites. Compared with the conventional method, this work provides an effort-saving and time-saving approach for finding and developing new inhibitors of XO from more protein. However, XO inhibitory peptides in vivo studies are needed in the future. In summary, peptides GL,

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**Table 4.** Residues of xanthine oxidase which interacted with 4 peptides (GL, PM, AL and AM) with higher xanthine oxidase inhibition rate by molecular docking

<table>
<thead>
<tr>
<th>Sample</th>
<th>Secondary structure</th>
<th>Molecular weight</th>
<th>Hydrogen bonding</th>
<th>Hydrophobic interaction</th>
<th>Van der Waals forces</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>[INSERT Figure 001]</td>
<td>188.20 Ser876, Arg880, Ser1008, Phe1009, Thr1010, Val1011</td>
<td>Gln802, Ala910, Ala1078, Ala1079</td>
<td>Leu648, Leu873, Leu1014, Pro1076, Glu1261</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>[INSERT Figure 002]</td>
<td>246.30 Arg880, Phe914, Lys771, Glu802, Glu912, Val1011</td>
<td>Gln767, Phe798, Gly799, Leu873, Ala910, Phe911, Gly913, Phe1005, Phe1009, Thr1010, Val1011, Ala1078, Ala1079, Ser1080, Gln1261</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL</td>
<td>[INSERT Figure 003]</td>
<td>202.20 Gln802, Arg880, Ala910, Phe914, Ala1078</td>
<td>Gln767, Phe798, Gly799, Leu873, Ser876, Phe911, Arg912, Gly913, Phe1009, Thr1010, Val1011, Ala1078, Ala1079, Ser1080, Gln1261</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>[INSERT Figure 004]</td>
<td>220.20 Gln802, Arg880, Ala1078, Ala1079</td>
<td>Gln767, Phe798, Gly799, Leu873, Ala1078, Phe911, Gly913, Phe1009, Thr1010, Val1011, Ala1078, Ala1079, Ser1080</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PM, AL, and AM could be beneficial candidates for preventing the development of HUA disease. This work provided new knowledge into screening novel XO inhibitory peptides from natural proteins economically and efficiently.

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Yaru Xu: Methodology, Investigation, Writing-original draft, Data curation. Xueying Mao: Conceptualization, Editing, Supervision, Funding acquisition, review & editing.

Declaration of Competing Interest The authors declare that there is no conflict of interest.

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