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# The Exploration of Bioactive Peptides that Docked to SARS-CoV-2 Spike Protein from Goats Milk Beta-Casein by In Silico

**Hermawan Setyo Widodo[1,2]*, Tridjoko Wisnu Murti[1], Ali Agus[1], Ambar Pertiwiningrum[1]**

[1]Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia.
[2]Faculty of Animal Science, Jenderal Soedirman University, Purwokerto, Indonesia.

*Corresponding author email: hsw@unsoed.ac.id

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**ABSTRACT.** Beta-casein in milk is known to be a bioactive peptide producer because of its amino acid sequence. Bioactive peptides have prospected molecules that can adhere with SARS-CoV-2 spike protein, so they can inhibit the virus from hooking up with human cell receptor protein. The research is aimed to find any peptides from goat's milk beta-casein that are prospective candidates as SARS-CoV-2 spike protein inhibitors. Goat's milk beta-casein was simulated as being digested by the digestive tract. Pepsin, trypsin, and chymotrypsin enzymes cut the beta-casein amino acids sequence into small peptides. Then, their bioavailability was predicted by Lipinski's Rules of 5 (Ro5), any most fitted peptides to the rules will be simulated to dock to SARS-CoV2 spike protein besides Curcumin as the control ligand. Peptides with the best bind activity with the spike protein will be selected as inhibitor candidates. Peptide QPK is selected as a SARS-CoV-2 inhibitor candidate because it has better affinity energy than Curcumin or other selected peptides.

**Keywords**: beta-casein, goat, in silico, milk, SARS-CoV-2

## INTRODUCTION

The year 2020 is challenging due to a global pandemic caused by the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-Cov-2) affecting Corona Virus Disease 2019 (COVID-19). The virus causes respiratory problems that lead to lethal conditions (Huang et al., 2020). In fact, it is estimated that the pandemic will not end until some time to come. Deaths conducted by the virus have been a concern to many parties. Efforts to end this pandemic have been carried out in various ways, including looking for potential biological materials as drugs that can inhibit the virus (Çakır et al., 2021; Hoffmann et al., 2020; Shanmugarajan et al., 2020).

Biological materials that can be used as inhibitors are bioactive peptides. This material is considered advantageous because it is unlike synthetic drugs, which sometimes have several side effects. Bioactive peptides come from biological components so that they can be used optimally by the human body (Karami & Akbari-adergani, 2019; Pillaiyar et al., 2020). Bioactive peptides can also be obtained by simple processing and are generally available in foodstuffs such as goat's milk.

Goat's milk and its beta-casein were known to have high potential as a source of bioactive peptides (Haque & Chand, 2008; Sun & Jensse, 2012). The potential occurs because the beta-casein of goat's milk has specific sequences that can produce bioactive peptides (Albenzio et al., 2017). In addition, goat's beta-casein does not have a variation that leads to the release of Betacasomorphine-7 (BCM-7) like in the cow's milk. The peptide was known to have an opioid effect of altering some hormonal mechanisms, causing non-generative diseases such as hypertension and heart attack (Park & Haenlein, 2021). Beta-casein from goats is also considered by its quantity. Goats milk is known to have more abundance of its beta-casein than alpha-casein. Contrary, cow's milk is dominated by alpha-casein rather than beta-casein (Widodo et al., 2021). The quantity of beta-casein in goat's milk will act as an advantage in line with the bioactive peptide production.

Casein in goat's milk is known to have bioactive peptides that can inhibit the performance of Angiotensin-I Converting Enzyme (ACE). Peptides that have many proline residues are highly potential as the bioactive peptides and mostly originated from beta casein. The peptides are produced by fragmenting the beta casein with the help of pepsin,trypsin and chymotripsin that naturally available in stomach (Ibrahim et al., 2017; Widodo et al., 2019). The peptide then can be referred as Angiotensin Converting Enzyme inhibitor (ACEi) that impede the conversion of Angiotensin I (Haque & Chand, 2008).

By taking the advantage of ACEi, the mechanisms can also has a promising effect to inhibit SARS-CoV-2 infection (Aminu et al., 2021; Chamata et al., 2021).

It is known that SARS-Cov-2 can enter human cells to replicate via the ACE2 receptor. The virus composed of spike, membrane, envelope and nucleocapsid proteins (Chen et al., 2020). Bioactive peptides can inhibit the viral spike protein's active side or Receptor-Binding Domain (RBD) that can link to the ACE2 receptor, so the virus cannot bind to the human cell (Shang et al., 2020). Curcumin is one of many ligands that intensively studied and has a good result in inhibiting the binding of SARS-CoV-2 spike protein to human cells (Patel et al., 2020; Rajagopal et al., 2020; Shanmugarajan et al., 2020). This natural compound ligand is an excellent example of control in docking with SARS-CoV-2 spike protein compared with many other predicted ligands. This was considered by the support of studies showing effectiveness of curcumin inhibiting SARS-CoV-2 infection (Rattis et al., 2021). In other hand, the spirit of exploring natural compound for medicine instead of conventional drugs must be encouraged primarily for the SARS-CoV-2.

The in silico method is one of many appropriate protocols to determine the potential of ligands. In this case, some selected peptides docked to SARS-CoV-2 spike protein virtually using a computer (Dallakyan & Olson, 2015; Nag et al., 2022). This method can be used to determine and promote the potency of beta-casein from goat's milk, as mentioned above. This study aimed to screen out and prove some bioactive peptides that could inhibit the SARS-CoV-2 spike protein from goat's milk beta-casein.

## EXPERIMENTAL SECTION

This research has been conducted by taking several steps, which refers to (Widodo et al., 2019). The overall steps in this research were carried out virtually, including (1) cutting the goat beta-casein protein sequences by digestive enzymes and then tabulating the results; (2) forming a 3-dimensional structure from every formed fragment; (3) ligand selection that meets Lipinski's Rule of 5 (Ro5) (Lipinski et al., 2001)and its bioactivity; (4) attachment of selected ligands to the SARS-Cov-2 protein spike.

This study uses some resources such as : a computer connected to the internet and installed programs to support in silico techniques. The programs were Avogadro v1.2.0, Discovery Studio v17.2.0.16349 (Dassault Systemes Biovia Corp.), OSIRIS Data Warrior v4.7.2. (Idorsia Pharmaceuticals Ltd.) (Sander et al., 2015), and PyRx-Python Prescriptions (v.0.8) (Dallakyan & Olson, 2015). The online programs were ExPASy-PeptideCutter (https://web.expasy.org/peptide_cutter), National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov), Protein Data Bank RCSB (http://rcsb.org), and Molinspiration (http://molinspiration.com). The goat beta-casein sequence (*Capra hircus*) was obtained from the NCBI website with accession code AAA30906 (https://www.ncbi.nlm.nih.gov/ protein/AAA30906), SARS-CoV-2 spike protein specifically on the Receptor-Binding Domain (RBD) sequence with accession code 6M0J retrieved from Lan et al.(2020)as well as Curcumin CC9 (https://www.rcsb.org/ligand/CC9 ) from RSCB-PDB website.

### Simulation of Protein Cutting by Digestive Enzymes

This stage begins by opening the amino acid sequence from the goat beta-casein protein downloaded from the NCBI website. The sequence is then uploaded to the online program ExPASy-PeptideCutter and followed by cutting the enzyme virtually. The digestive enzymes chosen are pepsin, trypsin, and chymotrypsin. The enzymes were choosen in consider the ease of application by just consume the milk without any furter proccess unlike fermentation. The pH of simulation is 2 to make sure the condition is similar with the real digestive tract condition. The result of the cutting is in the form of specific amino acid fragments due to specific enzyme activity. The each fragments then converted into a 3-dimensional shape using the Avogadro computer program. The 3-dimensional structure was minimized using the MMFF94 forcefield mode until the lowest value was achieved. The structure is then saved as a file in .pdb format and collected as ligand candidates.

The macromolecular structure in the form of SARS-CoV-2 virus spike proteins is processed by first deleting water molecules using the Discovery Studio program. The adhered ligands are then removed and then saved in .pdb format. The structure reopened in the Avogadro to minimize its energy until the minimum point is reached. This procedure is also carried out on the curcumin structure, which functioned as a control ligand.

### Ligand Selection

The collected ligands derived from peptide fragments, including Curcumin then calculated for their properties. The calculation includes molecular weight, cLogP value, cLogS, count of hydrogen donor, and hydrogen acceptor. Using Osiris Data Warrior, the drug properties were also predicted with parameters such as mutagenic, tumorigenic, reproductive effectiveness, and drug-likeness. The bioactivity value was also calculated on the Enzyme Inhibitor parameter for each ligand using Molinspiration. The ligand data was then selected for the peptides which do not violate Ro5 rules, have no side effects by drug properties data, and have high bioactivity. The results of this selection are used as ligand candidates for molecular docking.

### Docking Simulation

Docking simulations are carried out in the PyRx program and by the AutoDock Vina protocol which suitable for this research (Eberhardt et al., 2021; Trott & Olson, 2010). Ligand selection is made by inputting selected ligands in the previous step. The macromolecule selected was RBD from the SARS-CoV-2 virus spike protein. The grid setting is adjusted to a

3-dimensional volume covering both ligands and the SARS-CoV-2 RBD as macromolecules. This process will result in predicting the location of the link between them. By the simulation, the best position is assessed by the lowest energy affinity value. The docking results can be visualized in 2 and 3 dimensions using the Discovery Studio program. The results in 2-dimensional visualization will inform which molecules of the ligands and macromolecules are linked. The 3-dimensional visualization result will give information about ligand's rotational movement to interact with the cleavage of the macromolecule, and compare of simulation of any competing ligands.

## RESULTS AND DISCUSSION

### Virtual Digestion of Goat's Milk Beta Casein

The activity of pepsin, trypsin and chymotriypsin as human digestive enzymes to the casein are predicted to produce 81 peptide fragments. Goat's milk beta-casein, which has 207 amino acids, can be divided into 34 fragments due to the pepsin enzyme, 15 fragments due to the trypsin enzyme, and 32 fragments due to the chymotrypsin enzyme. The size of the resulting fragment varies from 2 up to 10 amino acids, however, dominated by four amino acid length fragments.

The performance of the pepsin enzyme is hydrolyze aromatic amino acids, specifically at the N-terminal of an amino acid (Johnston et al., 2007). The pepsin enzyme will work optimally at pH 2, so it is necessary to set these conditions during virtual cutting. Trypsin enzyme hydrolyzes the C end of the amino acid lysine and arginine. Sometimes, amino acid proline also can be cut by this enzyme (Rodriguez et al., 2008). Chymotrypsin works by hydrolyzing amino acids such as tyrosine, tryptophan, leucine, and phenylalanine. The selected peptide fragments are presented in **Table 1**. The selected peptides were mainly produced by trypsin enzyme activity and were then considered in Lipinski's Rule of 5 for the next step. The action could occur because trypsin activity can cut the C-end of the amino acid lysine. Four of the five selected peptides had Lysine on their end, based on the results obtained above. The result indicates that the Lysine residue in the end position of a peptide becomes one of the indicator characteristics of a bioactive peptide that can be produced from a protein (Karami & Akbari-adergani, 2019). However, PLP peptide was made by pepsin which has Proline residue at the end of peptide fragments. A peptide that has Prolin in the end of the sequence may also be a characteristic of a peptide with bioactive properties (Pak et al., 2005). The selected peptide fragments position has similar results from in vivo cow's casein digestion in the human digestive tract that was found by (Boutrou et al., 2013). This emphasizes that these peptides are available in real terms.

### The Ro5 Parameters

The Ro5 parameter inspects a peptide fragment to confirm its bioavailability implicates the appropiateness of the compound to be administered by oral. The bioavailability of a peptide indicates that the human body can properly utilize the molecule. The availability occurs because the peptide fragments can be absorbed by the digestive tract and distributed throughout the body (Benet et al., 2016). The opposite will happen if the peptide fragments do not meet the Ro5 criteria because the body will not absorb them. The properties of each selected peptide fragment on the Ro5 parameter and its energy affinity are presented in **Table 2**.

**Table 1.** Peptide fragments produced and their position on protein sequence

| Fragments | Sequence position | Hydrolyzing enzyme |
|---|---|---|
| GVPK | 94-97 | Trypsin |
| PLP | 151-153 | Pepsin |
| QPK | 166-169 | Trypsin |
| VK | 113-114 | Trypsin |
| VPK | 118-120 | Trypsin |

Note : G = Glycine, K = Lysine, L = Leucine, P = Proline, Q = Glutamine, V = Valine

**Table 2**. Selected ligands with their Lipinski's Rule of 5 properties and their affinity energy on docking simulation

| Ligands | Total Molecular Weight (Da) | cLogP | cLogS | H-Acceptors | H-Donors | Affinity Energy (kcal/mol) |
|---|---|---|---|---|---|---|
| GVPK | 401.506 | -1.0212 | -1.296 | 10 | 5 | -6.4 |
| PLP | 326.415 | -0.1805 | -1.722 | 7 | 3 | -6.4 |
| QPK | 373.452 | -1.5429 | -1.19 | 10 | 5 | -6.8 |
| VK | 247.338 | -0.1645 | -1.36 | 6 | 4 | -5.2 |
| VPK | 344.454 | -0.1049 | -1.529 | 8 | 4 | -6.2 |
| CC9 Curcumin | 368.384 | 2.949 | -3.622 | 6 | 2 | -6.7 |

Parameters for Ro5 include molecular weight, cLogP, cLogS, hydrogen donor, and hydrogen acceptor values. The Ro5 criterion in molecular weight is not more than 500Da, and all selected ligands have a molecular weight of less than 500Da. The cLogP or calculated partition coefficient is a value to predict lipophilicity. The value which fits on Ro5 is less than 5, and all fragments pass this criterion and considered as non liphopilic. The cLogS is a parameter to estimate the solubility of a molecule in water, and the more negative the value indicates that the molecule is insoluble. The range of 0 to -2 indicates a molecule is very soluble, and all ligands originating from goat milk beta-casein are at this stage. Curcumin molecule is soluble because its value is in the range between -2 to -4. The other stage is moderately soluble (-4 to -6), poorly soluble (-6 to -10), and insoluble (more than -10). Each selected ligand was fit to Ro5 that have no more than 5 hydrogen bonds donor and no more than 10 hydrogen bonds acceptor (Lipinski et al., 2001). These criteria reflect that all ligands are potential bioactive peptides and can be utilized by the body through absorption, distribution, metabolism, and excretion (ADME) (Benet et al., 2016).

### Bioactive Properties

Evaluation of the bioactive properties of each ligand is intended to determine the estimated side effects caused by the ligand if it is absorbed into the body. This evaluation includes side effects such as mutagenic, tumorigenic, reproductive effective, and irritant. All selected ligands were predicted to have no side effects while absorbed into the body. Considering peptides for its bioavailability is followed by side effects exploration to prevent any unfavorable behaviours (Srivastava, 2021). If it is predicted that side effects will occur, the selected ligand can be rejected or become a low priority for selection. The bioactive properties of each ligand are shown in **Table 3**.

The drug-likeness is a parameter that predicts a ligand has some similar effects like drug or not (Benet et al., 2016). The drug-likeness parameter shows promising results for several ligands except for PLP peptide. The peptide solely has positive drug-likeness value implicates its properties are similar to chemical drug molecules while absorbed into the body, however it still prospective because no predicted side effects following the result. The Proline-Leucine-Proline peptide is consisted of hydrophobic amino acids, this may cause the spesific molecular properties thus positive drug-likeness value (Thompson et al., 2006). Consideration on some ligands for its drug-likeness are essential, its examines any possibility of side effects if the drugs are consumed (Qureshi et al., 2022).

The protease inhibitor properties of each ligand showed varied results. The more excellent value obtained, the stronger the ligand will act as an inhibitor. The performance of protease inhibitors also means that a ligand can inhibit the mechanism of action of a protein (Morris et al., 2008). These considerations convincing selected ligands can compete with the existing ligand to attach their receptor.

### Docking Simulation and Affinity Energy

The docking simulation results are presented in **Figure 1** and **Figure 2**, below. The energy affinity of each ligand when simulated for docking with the SARS-CoV-2 spike protein, showed some variation. The best result is achieved by QPK ligands or a peptide consisting of Glutamine-Proline-Lysine amino acid residues. The lower energy affinity value indicates the easier for the ligand to dock with the receptor (Morris et al., 2008). Furthermore, the value obtained was lower than the energy affinity of Curcumin (-6.8vs.-6.7kcal/mol). These results may be due to the bonds between these molecules being more varied in QPK peptides than in Curcumin (**Figure 1**).

**Table 4** shows the interactions of QPK peptide is more diverse than curcumin. The bonds that make the QPK ligand attach to the site are 9 points of van der Waals bond, a salt bridge bond, 5 points of conventional hydrogen bond, a carbon-hydrogen bond, a Pi donor, a Pi alkyl, and a Pi-Pi stacked bond. However, Curcumin only has 7 points of van der Waals bond, 2 points of conventional hydrogen bond, a carbon-hydrogen bond, and 2 Pi-Pi stacked bonds. The interaction of QPK to spike protein is enhanced by van der Waals, rather than Curcumin. Van der Waals provides protein-ligand interaction, it helps the recognition of ligands to the binding pocket of target protein (Bitencourt-Ferreira et al., 2019). The hydrogen bond of QPK is more numerous than Curcumin, it may make lower affinity energy of QPK. The hydrogen bond is ubiquitous and plays general roles on ligand-protein interactions, the number of bond is more important than the strength (D. Chen et al., 2016). Salt bridge is a most substantial non covalent interaction of ligand-protein (Kurczab et al., 2018). However, salt bridge interaction only occurred in QPK ligand which may be causing stronger interaction. The docking positions of the two ligands are almost similar, so they are considered to work with the exact mechanism and capability to inhibit the attachment of the SARS-CoV-2 protein spike to human cells. The comparison of QPK and Curcumin interaction position is visualized on **Figure 1**.

In **Figure 2**, QPK peptide and Curcumin are presented in a 3-dimensional ilustration. Fortunately, both ligands have similar docking position implicates that the action of them are similiar in changing the macromolecule properties. The change of the properties will disrupt the activity of the spike protein to bind with ACE2 by allosentric mechanism (Shang et al., 2020; Yang et al., 2021). In further study, curcumin can inhibit the recent 'Omicron' variant of SARS-CoV-2 virus, this may enhance the prospect of QPK peptide as an alternative (Nag et al., 2022).

**Table 3**. Selected ligands and their bioactive properties

| Ligands | Drug-likeness | Mutagenic | Tumorigenic | Reproductive Effective | Irritant | Protease Inhibitor |
|---|---|---|---|---|---|---|
| GVPK | -7.8766 | None | None | None | None | 0.72 |
| PLP | 4.3661 | None | None | None | None | 0.85 |
| QPK | -7.7147 | None | None | None | None | 0.69 |
| VK | -12.263 | None | None | None | None | 0.18 |
| VPK | -7.9986 | None | None | None | None | 0.68 |
| CC9 Curcumin | -4.7745 | None | None | None | None | 0.15 |

**Table 4**. Ligands to macromolecule interactions

| Interactions | QPK | Curcumin |
|---|---|---|
| van der Walls | 9 | 7 |
| Salt bridge | 1 | - |
| Conventional hydrogen bond | 5 | 2 |
| Carbon hydrogen bond | 1 | 1 |
| Pi-donor hydrogen bond | 1 | - |
| Pi-Alkyl | 1 | - |
| Pi-Pi stacked | - | 2 |
| Unfavorable donor-donor | 1 | - |

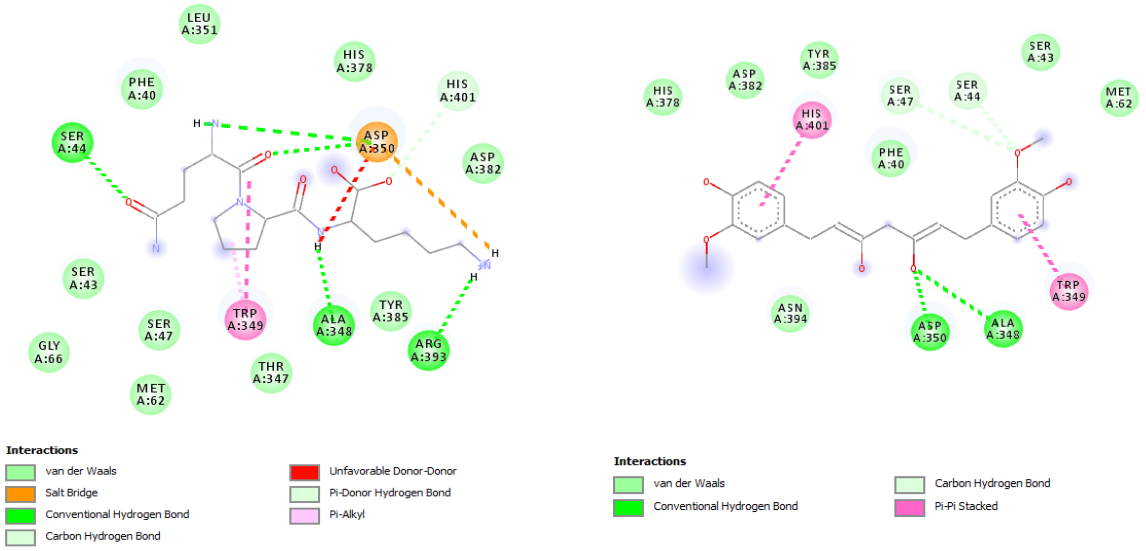


**Figure 1**. Prediction of molecule interaction between ligand and SARS-CoV-2 spike protein, QPK peptide (left) and Curcumin (right).

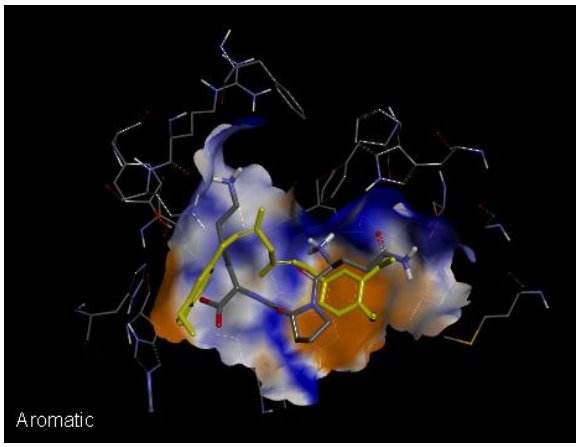


**Figure 2**. Prediction of docking site of QPK peptide (gray) and curcumin (yellow) on SARS-CoV-2 spike protein

Curcumin is considered one of the ligands that can inhibit the replication of the SARS-CoV-2 virus (Patel et al., 2020; Shanmugarajan et al., 2020). This suggests that the QPK peptide from goat's milk beta-casein can compete with better results to dock with the SARS-CoV-2 spike protein. The other advantage is that the peptide production from goat's milk beta-casein does not need further processing (Ahmed et al., 2015; Boutrou et al., 2013). It only needs enzymes that are naturally available in the gastrointestinal tract, than curcumin which needs treatments to yield the compound. The extraction of curcumin is needed to enhance its usability (Jiang et al., 2021).

## CONCLUSIONS

Goat's milk beta-casein has the potential as a bioactive peptide source that can inhibit the binding of SARS-CoV-2 to human cells. The QPK peptide becomes a potential ligand capable of interacting with that viral spike protein and competing with the curcumin. Further studies are needed on the performance of that peptide and its ADME properties by more specific parameters.

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