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Interaction of Sambiloto (*Andrographis paniculata*) Bioactive Compound with Milk Protein (Whey and Casein) Through Molecular Docking and Molecular Dynamics Simulation as a Basis for Encapsulation

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Abstract: This research aims for developing immune-boosting products necessary. The active ingredient in *Andrographis paniculate* (AP) acts as an immunostimulant which can improve the work of the immune system. The first stage of research was a collection of bioactive compounds from KnapSack database of Kanaya, Dr. Duke's Phytochemical and Ethnobotanical were compiled and selected based on the online pass results of each bioactive compound as an immunomodulator and 10 active compounds were obtained which will be continued. The second stage of research was a docking molecular between whey proteins (β-lactoglobulin and α-lactalbumin) with active compounds from AP and casein (α-Casein, β-Casein, and κ-Casein). The highest binding affinity was obtained for α-Casein with Neoandrographolide at -9.2 Kcal/mol. The results of the complex α-Casein with Neoandrographolide (CC) and α-Casein with Neoandrographolide ultraheat (CCT) support the research, namely to function α-Casein as an encapsulant well as a transporter or drug delivery of Neoandrographolide without changing the conformation of casein and disturbing its function. However, the conformation of casein will change drastically during ultraheat treatment to maintain the conformation and binding with the Neoandrographolide ligand. In addition, it supports the simulation results of single α-Casein at ultraheat temperatures which show conformational stability that is not much different from single α-casein and complexes at physiological temperatures.

Keywords: Bioactive; Casein; Docking; Encapsulant; Whey

Introduction

Immunity is a reaction in the body to foreign materials that enter either molecularly or cellularly. The cells involved in the immune system in the body are T cells produced by the thymus and B cells produced in the spinal cord. The development and activity of T cells can be stimulated by adding an immunomodulator. Immunomodulators are substances that can modulate the function and activity of the immune system. One effort that can be made is to look at herbal ingredients that have benefits as immunomodulators, but of course, clinical trials will be carried out in the future. One of the herbal ingredients that has this effect is Andrographis paniculate (AP) which contains a bioactive compound, namely andrographolide, which is a compound that acts an immunomodulator, especially an immunostimulant which can improve the work of the immune system (Ulvia et al., 2023). The andrographolide content in it can improve the function of the body's defense system such as white blood cells to attack bacteria and other antigens(immunomodulator), flavonoids as anti-inflammatory, and tannins as anti-diarrhea (Jayakumar et al., 2013).

Andrographolide can be obtained by extraction using the Microwave Assisted Extraction (MAE) method (Rahayu et al., 2019). In order for herbal products to provide maximum effect, coating materials are needed that can increase solubility, stability, bioavailability, and a targeted system so that the use of andrographolide is

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more effective. Apart from that, it is also used to protect and maintain its stability through nanoencapsulation. Nanoencapsulation is the process of coating core material in the form of solid particles, liquid, or gas using a coating material. This process aims to protect the core material in the form of bioactive compounds such as phenolic compounds from various environmental influences such as light, oxygen, water, and temperature (Bratovcic & Suljagic, 2019).Before proceeding to the encapsulation process, initial testing can be carried out regarding the interaction between the coating material and bioactive compounds. Apart from that, the right distributor is needed to protect the compound so that it does not reduce the effectiveness of the compound.

Phenolic compounds have a high ability to interact with milk proteins (Han et al., 2019), through non-covalent interactions between polyphenols and proteins resulting in complexation so that it can stabilize the protein structure (Li et al., 2015). The strength of this interaction depends on the size of the polyphenol, the structure of the polyphenol, and the amino acid sequence of the protein (Frazier et al., 2010) . Whey protein and casein are the delivery compounds that can be used as a nano-delivery system for bioactive compounds (Rahayu et al., 2015).The ability of whey protein to form gels and nanocapsules without high heat treatment and chemicals gives whey protein the potential to be used in nano delivery systems or natural nanovehicles for the bioactive compound andrographolide in the food system. Nanoparticles have many advantages compared to microparticles, including that nanoparticles can be dispersed homogeneously and more uniformly in the food system and are more easily absorbed.

Efforts made to determine a good distribution material are by using molecular docking computational methods to obtain an interaction model between the distribution materials used (Rahayu et al., 2019) . This will make it easier to carry out further research in creating milk protein complexes with stable andrographolide compounds as immunomodulator products. Application of protein-based nanoparticles to dairy product formulation based on hydrophobic and hydrophilic interactions. Based on the explanation above, in creating immunomodulator products, several studies are needed first to analyze the interactions between the best milk proteins as stable delivery agents.

This study aims to analyze the active components in sambiloto that have functional health benefits through in silico methods. Additionally, it will examine the interactions between milk proteins (whey and casein) and the active components of sambiloto, which are essential for the development of encapsulation products. Therefore, this study will evaluate the effectiveness of the bonds formed between whey and the active components of sambiloto, as well as between casein and the bioactive components of sambiloto.

Method

Collection of Target Bioactive Compounds

Various active compounds from Sambiloto (Andrographis paniculata) were obtained from the KNApSAck (www.knapsackfamily.com) and Dr Duke Phytochemical (www.phytochem.nal.usda.gov) databases. These two databases were created specifically to facilitate researchers searching for chemical compounds, bioactivity, and plant ethnobotany that are useful for pharmaceutical, nutritional, and biomedical studies. Next, the 3D and SMILE structures of the active compounds were obtained from the PubChem database (pubchem.ncbi.nlm.nih.gov) as samples.

3D Structure Modelling of Protein Targets

Protein sequences obtained from α-casein, β-casein, and κ-casein were obtained from the UniProt database (https://www.uniprot.org/) with IDs P02662, P02666, P02668 respectively. Next, the protein was modeled using ab initio principles, with I-TASSER software (https://zhanglab.dcmb.med.umich.edu/I-TASSER/). The best model, with the highest C-score and lowest RMSD, will be selected for further analysis. Meanwhile, the proteins α-lactalbumin and β-lactoglobulin have 3D structures crystallized in the PDB database (https://www.rcsb.org/), with IDs 1F6R and 1B0O, respectively.

QSAR Analysis

To determine the biological activity of each active compound, a Quantitive Structure-Activity Relationship (QSAR) prediction analysis was carried out using Way2Drug/PASS online (www.way2drug.com/PASSOnline). The score shown by the webserver varies from 0-1, which indicates the accuracy of the analysis (Jing et al., 2014). The terminology chosen for the parameters observed was antioxidant activity, anti-inflammatory, IL6 antagonist, cytokine release inhibitor, transcription factor NF Kappa B inhibitor, TP53 expression enhancer, immunostimulant, immunosuppressant, and immunomodulator.

Molecular Dooking

To determine the affinity bonds formed between the proteins α-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin with the selected active compounds, molecular docking analysis was carried out using PyRx software v.8.0, blind docking (Dantas et al., 2020). Next, visualization of interactions and complex docking results was carried out using Discovery Studio R17 and PyMol.

Molecular Dynamics Simulation

Molecular dynamics simulations were carried out with the YASARA Dynamic program developed by

Biosciences GmbH. The first step is to input the single protein casein (SC) file resulting from docking of the neoandrographolide- α-Casein (CC) complex into the program using Options, then select the Macro & Movie menu and finally select Set Target. Next, macro input is carried out to carry out molecular dynamics simulations which have previously been prepared in advance in the variable section, namely temperature and physiological pH, namely 310K and 7.4. In addition, ultraheat temperature (T) treatment (353K) was also used as a variable with a fixed pH. In the next step, the md_run macro also sets the running time, namely 10,000 ps (10 ns). This simulation uses the AMBER03 forcefield and snapshot storage every 25 ps (Delazar et al., 2012). RMSD and potential energy analysis are obtained by running the md_analyze macro. Next, the RMSF analysis is run with the md_analyzeres macro, while the running results by running the md_play macro. In the final step, the results are recorded with the IceCream Screen Recorder application with the output in the form of a file with the extension (.wmv).

Result and Discussion

Collection of Target Bioactive Compounds

Bioactive compounds obtained from the KnapSack database Kanaya and Dr. Duke's Phytochemical and Ethnobotanical were compiled and selected based on the online pass results of each bioactive compound as an immunomodulator (Tallei et al., 2024). There were 10 active compounds from Sambiloto selected for molecular docking tests and several follow-up tests (Table 1).

Table 1. Selected Active Compounds from AP

Compound	ID	SMILE
Caffeic acid	689043	C1=CC(=C(C=C1/C=C/C(=O)O)O)O
Apigenin 7,4'-dimethyl ether	5281601	COC1=CC=C(C=C1)C2=CC(=O)C3=C(C=C(C=C3O2)OC)O
Wogonin 5-glucoside	44258554	COC1=C2C(=C(C=C1O)O[C@H]3C([C@H]([C@@H](C(O3)CO)O)O)O)C(=O)C=C(O2)C4=CC=CC=C4
5-Hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside	44258544	COC1=CC=CC(=C1OC)C2=CC(=O)C3=C(O2)C(=C(C=C3O[C@H]4C([C@H]([C@@H](C(O4)CO)O)O)O)OC)OC
Cinnamic acid	444539	C1=CC=C(C=C1)/C=C/C(=O)O
14-deoxy-11-oxoandrographolide	101593061	C[C@@]12CC[C@H]([C@@]([C@H]1CCC(=C)[C@H]2C(=O)CC3=CC(=O)OC3)(C)CO)O
5-hydroxy-7,8,2',3'-tetramethoxyflavone	5319878	COC1=CC=CC(=C1OC)C2=CC(=O)C3=C(O2)C(=C(C=C3O)OC)OC
5-hydroxy-7,8,2'-trimethoxyflavone	5318506	COC1=CC=CC=C1C2=CC(=O)C3=C(O2)C(=C(C=C3O)OC)OC
Andrographolide	5318517	C[C@@]12CC[C@H]([C@@]([C@H]1CCC(=C)[C@H]2C/C=C/3\[C@@H](COC3=O)O)(C)CO)O
Neoandrographolide	9848024	C[C@]1(CCC[C@@]2([C@@H]1CCC(=C)[C@H]2CCC3=CCOC3=O)C)CO[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O

The α-Casein protein structure model has the best C-score value compared to other models, namely -4.23 and RMSD 15.8 +-3.2 A. Meanwhile, the β-Casein model has a C-score of -2.32 and RMSD 10.9 +- 4.6A. The κ-Casein structure model has a C-score of -2.5 and an RMSD of 10.9 +- 4.6A. The C-score shows the confidence score for each model. The greater the significance value or confidence score, the better the 3D prediction results.

QSAR Analysis

Based on the results shown by the Pass server for QSAR analysis, the 10 selected compounds have good antioxidant, anti-inflammatory, IL6 antagonist, cytokine release inhibitor, transcription factor NF Kappa B inhibitor, TP53 expression enhancer, and immunomodulatory potential, so they are continued for further analysis (Jing et al., 2014). Molecular docking and molecular dynamics (Figure 1).

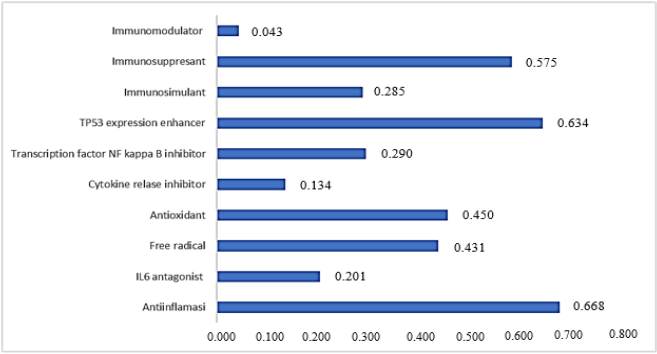

Figure 1. Potential of Bioactive Compounds as Immunomodulators Based on Pass Online Pa Values

Molecular Dooking

The docking results (Table 2) show that the complex between B-lactoglobulin and 14-deoxy-11-oxoandrographolide has a strong binding affinity, namely -7 Kcal/mol. Other compounds that bind strongly to B-lactobglobulin are Wogonin 5-glucoside and neoandrographolide with affinity scores of -6.9 and -6.8 Kcal/mol, respectively.

Table 2. Docking between B-lactoglobulin and selected active compounds from AP

Protein	ID Ligand	Compound	Binding affinity (kcal/mol)
β-lactoglobulin	101593061	14-deoxy-11-oxoandrographolide	-7.0
	44258554	Wogonin 5-glucoside	-6.9
	9848024	Neoandrographolide	-6.8
	44258544	5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside	-6.8
	5318517	Andrographolide	-6.7
	5319878	5-hydroxy-7,8,2',3'-tetramethoxyflavone	-6.2
	5281601	Apigenin 7,4'-dimethyl ether	-6.1
	5318506	5-hydroxy-7,8,2'-trimethoxyflavone	-6.1
	689043	Caffeic acid	-5.8
	444539	Cinnamic acid	-5.7

Molecular docking (Table 3) carried out between α-lactalbumin and the active compound AP shows that the strongest bond is on wogonin 5-glucoside with a score of -7 Kcal/mol. The other two compounds are neoandrographolide and 5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside with affinity scores of -6.5 and -6.4 Kcal/mol respectively

Table 3. Docking between α-lactalbumin and selected active compounds from AP

Protein	ID Ligand	Compound	Binding affinity (kcal/mol)
α-lactalbumin	44258554	Wogonin 5-glucoside	-7.0
	9848024	Neoandrographolide	-6.5
	44258544	5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside	-6.4
	5281601	Apigenin 7,4'-dimethyl ether	-6.1
	101593061	14-deoxy-11-oxoandrographolide	-6.0
	5318517	Andrographolide	-6.0
	5319878	5-hydroxy-7,8,2',3'-tetramethoxyflavone	-5.6
	5318506	5-hydroxy-7,8,2'-trimethoxyflavone	-5.5
	689043	Caffeic acid	-5.4
	444539	Cinnamic acid	-4.8

Furthermore, docking between the α-Casein protein and the active compound AP showed that the neoandrographolide compound had the strongest bond with a value of -9.2 Kcal/mol. The other two compounds are Apigenin 7,4'-dimethyl ether and Wogonin 5-glucoside, with bond strengths of -8.9 and -8.6 Kcal/mol (Tabe 4).

Table 4. Docking between α-Casein and selected active compounds from AP

Protein	ID Ligand	Compound	Binding affinity (kcal/mol)
α-Casein	9848024	Neoandrographolide	-9.2
	5281601	Apigenin 7,4'-dimethyl ether	-8.9
	44258554	Wogonin 5-glucoside	-8.6
	44258544	5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside	-8.4
	5318517	Andrographolide	-8.2
	101593061	14-deoxy-11-oxoandrographolide	-7.6
	5318506	5-hydroxy-7,8,2'-trimethoxyflavone	-7.6
	5319878	5-hydroxy-7,8,2',3'-tetramethoxyflavone	-7.3
	689043	Caffeic acid	-7.0
	444539	Cinnamic acid	-6.5

The interaction between β-Casein and 10 selected compounds from AP shows that 14-deoxy-11-oxoandrographolide, Neoandrographolide, and 5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside have the strongest bonds, respectively with scores of -8.1, -7.8, and -7.5 Kcal/mol (Table 5).

Table 5. Docking between β-Casein and selected active compounds from AP

Protein	ID Ligand	Compound	Binding affinity (kcal/mol)
β-Casein	101593061	14-deoxy-11-oxoandrographolide	-8.1
	9848024	Neoandrographolide	-7.8
	44258544	5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside	-7.5
	5318517	Andrographolide	-7.5
	44258554	Wogonin 5-glucoside	-7.4
	5281601	Apigenin 7,4'-dimethyl ether	-7.4
	5318506	5-hydroxy-7,8,2'-trimethoxyflavone	-7.4
	5319878	5-hydroxy-7,8,2',3'-tetramethoxyflavone	-7.3
	689043	Caffeic acid	-5.9
	444539	Cinnamic acid	-5.8

Furthermore, docking carried out between κ-Casein and 10 selected compounds from AP shows that Wogonin 5-glucoside had the strongest bond with a score of -8.5 Kcal/mol. The second and third sequences are Neoandrographolide and Apigenin 7,4'-dimethyl ether with an affinity of -8.2 and -8.1 Kcal/mol (Table 6).

Table 6. Docking between κ-Casein and selected active compounds from AP

Protein	ID Ligand	Compound	Binding affinity (kcal/mol)
κ-Casein	44258554	Wogonin 5-glucoside	-8.5
	9848024	Neoandrographolide	-8.2
	5281601	Apigenin 7,4'-dimethyl ether	-8.1
	5319878	5-hydroxy-7,8,2',3'-tetramethoxyflavone	-7.5
	44258544	5-hydroxy-7,8,2',3'-tetramethoxyflavone 5-glucoside	-7.0
	5318517	Andrographolide	-7.0
	101593061	14-deoxy-11-oxoandrographolide	-6.8
	5318506	5-hydroxy-7,8,2'-trimethoxyflavone	-6.7
	444539	Cinnamic acid	-5.4
	689043	Caffeic acid	-5.4

Based on the results of docking five proteins with 10 active compounds from AP, the Neoandrographolide compound is always in the top three positions with high affinity. So, the stability of the interaction between α-Casein and neoandrographolide will be analyzed further using molecular dynamics. Visualization between each protein and compound with the best affinity is shown in Figure 2. The interactions of the amino acids involved in the protein and compound-complex are show in Table 7.

Table 7. Amino acid interactions involved in complex docking

Complex	Interaction
α-lactalbumin dan Wogonin 5-glucoside	Van Der Waals: His32, Thr33, Val42, Ile55, Glu49, Phe53, Trp104, Asn56, Tyr103, Hoh128, Leu105 Hydrogen: GLN54 Pi Sigma: Leu110 Pi Alkyl: Ala106
β-lactoglobulin dan 14-deoxy-11-oxoandrographolide	Van Der Waals: Glu157, Glu44, Gln159, Cys160, Tyr42, Val43, Ser21, Leu156, Glu158, Thr18, Trp19, Gln155 Hydrogen: GLN68, GLN59 Pi Alkyl: Tyr20, His161
α-Casein and Neoandrographolide	Van Der Waals: Lys51, Gly48, Pro44, Glu45, Ala41, Glu33, Asn89, Val87, Glu78, Ile86, Pro88, Val91, Ser90, Glu92, Ser103, Asp100, Gln97, Glu111, Gln74 Hydrogen: Glu85 Alkyl: Leu144, Leu110, Leu107
β-Casein dan 14-deoxy-11-oxoandrographolide	Van Der Waals: Ile38, Ser37, Glu36, Ser34, Glu35, Lys43, Lys44, Leu31, Lys47, Asp58, Ser33 Hydrogen: ASN42, SER32 Pi Sigma: Phe102
κ-Casein dan Wogonin 5-Glucoside	Van Der Waals: Ser153, Pro155, Pro105, Glu172, Val104, Ile94, Ser108, Ile174, Ser176, Pro151, Lys107, Pro177, Met116 Hydrogen: Glu175, Thr154 Pi Sigma: Ala106 Pi Alkyl: His123

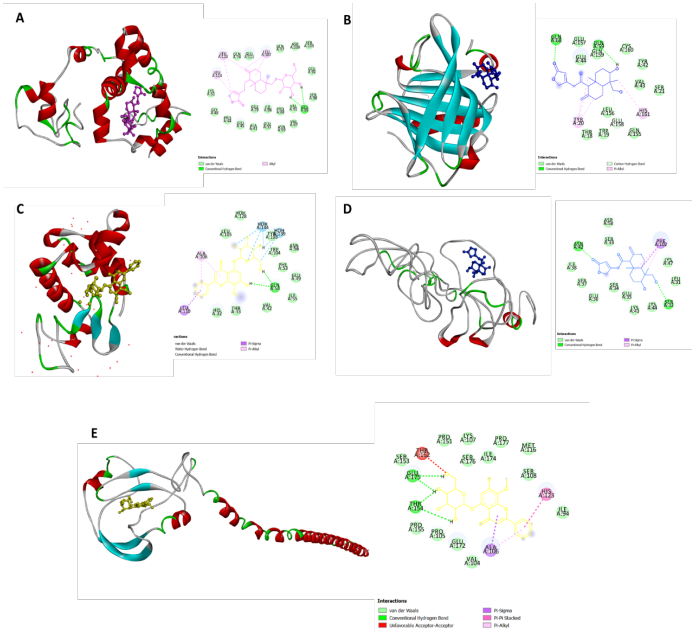

Figure 2. Visualization of interactions between α-lactalbumin and Wogonin 5-glucoside (A), β-lactoglobulin and 14-deoxy-11-oxoandrographolide (B), α-Casein and Neoandrographolide (C), β-Casein dan 14-deoxy-11-oxoandrographolide (D), κ-Casein and Wogonin 5-glucoside (E)

Molecular Dynamics Simulation

- Potential energy analysis

Molecular dynamics simulations are carried out using the YASARA dynamic program, a method used to see the dynamic stability of singles and complexes. So, several parameters used are, running temperature, namely 310K or 37°C which is a physiological temperature and 353K or 80°C as an ultraheat treatment. Furthermore, the level of NaCl or physiological salt used was 0.9% which was deemed sufficient to represent physiological conditions to maintain the viability of the two samples, then treatment was also given in the form of pH 7.4 which is physiological pH. Furthermore, the running time used is 10,000 ps (10 ns). In addition, the AMBER03 forcefield type was chosen because it is one of the forcefields prepared by the YASARA program and is most commonly used for simulating biological materials. This type of forcefield is also very suitable for simulating peptides that have secondary structures and bonds with ligands, because it is optimal in measuring the dynamic stability of bonds, especially intrapeptide bonds (Li et al., 2015)

The total single and complex potential energy analysis is obtained after running the md_analyze macro. The analysis results of the four samples generally show that the potential energy will experience a very significant increase from 0 ns to 0.5 ns running time (Figure 3). This shows that the energy initiation process occurs to achieve energy stability. However, fluctuations began to appear at 0.25 ns of running time, indicating that the four types of samples experienced changes in molecular bond energy. This fluctuation continues to occur even though it has reached a stable potential energy range (equilibrium phase) at ± -7,28,105 kJ/mol (single α-Casein/SC and neoandrographolide- α-Casein/CC complex) up to ± -6,78,105 kJ/ moles (single α-Casein/SCT and neoandrographolide-casein alpha CCT complex at ultraheat temperatures). Rising fluctuations can be interpreted as a strengthening of bonds in molecules, whereas conversely a decrease in potential energy can be interpreted as a result of the relaxation of molecular bonds (Bakker & Skinner, 2010). In addition, the fluctuation tendency between single alpha casein and the complex is quite different, so it can be concluded that the neoandrographolide ligand causes quite significant conformational changes. Meanwhile, ultraheat temperature treatment does not change the potential energy significantly.

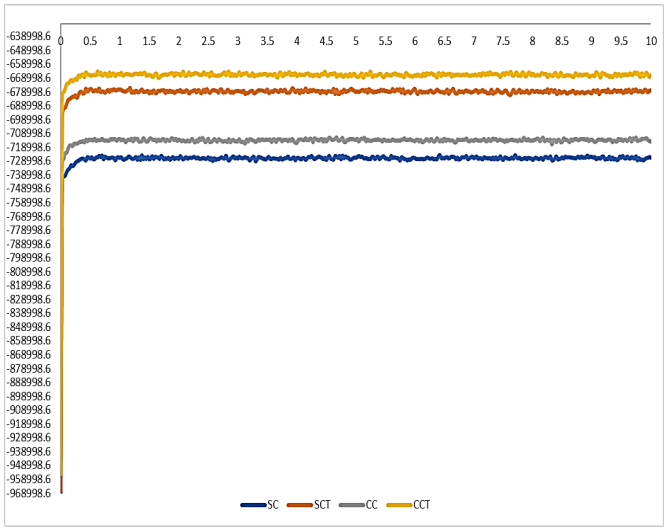

Figure 3. Single and complex potential energy at physiological and ultraheat temperatures. SC: single α-Casein; SCT: single α-Casein+ ultraheat; CC: complex compound (α-Casein+ neoandro); CCT: complex compound (α-Casein+ neoandro) + ultraheat

- Total RMSD analysis, ligand configuration, and superimposed visualization results

RMSD (Root Mean Square Deviation) is a score resulting from analysis that provides information on conformational changes in a macromolecule that acts as a receptor after the interaction process with a particular ligand (Naqvi et al., 2019). This RMSD value analysis was obtained after running the md_analyze macro. RMSD can also be used as a standard deviation of conformational changes, with standards < 2 nm and > 2 nm generally applied to docking results (Bell & Zhang, 2019) . RMSD is data that adequately represents the stability of the sample under simulation conditions. The dynamic stability in question is the absence of significant conformational changes, which is better known as the unfolding process.

The standard RMSD for a protein receptor from simulation results is 3 nm, where if the RMSD value of a protein is ≥ 3 nm, it is a sign that the protein has undergone a conformational change that is very different from its native condition. The RMSD values of the four samples generally have a tendency to continue to increase with several fluctuations and have passed the maximum limit of conformational stability, namely 3 nm at around 0.15 ns (Figure 4). Based on the total RMSD of single alpha casein samples at ultraheat (SCT) and complex (CC) temperatures, they have the best conformational stability with an average total RMSD value of 7,358 nm and 7,827 nm respectively. Then followed by single alpha casein (SC) with a total RMSD value of 8,023 nm and the complex at ultraheat temperature (CCT) became the sample with the most unstable total RMSD with an average value of 16,041 nm and a maximum of 21,278 nm.

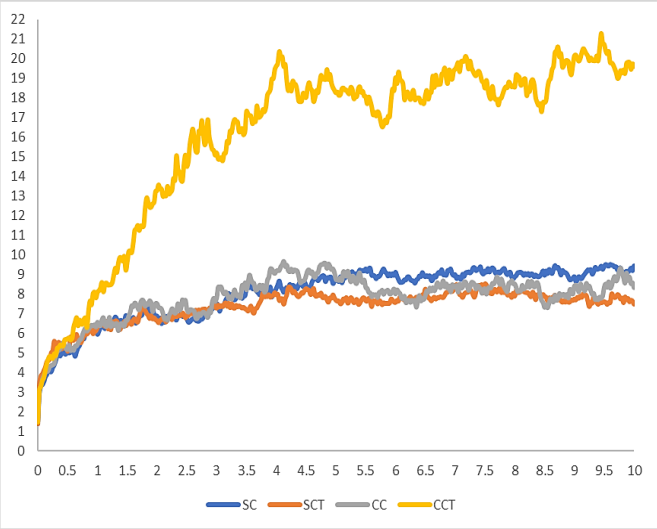

Figure 4. Single and complex RMSD at physiological and ultraheat temperatures. SC: single α-Casein; SCT: single α-Casein+ ultraheat; CC: complex compound (α-Casein+ neoandro); CCT: complex compound (α-Casein+ neoandro) + ultraheat

In addition to the RMSD of the entire molecule, the YASARA program can also be used to view the RMSD of the ligand configuration, which in this study is the compound neoandrographolide. The RMSD value of the neoandrographolide ligand CC complex shows quite stable fluctuations with a tendency to increase at the 0 ns to 0.5 ns simulation time, namely from initially having a value of 0.94 nm, increasing to 1.78 nm, which means that it undergoes an undesirable conformational change much different from the initial conformation in the CC complex. In addition, the average conformational change of the ligand was 1.61 nm or almost no conformational change at the end of the simulation time (Figure 5). Meanwhile, the RMSD value of the neoandrographolide ligand CCT complex that was treated at ultraheat temperatures showed high fluctuations with a tendency to increase at the 0 ns to 2 ns simulation time, namely from initially having a value of 0.98 nm , it increased to 2.93 nm, which means there was a change conformation that is very different from the initial conformation in the CCT complex or it is also possible that the bond is almost untied. However, the average conformational change of the ligand is 1.99 nm or almost no conformational change at the end of the simulation time. The RMSD value of the ligand configuration is also in line with the total RMSD value, where the CC complex has a more dynamically stable fluctuation value than the CCT complex.

The results of the CC and CCT complexes support the research objective, namely to function alpha casein as an encapsulant as well as a transporter or drug delivery of neoandrographolide without changing the conformation of casein much and without disturbing its function under physiological conditions, however the conformation of casein will change drastically during ultraheat treatment to maintain conformation and binding with the ligand neoandrographolide. Apart from that, supporting the simulation results of single alpha casein at ultraheat temperatures which show conformational stability that is not much different from single alpha casein and complexes at physiological temperatures, Kenkare and Hansen in 1967 also concluded that alpha casein protein has high conformational or thermodynamic stability because it has amino acid groups that can reversibly return to their original conformation even after undergoing high temperature treatment. Furthermore, the superimposed results of the four samples from before and after the simulation can also show in more detail the effect of ultraheat temperature treatment on changes in alpha casein conformation (Figure 6).

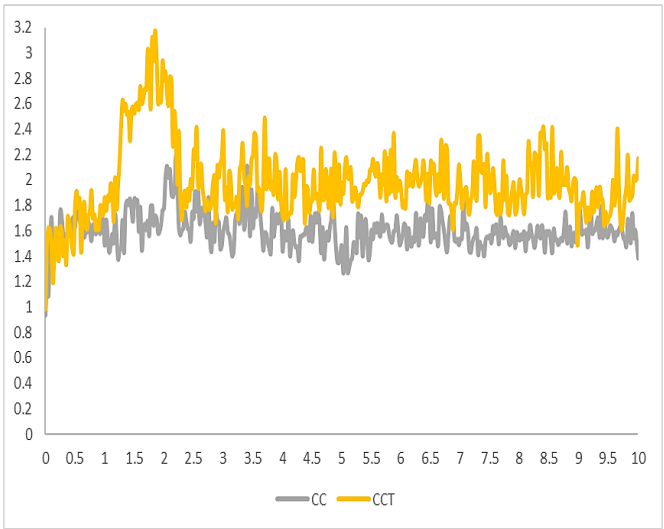

Figure 5. RMSD of ligand configurations of CC and CCT complexes at physiological and ultraheat temperatures. CC: complex compound (α-Casein+ neoandro); CCT: complex compound (α-Casein+ neoandro) + ultraheat

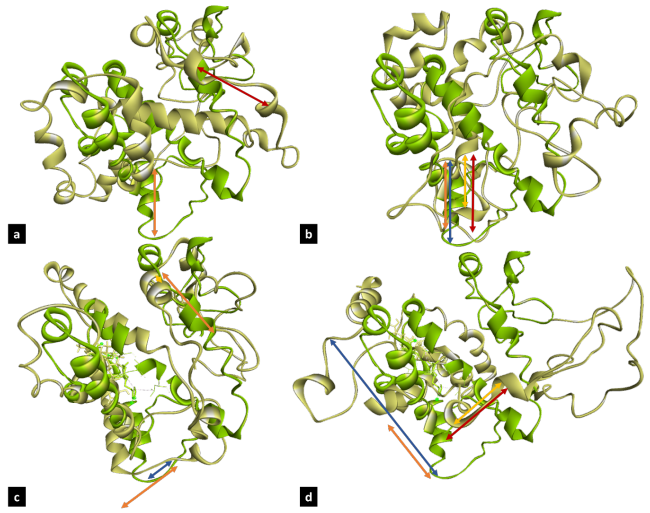

Figure 6. Superimposed (a) SC, (b) SCT, (c) CC, and (d) CCT before (green) and after (yellow) molecular dynamics simulations. SC: single α-Casein; SCT: single α-Casein+ ultraheat; CC: complex compound (α-Casein+ neoandro); CCT: complex compound (α-Casein+ neoandro) + ultraheat

- RMSF analysis of amino acid residues

RMSF (Root Mean Square Fluctuation) is a score that provides information on conformational changes in more detail because it is linked to fluctuations that occur at the amino acid residue level (Abhinand et al., 2016). Therefore, RMSF analysis was carried out more specifically, namely only looking at several alpha casein amino acid residues that interact with the neoandrographolide ligand. RMSF analysis of amino acid residues from singles and complexes is obtained after running the macro md_analyzeres. The results RMSF analysis between the control (single casein/SC) and the three treatment samples showed that several amino acid residues experienced conformational changes and large binding distances with the ligand. SCT and CC have an average RMSF of binding site residues that are lower or more stable, namely 3.52 nm and 3.51 nm, respectively, when compared to the control with an average RMSF value of 4.29 nm. This shows that by being an encapsulant, alpha casein protein also becomes more stable than when it is a free or single protein. Apart from that, it again confirms that the dynamic stability of alpha casein is better at high temperatures, so it is highly recommended as an encapsulant. This is because alpha casein is a type of unfolded protein that has a globular structure and is suitable for use as a surfactant. Apart from that, alpha casein contained in milk, especially cow's milk, can easily form amyloid fibril structures which are also called carrier micelles, which are structures that are suitable for use in drug delivery. On the other hand, this protein also shows characteristics that are almost similar to small heat-shock protein (sHSP) which has high thermodynamics (Hilton et al., 2012).

Furthermore, three residues of the 23 amino acids whose RMSF values were analyzed are amino acids that bind hydrophobically to neoandrographolide in the CC and CCT complexes, namely Leu107, Leu110, and Leu114. Then one amino acid in the form of Glu85 forms a conventional hydrogen bond with the ligand. Meanwhile, the other 19 amino acids interact in a van der Waals manner which increases the stability of the protein bond with the ligand (Figure 7). The four amino acids, namely Leu107, Leu110, Leu114, and Glu85, which were initially free because they had an RMSF > 3, were bound by ligands in the CC so that their RMSF value was < 3 and became more stable. Meanwhile, in SCT the amino acids Leu107, Leu110, and Leu114 also became more stable than the control, but the amino acid Glu85 was still above 3 even though it was still more stable than in physiological conditions.

CCT is the sample with the highest RMSF value of binding site amino acid residues with an average value of 6.72 nm which can indicate the release of the bond between the alpha casein protein and the neoandrographolide ligand after 10 ns simulation. However, based on the results of a comparison of 4 amino acids that are bound to 19 amino acids that interact in a van der Waals manner, it can be predicted that although in the end the ligand bond with the encapsulant is released and overall unfolding of the protein occurs, this release process will be slowed down by the bond with the ligand. Apart from that, this unfolding process is in accordance with the research objectives which require the release of ligands in the form of bioactive compounds of the bitter herb from the encapsulated protein when it reaches the therapeutic target.

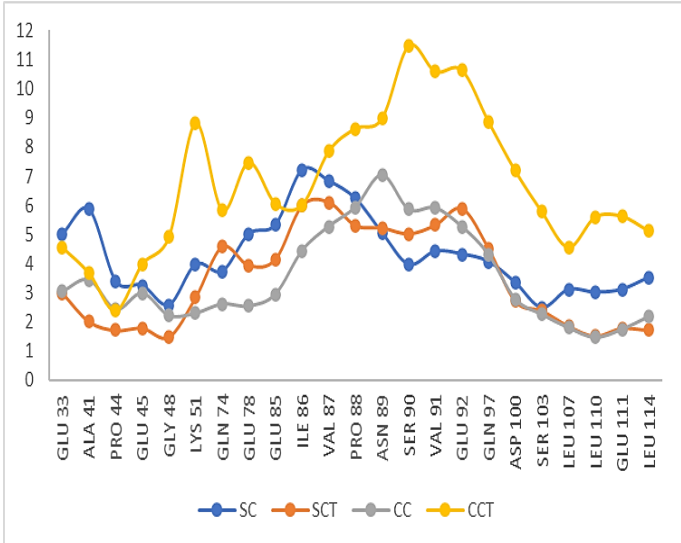

Figure 7. RMSF of receptor binding site amino acid residues from complex ligands. SC: single α-Casein; SCT: single α-Casein+ ultraheat; CC: complex compound (α-Casein+ neoandro); CCT: complex compound (α-Casein+ neoandro) + ultraheat

Conclusion

α-Casein is the ideal encapsulation material for the bioactive compound AP because the resulting CC and CCT complexes have been shown to not significantly change the structure of casein and not to disrupt its function its function under physiological conditions. However, the structure of casein will change significantly during superheat treatment to maintain shape and bind to the ligand neoandrographolide. Furthermore, it confirms the simulation results of single alpha casein at extremely hot temperatures showing that the conformational stability is not significantly different from the results of single α-Casein and complexes at biotemperatures physical.

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Author Contributions
Conceptualization P.P.R., D.S. and M.E.S.; Conducting research P.P.R.; Writing—original draft preparation P.P.R. and M.E.S.; Writing—edit draft C.N.Y; Supervision P.P.R. and D.S.

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Conflicts of Interest
The authors declare no conflict of interest

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