A Study of Encapsulation and Antioxidant Properties of Genistein in Caseinate and Liposome Systems

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Gemala Anjani

JULY 2014

DISSERTATION

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Graduate School of Natural Science & Technology

Kanazawa University

Division of Material Sciences

Course of Material Information Analysis

Student ID : 1123132316

Name : Gemala Anjani

Chief Supervisor : Assoc.Prof. Akio Ohta

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Chapter 1

GENERAL INTRODUCTION

1.1 BACKGROUND AND RESEARCH OVERVIEW

Genistein is one of isoflavone present in soybean and soy-products. Genistein has well known has estrogen-like chemicals, or phytoestrogen. This estrogen-like has the same receptor to that the original, therefore it will replace the estrogen role in the body, without negative effect as increasing the hormone related cancer risk. Furthermore it also beneficial as its phytoestrogen properties affected to hormonal and metabolic changes [1] with prevention of hormone-dependent cancers as breast cancer [2] [3] and prostate cancer [3]. The positive effect of genistein also inferred from the demonstrated capacity of genistein to relieve menopausal symptoms and enhance bone mineral density in women [4].

In addition, genistein is important related to its antioxidant capacity. Genistein fulfills many of the structural requirements considered essential for effective radical scavenging by flavonoids and isoflavonoids. Similar to other phenolic antioxidants, genistein as an antioxidant would act by scavenging peroxyl radicals, thereby suppressing radical chain auto-oxidations [5]. Antioxidant actions of the soy isoflavone genistein are believed to contribute to its overall chemo-preventive activities [5]. Genistein has capability to prevent

oxidative damage in lipid [6]. Other than the application of genistein as a complex food, we prefer to use genistein in pure form to increase to applicability of it in the wider system.

Genistein, as can be seen in **Fig. 1** is structurally hydrophobic, consequently poorly soluble in water system. This hydrophobic property limits the wide range applicability of genistein. As the other researchers also try to solve this sub optimal property of genistein by considerable synthetic effort made towards “better genistein” or “more efficient pro-genistein” [7]. Furthermore other studies recommended attaching these materials to amphipilic chemicals; as Crupi, *et al*. who attached these materials to amphiphlic chemicals as β-cyclodextrins. [8].

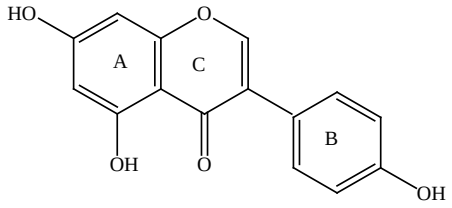


Fig. 1 Genistein (4`,5,7-trihydroxyisoflavone, Gen)

In this study, we used caseinate and liposome as encapsulate materials of genistein. Casein, the major protein found in milk, is inexpensive, nontoxic, and highly stable. Being a natural food product, it is generally recognized as safe protein and is biocompatible and biodegradable [9]. Furthermore, because of their structural and physicochemical properties, caseins are used to deliver medicines and nutraceuticals. The self-assembling capability of caseins is widely recognized, and has been exploited by previous researchers to encapsulate various nutraceuticals and medicines for many purposes. Native bovine caseins or caseinates, comprise mixtures of α_{s1}-,α_{s2}, β, and κ-caseins in the ratio 4:1:3.5:1.5

[10]. Extensive secondary structures and permanent, well-defined tertiary structures have not been reported in caseins [11]. All caseins are amphiphilic and have been described as rheomorphic or natively disordered, implying high adaptability. Native casein exists in micelle form. However, unlike typical micelle systems, casein micelles do not assemble with their hydrophilic heads exposed and their hydrophobic tails buried. Instead, the α_s- and β-casein fractions assemble with calcium phosphate and the surface is stabilized by κ-casein [12].

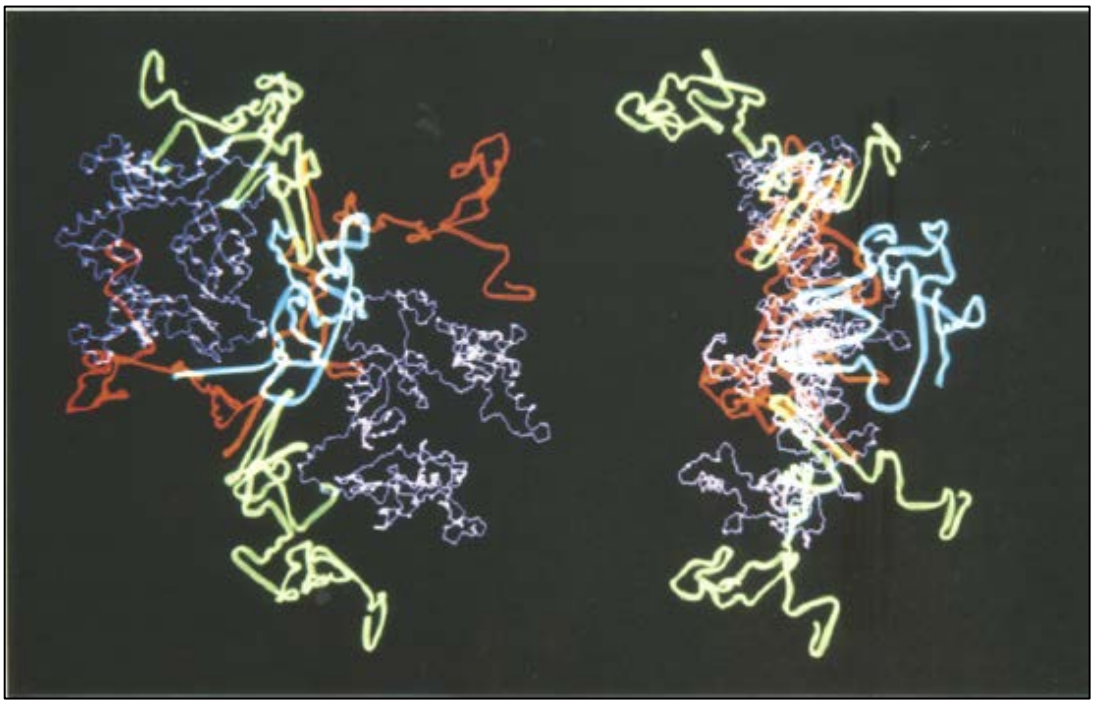


Fig.2 Energy-minimized casein asymmetric sub-micelle structure i.e. one κ-casein variant B, four α_{s1}-casein, two β-casein variant A^2 asymmetric dimers. Orthogonal view of ribboned backbone without side chains; κ-casein B cyan, α_{s1}-casein red and green, β-casein variant A^2 backbone without O and H atoms are magenta [13].

Kumosinski *et al.* proposed a computational study for sodium caseinate that constructed from κ-casein, α_{s1}-casein and β-casein molecules as can be seen in **Fig. 2** [13]**.** This model predicted sub-micelle and micelle formation structures were built from the various casein monomer structures previously refined via

energy minimization.

The self-assembling capabilities of caseins have been demonstrated in numerous studies. Huppert *et al*. prepared microgel and nanogel particles from casein micelles [14]. Semo *et al*. exploited casein micelles as natural nanocapsular vehicles for delivering lipophilic nutraceutical vitamin D [15], while Sahu *et al*. proposed curcumin–casein micelles as drug nanocarriers to cancer cells [16]. β-casein assemblages have also been considered as nano-vehicles for hydrophobic bioactive molecules.

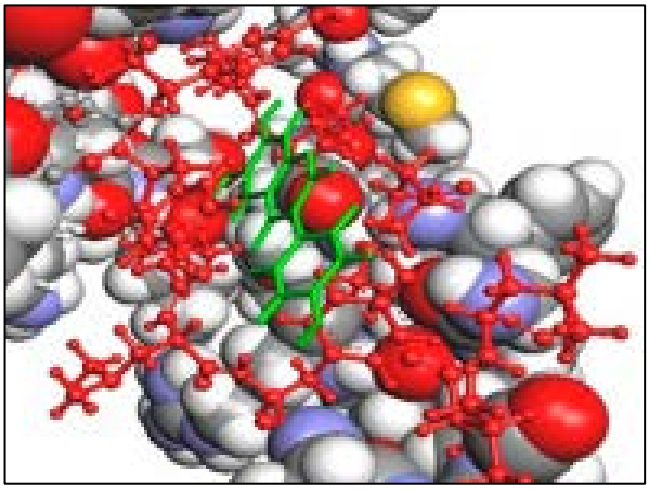


Fig. 3 Best docked conformations of genistein attached to β casein. Amino acids residues are shown in red color and polyphenol in green color [17].

Bourassa *et al.* argued the model attachment of geinistein to individual caseins as can be seen in **Fig. 3** [17]. Genistein binds to caseins in hydrophobic and hydrophilic interaction and at different binding site to α casein and to β casein. However, the free binding energy for genistein-β casein is -9.97 kcal/mol, higher than genistein-α casein that is -9.68 kcal/mol respected of the more hydrophobic property of β casein.

Liposome, in the contrary, is derives from double-chained phospholipid that

driven into bilayers, and resulting lamellar structures form closed vesicles. Liposome has well known to be encapsulating nutrients and pharmaceuticals drugs because of its unique structure properties as it solubilized and delivered hydrophobic, hydrophilic and amphiphilic materials [18]. Amphiphilic and hydrophilic compounds are entrapped within aqueous interior of the liposome, while hydrophobic compound are attached within hydrophobic region of lipid bilayer [19]. Liposome entrapment has been shown to stabilize the encapsulated materials against a range of environmental and chemical changes, including enzymatic and chemical modification, as well as buffering against extreme pH and temperature [20]. Previous study has been verified the efficiency of encapsulation of liposome to antimicrobial [21] and antioxidant [22].

In this study we used both DPPC and DOPC as materials encapsulation on genistein. DPPC, dipalmitoyl is neutral saturated phospholipid consisting two palmitic acids, while DOPC, 1,2-dioleyl phosphatidylcholin composed from two monounsaturated fatty acids. Saiz *et al.* said that, basically, the outermost layer of lipid bilayer consist perturbed water, which has a considerably lower dielectric constant that the bulk and is less capable of forming hydrogen bonds with approaching compounds. The second layer contains water, lipid headgroups and the upper path of acyl chain, as the hydrophilic/hydrophobic interface is smeared out over a significant depth. This layer is available for non-covalent interaction with drugs. The third layer consists of conformationally ordered acyl-chain segment, which impose an anisotropic potential on hydrophobic molecules penetrating the membrane. Finally, the innermost layer consists of the acyl chain termini and is as conformationally disordered as liquid for DOPC and gel form for DPPC at room temperature [23].

We expected that by this study genistein was embedded in the protein-base, micelle-like protein system and small unilamellar vesicle liposome and was retained its primarily antioxidant capacity. In this doctoral thesis we divide the discussion to be two chapters. We start this thesis discussing about the genistein solubility in caseinate system with and without calcium ion [24]. In this part, we began our study by estimating the CAC, critical aggregation concentration of caseins with and without calcium ion. Then, the solubility of genistein in caseinate system was studied by HPLC. To get the description of size and morphology of genistein in this system, we observed zeta potential and the structure of particle were observed by AFM then were confirmed by cryo-TEM.

In the next chapter, we focused on the antioxidant capacity measurement of genistein in caseinate system and we compared it in liposomal composed by saturated DPPC and unsaturated DOPC. The method that we applied in this study is TEAC (trolox equivalent antioxidant capacity) with ABTS* cation (ABTS:2,2`-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) as radical scavenger [25]. We compared antioxidant capacity of genistein in its minimum soluble solvent, phosphate buffer, and in ethanol. The estimating of TEAC valued was obtained by comparing the IC50 (concentration that provide 50% inhibition of the antioxidant) at 6 minutes after mixture to standard trolox. All of the discussions in this study were summarized in the last chapter in this doctoral thesis.

1.2 REFERENCES

[1] K. Szkudelska and L. Nogowski, "Genistein—A dietary compound inducing hormonal and metabolic changes," *Journal of Steroid Biochemistry & Molecular Biology,* vol. 105, pp. 37-45, 2007.

[2] V. Cappelletti, P. Fioravant, Miodini and G. Fronzo, "Genistein blocks breast cancer cells in the G2M phase of the cell cycle," *Journal of Cellular Biochemistry,* vol. 79, p. 594–600, 2000.

[3] C. A. Lamartiniere, M. Cotroneo, W. A. Fritz and J. Wang, "Genistein chemoprevention: timing and mechanisms of action in murine," *The Journal Nutrition,* vol. 132, no. 3, pp. 552S-558S, 2002.

[4] V. Kumar, A. Rani, A.K.Dixi, D.Bhatnagar and G. Chauhan, "Relative changes in tocopherols, isoflavones, total phenolic content and antioxidative activity in soybeen seeds at different reproductive stages," *Journal of Agricultural and Food Chemistry,* vol. 57, no. 7, pp. 2705-2710, 2009.

[5] A. Arora, S. Valcic, S. Cornejo and M. G. Nair, "Reactions of genistein with alkylperoxyl radicals," *Chem. Res. Toxicol.,* vol. 13, no. 7, pp. 638-645, 2000.

[6] R.M.han, Y.X.Tian, Y.Liu, C. Chen, X.Ch.Ai, J.Zhang and L.H.Skipsted, "Comparison of flavonoids and isoflavonoids as antioxidants," *J.Agric. Food Chem.,* vol. 57, no. 9, pp. 3780-3785, 2009.

[7] A. Rusin, Z. Krawczyk, G. Grynkiewicz and G. A, "Synthetic derivatives of genistein, their properties and possible," *Acta Biochimica Polinica,* vol. 57, no. 1, pp. 23-34, 2010.

[8] V. Crupi, R. Ficarra, M.Guardo, D. Majolino, R.Stancanelli and V.Venuti, "UV–vis and FTIR–ATR spectroscopic techniques to study the inclusion complexes of genistein with β-cyclodextrins," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 44, pp. 110-117, 2007.

[9] L. Chen and G. Remondetto, "Food protein-based materials as nutraceutical delivery systems.," *Trends Food Sci. Technol,* vol. 17, pp. 272-283, 2006.

[10] H. Swaisgood, "Chemistry of the caseins," in *Advanced dairy chemistry-1 proteins part A. P.F. Fox, P.L.H. McSweeney (Eds.).,* New York, Kluwer Academic/Plenum Publishers, 2003, p. 139–202.

[11] A. Gaspar, M.S.Appavon, S.Busc, T.Unruh and W.Doster, "Dynamics of well-folded and natively disordered proteins in solution: A time-of-flight neutron scattering study," *Eur. Biophys Journal,* vol. 37, pp. 573-582, 2008.

[12] P. Fox and A. Brodkorb, "The casein micelle:historical aspects, current concepts and significance," *International Dairy Journal,* vol. 18, pp. 677-684, 2008.

[13] T. Kumosinski, G.King and H. F. Jr., "An energy minimized casein submicelle working model," *J. Protein Chem.,* vol. 13, no. 8, pp. 681-700, 1994.

[14] T. Huppertz, M.Z.Smiddy and C.deKruif, "Biocompatible micro-gel particles from cross-linked casein micelles," *Biomacromolecules,* vol. 8, no. 4, pp. 1300-1305, 2007.

[15] E. Semo, E.Kesselman, D.Danino and Y. Livney, "Casein micelle as a natural nano-capsular vehicle for nutraceuticals," *Food Hydrocolloids,* vol. 21, pp.

936-942, 2007.

[16] A. Sahu, N.Kasoju and U.Bora, "Fluorescence study of the curcumin -casein micelle complexation and its application as a drug nanocarrier to cancel cells.," *Biomacromolecules,* vol. 9, pp. 2905-2912, 2008.

[17] P. Bourassa, J.Bariyanga and H.A.Tajmir-Riahi, "Binding sites of resveratrol, genistein, and curcumin with milk α-and β-caseins.," *J. Phys. Chem. B.,* vol. 117, pp. 1287-1295, 2013.

[18] L. Jubo, H. Lee, M. Huesca and A. Young, "Liposome formulation of a novel hydrophobic aryl-imidazole compound for anti-cancer therapy," *Cancer Chemother Pharmacol,* vol. 58, p. 306–318, 2006.

[19] J. A.S, Liposomes: rational design, Marcel Dekker, 1999.

[20] T. M. Taylor, P. Davidson, B. Bruce and J.Weiss, "Liposomal nanocapsules in food science and agriculture," *Critical Reviews in Food Science and Nutrition,* vol. 45, no. 7–8, p. 587–605, 2005.

[21] L.M.Were, B. Bruce, P. Davidson and J. Weiss, "Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials," *Journal of Agricultural and Food Chemistry,* vol. 51, no. 27, p. 8073–8079., 2003.

[22] B. Maherani, E. Arab-Tehrany, A. Kheirolomoom and F. Cleymand., "Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant L-carnosine," *Food Chemistry,* vol. 134, p. 632–640, 2012.

[23] L. Saiz, S.Bandyopadhyay and M.L.Klein, "Towards an understanding of

complex biologycal membranes from atomistic molecular dynamics simulations," *Biosci.Reps.,* vol. 22, no. 2, pp. 151-173, 2001.

[24] G. Anjani, A.Ohta, K.Yazuhara and T.Asakawa, "Solubilization of genistein by caseinate micellar system," *Journal of Oleo Science,* vol. 63, no. 4, pp. 413-422, 2014.

[25] R. Re, N. Pellegrinia, A. Proteggente, A. Pannala, M.Yang and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine,* vol. 26, no. 9–10, p. 1231–1237, 1999.

Chapter 2

SOLUBILITY OF GENISTEIN IN CASEINATE SYSTEM

2.1 INTRODUCTION

In the current study, caseinate was investigated as a model casein solution for encapsulating gensitein. Caseinate comprises natural mixtures of milk caseins with their inorganic calcium and phosphate removed. According to the "sub-micelle" hypothesis [1], four casein proteins form small aggregates of tens of molecules, weighing a few hundred thousand Daltons. These assemblages are called the "core polymers" of casein micelles. They contain a mixture of α_s and β caseins polymerized via hydrophobic interaction. Further aggregation, aided by calcium phosphate, constructs the super polymers known as casein micelles. Interactions among core polymers are surface-limited by κ-casein binding to the four aggregate surfaces. Alternative mechanisms of micelle formation include calcium phosphate nano-clustering proposed by Holt [2] and the dual binding model of Horne [3].

As purposed by Kumosinski *et al*. sodium caseinate system that composed of one κ-casein, four α_{s1}-casein and four β-casein molecules attached each other in the following interaction [4]. Primary, two hydrophobically dimers of α_{s1}-casein

interact with β-sheet `legs` of κ-casein, thus preventing amyloid formation (a 4:1 ratio); secondly, two dimers of β-casein, held together by their C-terminal peptides, interact at later time with κ- and α_{s1}- complex [4]. However sodium caseinate might contain particles of different composition based upon mixed association of the casein in sedimentation velocity studies at elevated ionic strength [1].

Despite numerous studies and debate on the interior structures of caseins and their assembling process, native caseins and caseinate remain incompletely understood. Acharya *et al*. [5] suggested that resveratrol (a polyphenol) strongly binds to sodium caseinate through hydrogen bonding and hydrophobic interactions. Furthermore, Bourassa *et al*. proposed that genistein bound the individual casein as α- and β-casein in hydrophilic and hydrophobic interaction. This indicates a potential role for sodium caseinate as a carrier of oil and water insoluble nutraceuticals [6].

In this experiment, genistein was applied as a model casein nano-encapsulation agent with and without calcium ions, to enhance its solubility in a buffer system. At certain concentrations, calcium ions are believed to influence the solubility of caseinate. Adding small quantities of ionic calcium to sodium caseinate may increases the aggregation of a particular protein composition that can remain in colloidal suspension [7].

2.2 EXPERIMENTAL

2.2.1 Materials

The materials that used in this study can be described as followed. Sodium caseinate and genistein were bought from Sigma-Aldrich (St. Louis, US) and from the Tokyo Chemical Industry (Tokyo, Japan), respectively. The probe that used for binding to the hydrophobic site of casein 8-anilino-1-naphthalene sulfonic acid (ANS) was purchased from MP Biomedicals LLS, (CA, US). The caseinate solution was made by dissolving sodium caseinate powder in 1 mM phosphate buffer (pH 7.4) prepared by diluting phosphate buffer stock (0.1 M, pH 7.4, 20 ℃) purchased from Nacalai Tesque (Kyoto, Japan). Furthermore, in the study of HPLC, we used HPLC-grade methanol and acetic acid solvent that obtained from Kanto Chemicals (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. The caseins of molecular weight 100 kD (1 Da = 1.66 10^{-27} kg) was used.

2.2.2 Sample preparation

The measuring of solubility of genistein in caseinate was accomplished by dissolving sodium caseinate in 1 mM phosphate buffer (pH 7.4) solution. The genistein-casein complex was prepared by mixing 0.5 mM genistein with various concentrations of caseinate in the buffer solution. This mixture was stirred in a water bath at 25 ℃) until equilibrium reached (approximately 48 h). In addition, to investigate the influence of calcium in the complex system, genistein and 0.6 mM of calcium chloride were added to the caseinate solution and stirred in water bath with the same condition as it without calcium until reached the equilibrium

state and filtrated before next assessments.

2.2.3 Hydrophobicity test (ANS fluorescence)

The hydrophobicity of caseinate with and without calcium ion was measured by attaching this chemical to ANS probe with following steps. A 1 mM ANS stock solution was prepared by dissolving ANS in ethanol. Furthermore, a 1 ml of ANS solution was evaporated and then dissolved in the phosphate buffer solution to obtain a final concentration of 10 µM. The caseinate (0-5 µM) were dissolved in 1 mM phosphate buffer at pH 7.4 prior to luminescence spectrometry. Then, those mixed to 10 µM of ANS, and incubated for 60 min in the dark at room temperature (25°C). Fluorescence emission from ANS was measured using 10/10 nm bandwidths in excitation/emission channels. Following excitation at 350 nm, the emission was measured from 345 nm to 620 nm.

2.2.4 Genistein solubility

The core of this study, that are solubility of genistein in caseinate and caseinate-calcium system was determined by HPLC (Hitachi L-6320; Japan) with a UV detector operating at 262 nm. This assessment was confirmed by fluorescence spectrophotometry (Hitachi F-1050; Japan) at excitation and emission wavelengths of 262 nm and 320 nm, respectively. The sample was extracted by a 100 µL syringe (SGE Analytical Science; Melbourne, Australia) and was applied to the HPLC column (Nucleosil 100-5 C18, 4.6 x 250 nm, GL Science Inc. Japan). Mobile phase was programmed during 17 min. At the first 3 min we used 75 % acetic acid : 25%, followed by 100% methanol during 3 min until 11 min run and 75% acetic acid in water : 25% methanol from 11 min until

17 min run, with the flow rate 1 ml/min.

Genistein and calcium ions were mixed with the caseinate solution of varying concentration in phosphate buffer (1 mM, pH 7.4), stirred in the water bath at 25°C until equilibrium was reached at 48 h, then applied to the HPLC column. Two sizes of syringe filter were used. The 1 μm filter was used for lower caseinate concentrations (0-2 μM), while the 0.45 μm filter was used for higher caseinate concentrations (2-20 μM). The genistein concentration in the system was estimated from a genistein standard curve Y=41214 x, R^2= 0.9995.

2.2.5 Zeta potential, size and morphology

Before measurement, the samples were filtered through 1.0 μm syringe filter. The average size and zeta potential of caseinate sub-micelles were measured by Nano Particle Analyzer SZ-100 (Horiba Scientific; Japan) with 100 μL disposable-cell featured by carbon-coated electrodes. The scattering angle applied was 173°.

2.2.5.1 AFM (Atomic Force Microscopy)

Atomic force microscopy studies were carried out on 2 μM of caseinate solution in phosphate buffer with and without genistein (0.5 mM). The caseins were immobilized on a mica surface. Caseinate imaging was performed by an SII SPA 400 microscope (Japan) operating in DFM mode at room temperature. Images in dry air were obtained from an Al coated probe (SI-DF20, SII; Japan). The probe had a nominal spring constant of 14 Nm-1, and a nominal tip radius of a curvature of 10 nm under an applied force of 0.5-2 nN. The face figure, height, and structure figure of the caseinate sub-micelles were measured by particle analysis implemented in the AFM software.

2.2.5.2 TEM (Transmission Electron Microscopy)

The morphologies of caseinate and caseinate mixed to genistein were determined by cryo-TEM (JEM-3100FEF, JEOL Ltd.; Japan) under an accelerating voltage of 300 kV. The 10 μM caseinate and 0.5 mM genistein were mixed in 1 mM phosphate buffer (pH 7.4) and stirred for 48 h at 25 ℃ until equilibrium was reached, then was filtrated before examination.

2.3 RESULTS AND DISCUSSION

2.3.1 Hydrophobicity of caseinate

In this study we used ANS as a probe in assessing caseinate hydrophobicity. It is generally accepted that pre-existing hydrophobic surface (non-polar) of proteins can be attached by ANS anion primarily via aniline-naphthalene group. As a consequence, the ANS fluorescence intensity increases with the existing protein in the system. Furthermore, the binding of ANS probe to caseins result in blue shift in the ANS fluorescence spectrum, which can be seen clearly in **Fig.1, Peak A.** In this picture, the ANS alone at excitation wavelength of 350 nm shows fluorescence emission spectrum at 500 nm. In comparison, 5 μM caseinate mixing to ANS induces fluorescence spectrum shifting to 487 nm. Semisotnov *et al*. mentioned that the strong affinity of ANS to proteins in the "molten globule" state is because of the lack of tertiary structure [8]. And because caseins is a kind of protein that lacks of a well-defined tertiary structure, therefore the spectrum of ANS should be blue shifted following to its binding [9]. Together with **Peak A** in **Fig.1**, we can see another peak at lower wavelength, 433 nm, and indicate it as **Peak B.** This peak looks like a shoulder in the lower caseinate concentrations. Because of this shoulder cannot be observed in the spectra of κ-casein, [10] [11] α_s-, and β-casein [11]. Thus, we suggest that Peak B belong to caseinate or to ANS-caseinate complex spectrum.

Both of these peaks, Peak A that belong to ANS and Peak B has the same tendency to increase as casein is added. We assume that more hydrophobic regions were bound to ANS in the presence of additional caseinate. In addition, the more caseinate is added, the more ANS-caseinate spectrum is blue shift,

indicating the more hydrophobic patches of caseinate are binding to ANS. However, the shoulder emission band (Peak B) is unaffected (remaining in the 445-450 nm wavelength regions regardless of casein concentration).

Alaimo *et al.* mentioned that hydrophobic interactions are important in the self-association of milk proteins. Caseins have a high content of hydrophobic amino acids. The high hydrophobicity and proline contents of caseins are considered to prevent the formation of globular structures in which the nonpolar groups are completely buried in the hydrophobic interior of the protein. Thus, a proportion of the hydrophobic amino acids in caseins are found on the outer molecular surface of the casein monomers [12]. Moreover, caseins are unique protein refers to unfolded structure under native conditions, brought about their high net charge and low intrinsic hydrophobicity [13]. These hydrophobic surfaces are essential in casein–casein interactions and may account for the self-associative properties that enable colloid formation [12].

The hydrophobicity assessment of caseinate brings to the determination of CAC (critical aggregation concentration) in this system. **Figure 2** presents the plotting of caseinate against intensity of ANS fluorescence at 500 nm. As the caseinate concentration increases, two trends emerge in the graph. The changing of gradient in this graph occurs at 0.68 μM of caseinate as the caseins begin to aggregate (CAC is reached). In this point, the sharper gradient change to be more plateau that can be explain as follow. At higher caseinate concentrations, the hydrophobic surfaces of caseins attach to each other, forming aggregates. Consequently, fewer hydrophobic sites are exposed to ANS compare to free caseins in lower concentrations.

In comparison, the critical micellar concentration (CMC) of β-casein is 0.5–2

mg/mL (0.021–0.083 mM), at pH approximately 7 and 25 °C [14]. The CMC of α-casein, measured by surface tension analysis, is approximately 0.14 mg/mL [15].

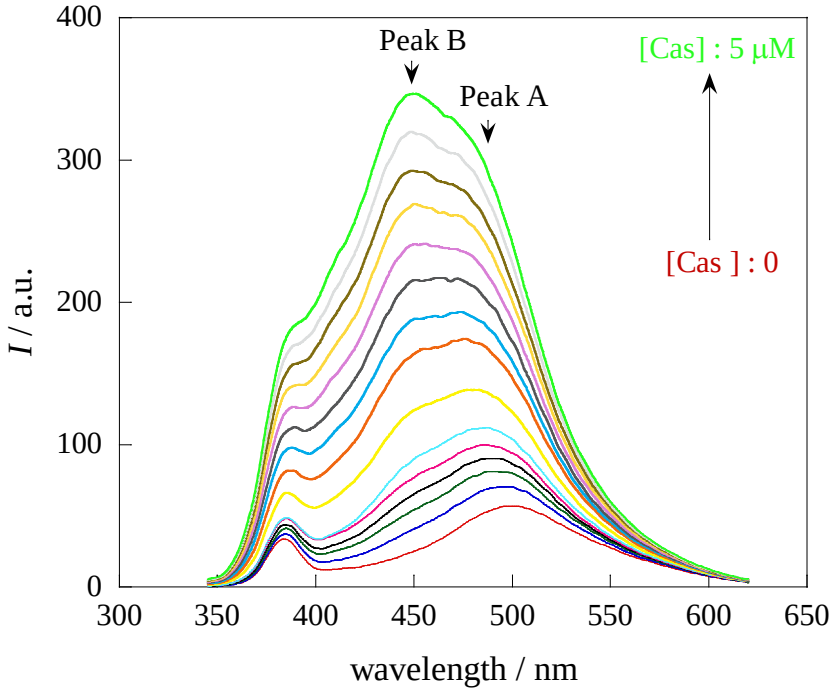


Fig. 1 Fluorescence spectra of ANS at various concentration of caseinate at 25°C in 1 mM of phosphate buffer at pH=7.4. Peak A belong to ANS peak at around 500 nm, without caseinate. Peak B belong to ANS with caseinate micelle at around 445 nm.

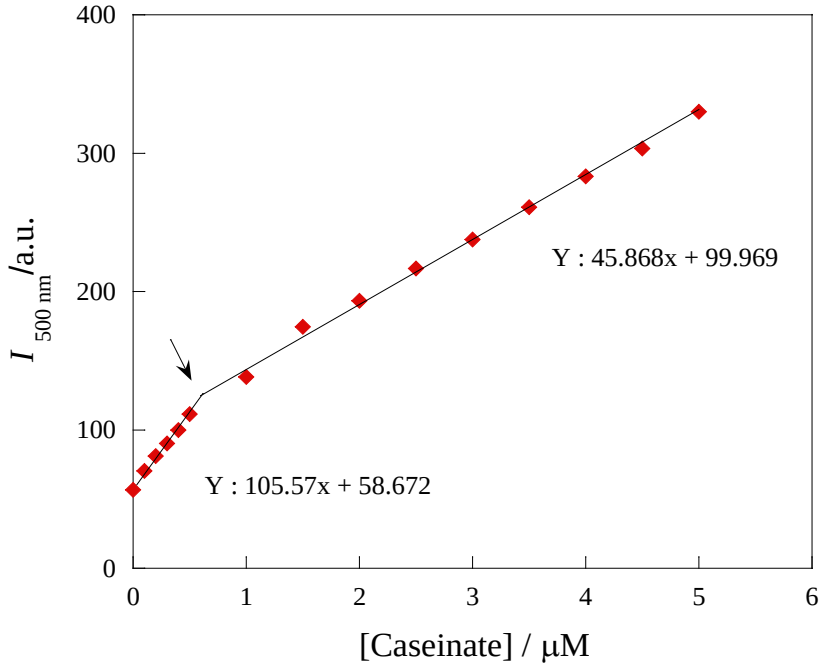


Fig. 2 Fluorescence intensity of ANS at 500 nm in different concentration of caseinate systems.

2.3.2 Solubility of genistein in caseinate

The solubility of genistein in caseinate system was examined by HPLC. Prior to this assesment, we determined the maximum-intensity monitoring wavelength of genistein by spectrometer and got the result as 262 nm. In order to determine the genistein solubility, a fixed amount of genistein was dissolved in phosphate buffer containing various concentrations of caseinate, and then mixed it until equilibrium. The mixture was filtered to remove insoluble materials. The genistein–caseinate complex filtrates were directly injected into HPLC and read as a chromatogram.

We decided the equilibrium phase by dissolving the different concentration of genistein in fixed caseinate, then mixed it and checked the genistein amount by spectrometer every 24 hours from 0-72 hours. The equilibrium state was

completed when the genistein amount reached steady state, which is 48 hours.

The content of genistein dissolved in caseins was evaluated by measuring the area under the maximum emission peak. All chromatograms showed a single clear peak at approximately 10 min retention time. The concentration of soluble genisten was estimated from the standard curve of genistein in methanol that is: Y: 41214 X; Y is area under peak and X is genistein /μM. The number of genistein molecules in caseinate solution was calculated by comparing genistein concentration with caseinate concentration in the system. The MW of casein sub-micelle was assumed as 100 kDa.

In this study, we found that low concentrations of genistein (below 25 μM) are soluble in phosphate buffer. This is probably because the 3- hydroxyl group of genistein enables to bind to a water-based system. However, the solubility of genistein in the evaluated system is increases with increasing caseinate concentration (**Fig 3**). Again, two slopes emerge; the gradient change occurs at approximately 0.83 μM, higher than that of **Fig. 2** (0.68 μM). This result indicates that adding genistein to caseinate slightly increases the CAC of the system.

The aggregation behavior of among caseins can be explained as follows. Caseins have unique open structure and hydrated despite of their high hydrophobic amino acid content. This protein also has regions of high net protein. The combination of electrostatic and hydrophobic properties forms large colloidal aggregates. However, caseins are low internal hydrophobic proteins. Thus, the number of hydrophobic amino acids residing on the outer molecular surfaces of casein monomers is largely responsible for the interaction and self-association properties of caseins [12].

At low concentrations, casein monomers (α β, and κ) self-attach and form small

aggregates, known as sub-micelles. In this study, the term sub-micelle defines as casein monomers aggregating in response to caseinate dilution in the buffer system. The sub-micelles then form sub-micelle aggregates.

In order to calculate the number of genistein molecules bound to caseins, total concentration genistein that dissolved in caseinate (μM) is compared to caseinate concentration (μM) in the system. We found that below CAC, in average 10 molecules of genistein attach to 1 molecule of caseinate. As the caseinate concentration increases, fewer genistein molecules attach to a given casein molecule.

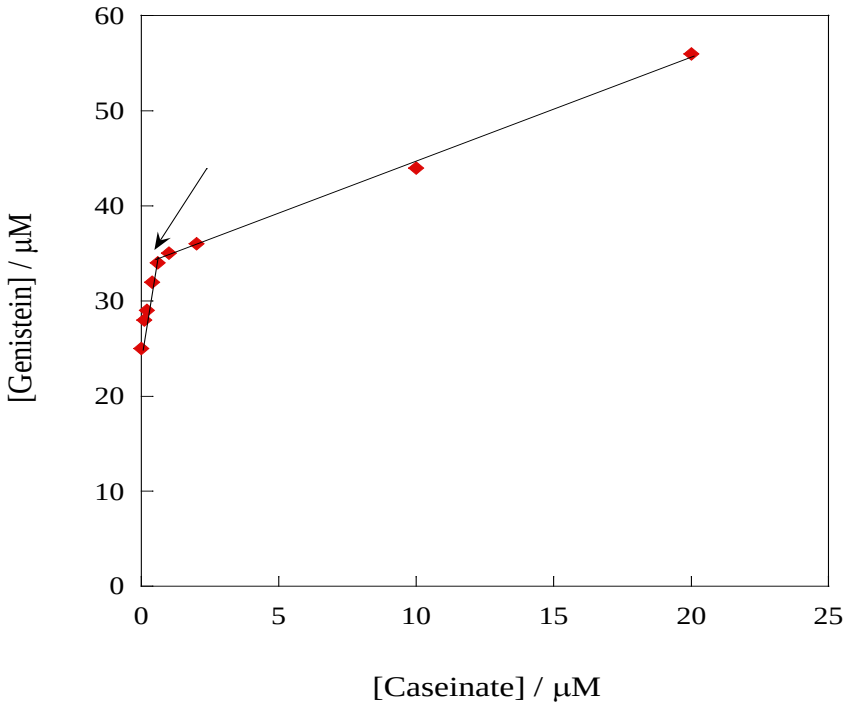


Fig. 3 Solubility of genistein against caseinate concentration. Slope of the graph changes at 0.83 μM. The slopes below and above CAC are 9.3 and 1.1 respectively.

At the caseinate concentrations exceeding CAC, only one molecule of genistein attach to single sub-micelle aggregate **(Fig.3)**. This behavior can be explained that the available hydrophobic site of caseins reduced respected to the

casein casein binding to form bigger aggregate. Therefore the attachment site of caseins to that of genistein reduced. Structural analysis showed that genistein binds to casein via hydrophobic and hydrophilic interactions. The number of genistein molecules (*n*) bound to α-casein and β-casein is 1.42 and 1.27, respectively. Polyphenol binding disrupts the α-helical structure of casein, indicating partial protein destabilization [6].

2.3.3 Solubility of genistein in caseinate-calcium ion system

Some studies have demonstrated that calcium ions supplied at certain concentrations can assist casein molecules to aggregate and remain in the colloidal form without precipitating. The addition of sodium caseinate and low ionic calcium (insufficient to form calcium caseinate precipitates) encourages the aggregates of a particular protein composition that can remain in colloidal suspension [16]. In the present study, caseinate-calcium ion aggregates are expected to increase the binding capacity of genistein to casein.

2.3.3.1 Hydrophobicity of caseinate-calcium ion

Fluorescence spectrometry with the ANS probe was used to estimate the hydrophobicity of caseinate mixed to calcium ion at 0.01 mM, 0.05 mM, 0.1 mM and 0.6 mM. To establish an equilibrium state of caseinate-calcium ions, the calcium ions were mixed with caseinate in the buffer system for 48 h in a water bath at 25 °C, followed by incubation with 10 μM ANS for 60 min.

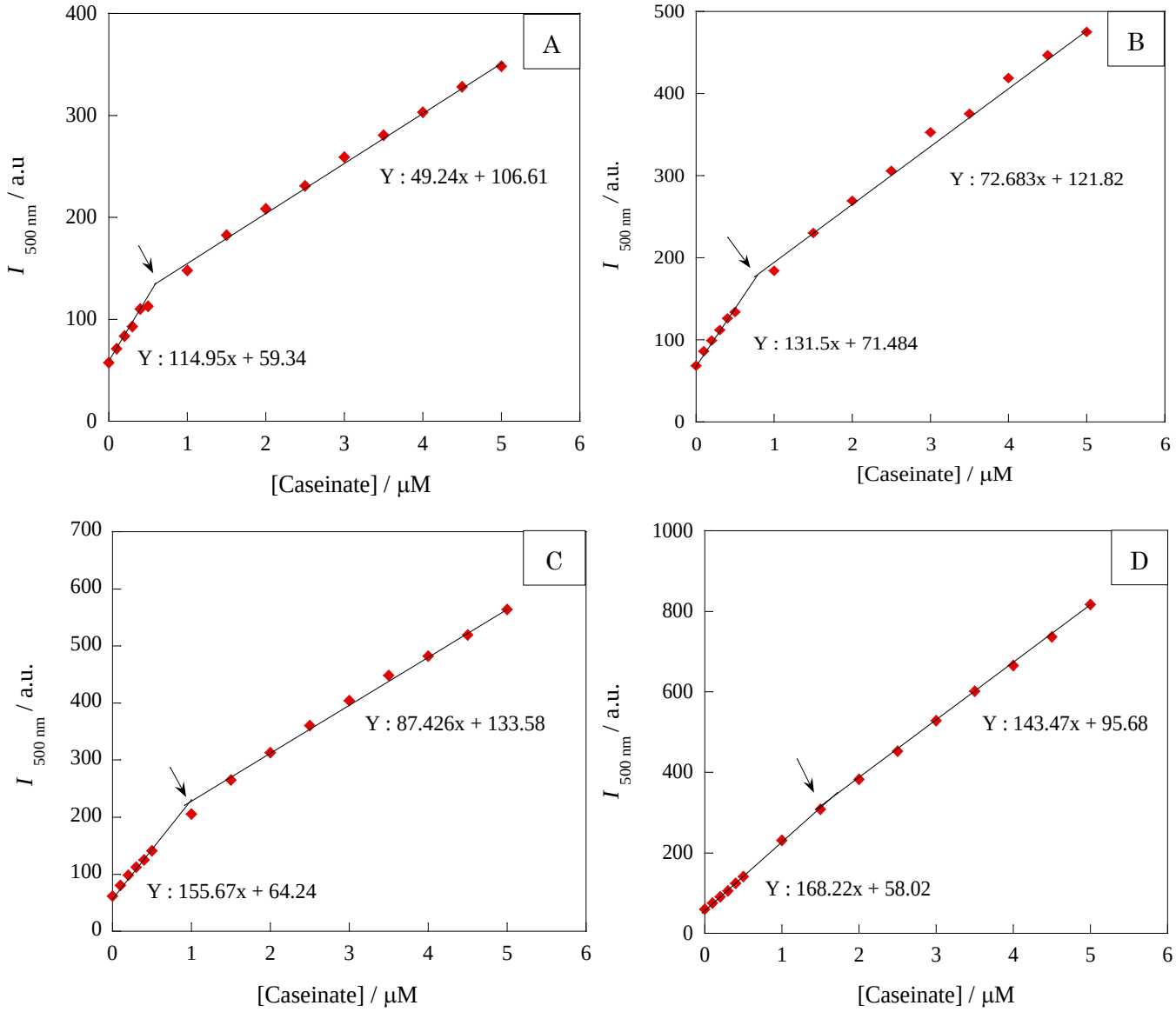


Fig. 4 Fluorescence intensity of ANS vs. caseinate mixed to various concentration of calcium ion. Obtained CAC values are 0.72 μM, 0.86 μM, 1.02 μM, and 1.52 μM at 0.01 mM (A), 0.05 mM (B), 0.1 mM (C) and 0.6 mM (D) of calcium ion, respectively.

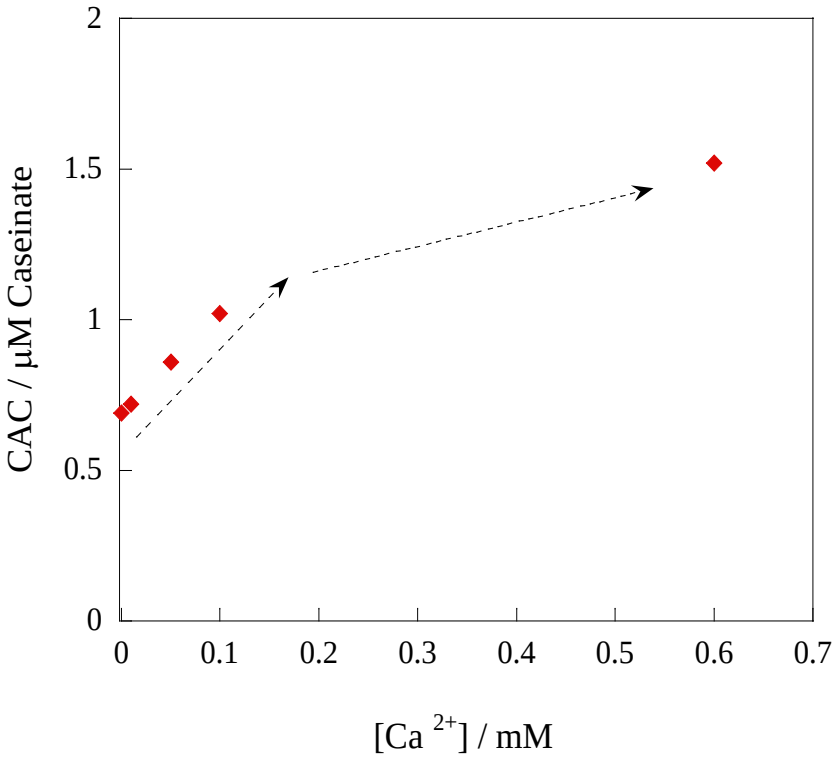


Fig. 5 Variation of CAC that are influenced by calcium ion addition in caseinate system.

Figure 4, describes the fluorescence intensity at 500 nm against caseinate mixed to different calcium ion concentrations. These graphs show that fluorescence intensity increases as more calcium ions are added to the caseinate system. A sharply increasing of intensity is shown at the highest presence of calcium ions, 0.6 mM in caseinate system. This intensity results were obtained by use of the cutoff filter (390 nm), beyond which the emission spectrum becomes very intense. As the consequences of ANS-caseinate-calcium ion binding, calcium ion also induce a blue shift of the emission peak spectrum of fluorescence. As Philippe *et al*. [17] reported a protein hydrophobicity increase of 6.7% following the addition of 13.5 mmol kg^{-1} of calcium chloride to milk.

Calcium ions might activate the binding site of caseins and ANS in the system.

ANS can interact with other molecules in two ways: (1) by hydrophobic binding via its naphthalene backbone or aniline ring or (2) through the negative charge on its sulfonate group [18]. In casein molecules, the phosphoserine residue and carboxylate groups bind calcium with strong and weak affinity, respectively [19]. Once calcium binds to caseins, fluorescence intensity might be enhanced by one of two binding possibilities to ANS. More specifically, calcium ions can bind to ANS anions, or ANS can hydrophobically bind to caseins. Calcium ions neutralize the casein anions and decrease the electrostatic repulsions. Consequently, the hydrophobic patches exposed to ANS in the system are increased, as reflected in the range of fluorescence intensity.

2.3.3.2 Critical aggregation concentration of caseinate-calcium system

The CAC estimation in the caseinate-calcium system can be acquired by plotting the emission peak intensity of ANS against a fixed calcium ion in caseinate system. The CAC at four calcium ion concentrations, namely, 0.01, 0.5, 0.1, and 0.6 mM, were 0.72, 0.86, 1.02, and 1.52 μM, respectively (**Fig. 4**). As we mentioned before, the CAC of the caseinate system only without calcium ions is 0.68 μM. It is widely accepted that CAC decreases as hydrophobicity increases. However, this study identifies an increasing tendency of CAC with increasing calcium ion concentration (**Fig. 5**). We suggest that different binding mechanisms control in the presence and absence of calcium ions.

Casein molecules have tendency to bind each other and aggregates via varying interaction sites on caseins. Caseins contain phosphoserine cluster and hydrophobic sites through which protein–protein interactions can occur. The ANS assay suggests that calcium ions bind to casein molecules before they aggregate, as evidenced by the increasing hydrophobicity in the presence of calcium ions

below the CAC **(Fig 4)**. The existence of calcium in the system would probably change the local environment and influence the binding capacity of caseins. If only the hydrophobic binding dominates and encourages aggregation among the caseinate molecules, the CAC should decrease by presence of calcium ions. However obtained result in this study was reversed contrary to expectation.

In the absence of calcium ions, the phosphoserine sites on the caseins easily interact with the basic amino acid side-chains on the other caseins. Calcium ions on the surface of casein molecules may reduce the number of these available binding sites for casein–casein attachment and aggregation. Consequently, the CAC trends are inconsistent with the ANS hydrophobicity results. We would like to suggest that aggregation is driven by electrostatic interactions as well as by hydrophobic binding.

2.3.3.3 Zeta potential of caseinate-calcium system

In this experiment, calcium ions of varying concentration (0–30 mM) were added to caseinate at fixed concentration (20 μM). **Figure 6** clearly displays the increasing zeta potential as the calcium ion concentration increases in the caseinate system. In the absence of calcium ions, the zeta potential is approximately −54 mV. Its gradual increase reflects the neutralization of electronegative charges and/or redistribution of charged amino acid chains, possibly the redistribution of phosphorylated amino acids at the casein surface, with possible changes in the overall thickness of the steric layer [20]. However, as shown in **Fig. 6**, the zeta potential remains negative (approximately −10 mV) until 5 mM calcium has been introduced to the caseinate, implying that in this system, the formed colloids are highly stable. This results support the previous suggestion that calcium ions on the surface of casein molecules reduce some of available

binding sites on the caseinate.

2.3.3.4 Solubility of genistein in the caseinate-calcium system

The solubility of genistein in caseinate-calcium in the phosphate buffer system was measured by HPLC. Samples for this system were prepared in the same way to the study of genistein solubility in caseinate solution. Calcium ions of varying concentration were directly dissolved in 20 μM caseinate, then mixed with 0.5 mM genistein in the water bath for 48 h. Following incubation, the mixture was centrifuged to remove undissolved materials and filtered before application to HPLC. Low concentrations of calcium ions in caseinate solution were expected to encourage large aggregates and increase the binding capacity of genistein to casein. However, as shown in **Fig. 7**, genistein solubility in caseinate system independent of calcium ion concentration. Although hydrophobicity increases as calcium ions are added, the attachment of casein molecules to genistein is unaffected.

As mentioned above, the driving force of casein aggregation is altered by calcium ions. Instead of hydrophobic binding, calcium ions on the casein surfaces induce electrostatic aggregations. This phenomenon explains the less hydrophobic caseinate surface, on where genistein molecule can be attached each other. Under these conditions, hydrophobic chemicals such as genistein are excluded from the calcium ion–casein aggregates. On the other hand, the addition of calcium ion increases the CAC, and then increases the number of caseinate monomer. The increase of caseinate monomer produces a gain of solubility of genistein. Therefore, genistein solubility in caseinate system might be independent of calcium concentration because the both effects may compensate.

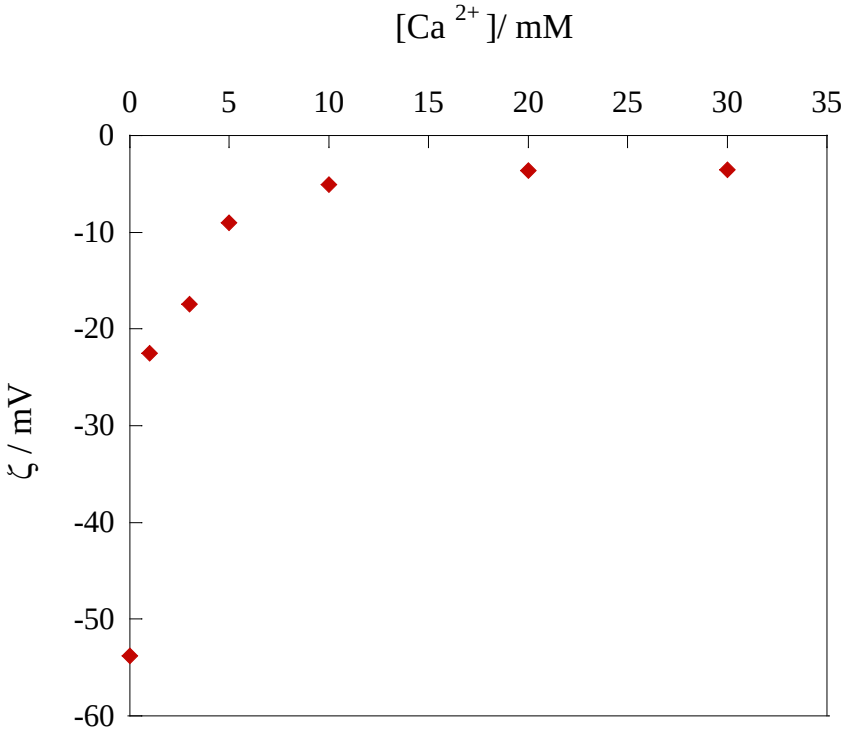


Fig. 6 Zeta potential of 20 μM caseinate vs. calcium ion concentration plots.

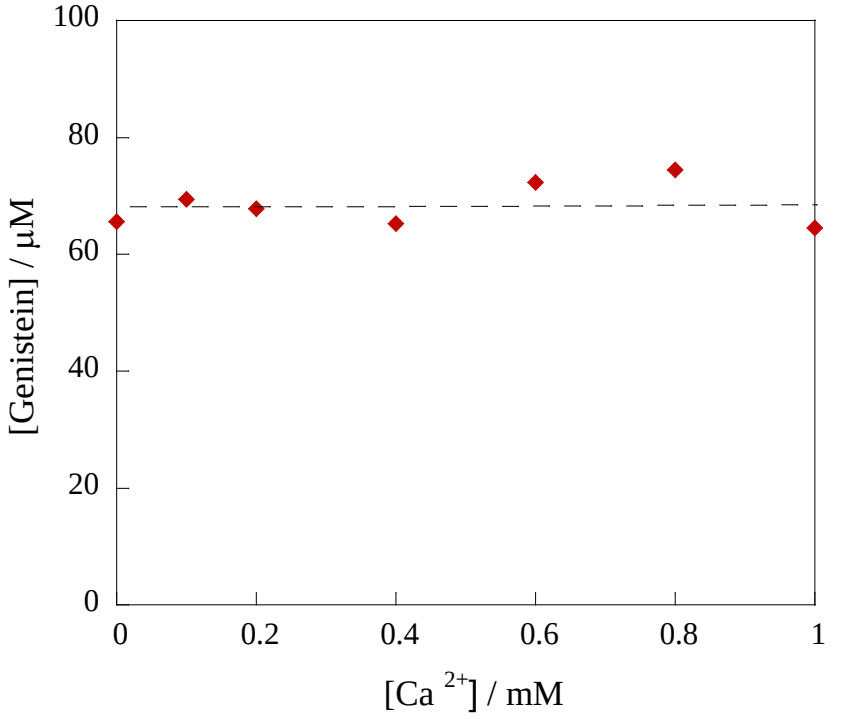


Fig. 7 Effect of calcium ion concentration on the solubility of genistein in 20 μM of caseinate system.

2.3.4 Size and Morphology

The morphology of caseinate sub-micelles was investigated by AFM. Samples of 2 μM caseinate and/or 0.5 mM genistein in caseinate solution were prepared under dry conditions on a mica surface. As revealed in **Fig 8A and 8B**, all of the immobilized casein sub-micelles are wider than their height. However, the diameters of casein sub-micelles are defined by height rather than by width, because the soft micelles can deform under surface tension, and become flattened along the observation direction (width). Consequently, their apparent diameters may be larger than their true diameters.

In the presence of genistein, the sub-micelle diameters are twice those of caseinate alone (24 nm versus 12 nm; **Figs. 8C and 8D**), although the sub-micelles are of similar shape (**Figs. 8A and 8B**). In comparison, Pitkowski *et al.* [21] characterized soluble casein by light scattering, and concluded that casein forms small aggregates of approximately 15 molecules with radii of approximately 12 nm. Farrer and Lips modeled the self-assembly of sodium caseinate in the dilute, semi-dilute, and highly concentrated regimes [22]. They identified small-micellar building blocks of 4–5 molecules, aggregated into structures of approximately 11 nm in diameter.

The phase image reveals contrasting dark and bright areas over a scale of 5 μm. **Figure 8E** is a phase image of a dry caseinate sample in the absence of genistein, showing dark spots in the bright area. On the contrary, the phase image of casein with genistein (**Fig. 8F**) reveals bright spots on dark areas. The assignment of bright or dark contrast in phase images to hard or soft domains is not always straightforward [23]. However, in this study, dark and bright spots clearly indicate softer and more rigid materials, respectively. The more rigid

bright spots might signify genistein molecules attached to the surface or to the narrow part of the sub-micelle.

The caseins structure was also observed by cryo-TEM. The cryo-TEM image shows the sub-micelles of caseins (mean diameter 20 nm) uniformly distributed in the phosphate buffer solution (**Fig. 9A**). Casein attached to genistein forms larger structures than casein alone (mean diameter 30 nm; **Fig. 9B**), consistent with the AFM measurements. The sub-micelles are not appreciably larger than those of pure casein, because few genistein molecules are bound to the sub-micelles. This microscopic observation was performed on 10 μM caseinate. From the genistein solubility measurement, we know that at this concentration, each caseinate in the sub-micelle will bind a single molecule of genistein.

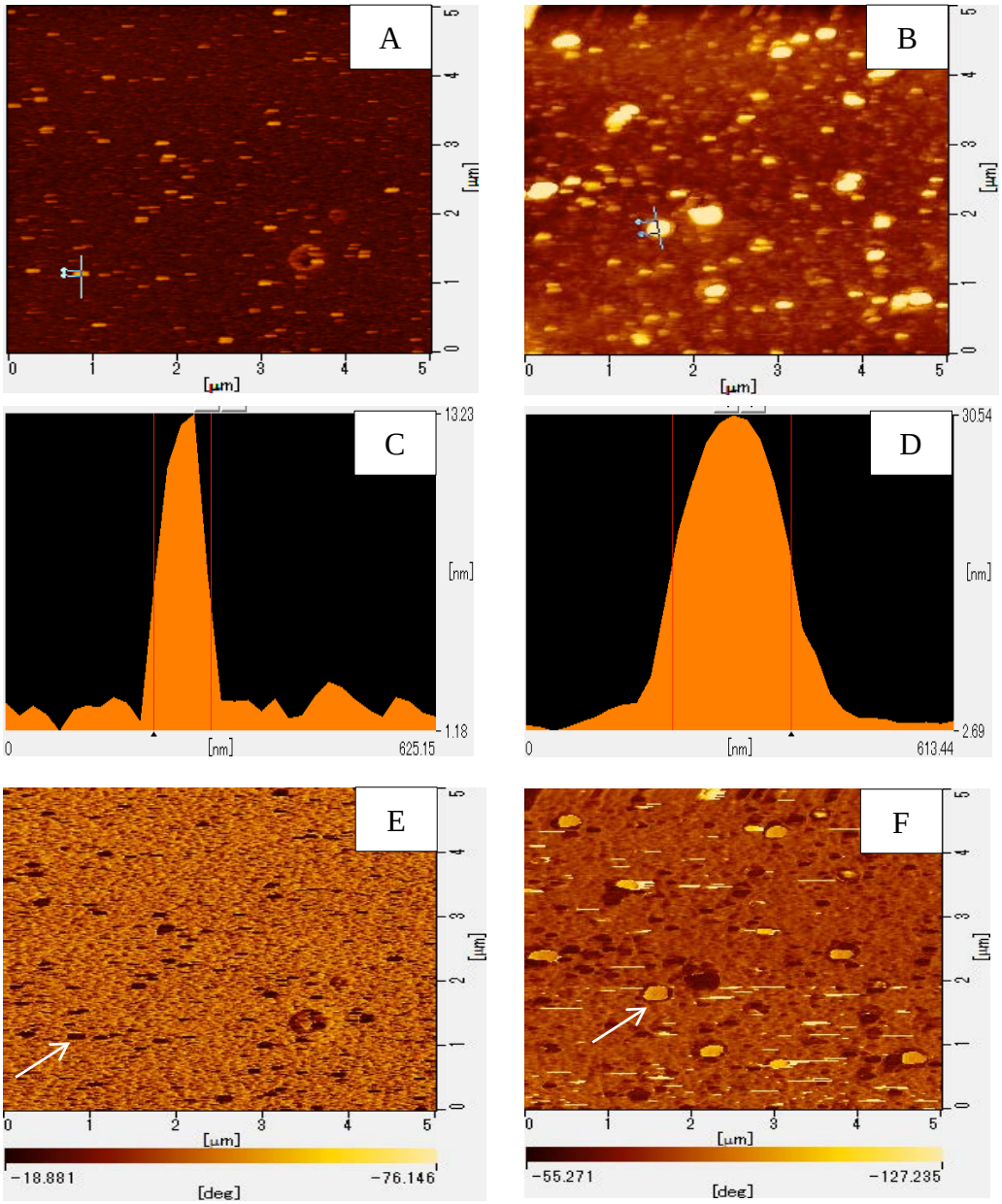


Fig. 8 Atomic force microscope images of sub-micelle of caseinate (A,C, and E) and sub-micelle caseinate with genistein (B,D and F). A and B are stucture images, C and D show sub-micelle height indecaded in the structure images. While E and F are phase images of sub-micelle. The white arrow point to the sub-micelle. Samples were 2 µM of caseinate with/without 0.5 mM of genistein in 1 mM of phosphate buffer at pH=7.4.

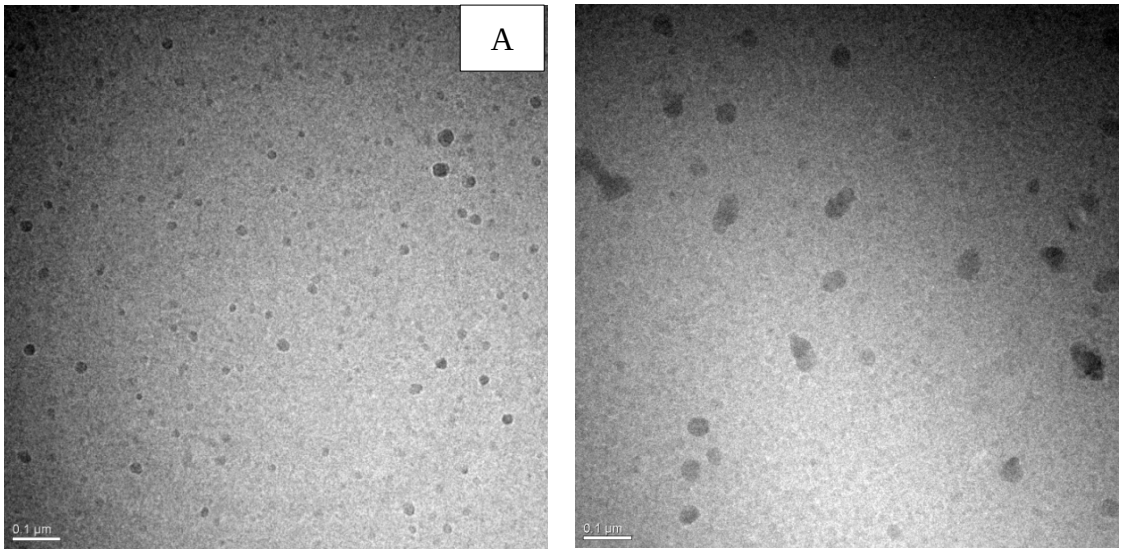


Fig. 9 The cryo-TEM images of casein sub micelle (A) and genistein-sub micelle binding (B). The sample is a 10 μM caseinate or and 0.5 mM genistein in 1 mM phosphate buffer pH 7.4. The scale bar is 100 nm.

2.4 REFERENCES

[1] C. Slattery and R. Evard, "A model for formation and structure of casein micelles for subunits of variable composition," *Biochim. Biophys. Acta.,* vol. 317, pp. 529-538, 1973.

[2] C. Holt, "Casein micelle substructure and calcium phosphate interactions studied by sephacryl column chromatography," *Journal of Dairy Science,* vol. 83, no. 11, pp. 2994-3003, 1998.

[3] D. Horne, "Casein Interactions: casting light on the black boxes, the structure in dairy products.," *International Dairy Journa,* vol. 8, pp. 171-177, 1998.

[4] T. Kumosinski, G.King and H. F. Jr., "An energy minimized casein submicelle working model," *J. Protein Chem.,* vol. 13, no. 8, pp. 681-700, 1994.

[5] D. P. Acharya, L.Sanguansri and M.A. Augustin, "Binding of resveratrol with sodium caseinate in aqueous solution.," *Food Chemistry,* vol. 141, pp. 1050-1054, 2013.

[6] P. Bourassa, J.Bariyanga and H.A.Tajmir-Riahi, "Binding sites of resveratrol, genistein, and curcumin with milk α-and β-caseins.," *J. Phys. Chem. B.,* vol. 117, pp. 1287-1295, 2013.

[7] E. Alvarez, P. H.Risso, A. G.Carlos, M. Burgos and V. S. Sala, "Calcium-induced aggregation of bovine caseins: effect of phosphate and citrate," *Colloid Polym Sci.,* vol. 285, p. 507–514, 2007.

[8] G. Semisotnov, N.A.Rodionova, O.I.Razgulyaev, V. Uversky, A.F.Gripas and

R. Gilmanshin, "Study of the “molten globule” intermediate state in protein folding by a hydrophobic fluorescent probe," *Biopolymers,* vol. 31, no. 1, pp. 119-128, 1991.

[9] Y. Stroylova, J.Zimny, R.Yousefi, J.Chobert, H.Jakubowski, V. I. Muronetz and T.Haertlé, "Aggregation and structural changes of αs1, β-and κ-caseins induced by homocysteinylation," *Biochimica et Biophysica Acta (BBA)-proteins and Proteomics,* vol. 1814, no. 10, pp. 1234-1245, 2011.

[10] P. H. Risso, D. M.Borraccetti, C. H.Araujo, E.María and C.A.Gatti, "Effect of temperature and pH on the aggregation and the surface hydrophobicity of bovine κ-casein," *Colloid and Polymer Science,* vol. 286, pp. 1369-1378, 2008.

[11] A. Chakraborty and S.Basak, "PH-induced structural transitions of caseins," *Journal of Photochemistry and Photobiology B: Biology,* vol. 87, no. 3, pp. 191-199, 2007.

[12] M. Alaimo, H. M. Jr. and M. W. Germann, "Conformational analysis of the hydrophobic peptide α-s1 casein (136–196).," *Biochimica et biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* vol. 1431, pp. 410-420, 1999.

[13] C. Phadungath, "Casein Micelle Structure:A concise review," *J.Scie.Technol.*, vol. 27, no. 1, pp. 201-212, 2005.

[14] I. Portnaya, U. Cogan, Y.D.Livney, O. Ramon, K. Shimoni, M.Rosenberg and D.Danino, "Micellization of bovine beta casein studied by isothermal titration microcalorimetry and cryogenic transmission electron microscopy," *J Agric Food Chem,* vol. 54, p. 5555–5561, 2006.

[15] M. Sakono, M. K., T. Maruyama, K. N. and M. Goto, "Alpha casein micelles show not only molecular chaperone-like aggregation inhibition properties but also protein refolding activity from the denatured state," *Biochemical and Biophysical Research Communications,* vol. 404, no. 1, pp. 494-497, 2011.

[16] E. Alvarez, P. H. Risso, A.G.Carlos, M.Burgos and V.S.Sala, "Calcium-induced aggregation of bovine caseins: effect of phosphate and citrate," *Colloid Polym Sci.,* vol. 285, p. 507–514, 2007.

[17] M. Philippe, F.Gaucheron, Y. L. Graet, F. Michel and A. Garem, "Physicochemical Characterization of calcium-supplemented skim milk," *Le lait,* vol. 83, no. 1, pp. 45-49, 2003.

[18] L. Stryer, "The Interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescence probe of non-polar binding sites.," *J Mol Biol,* vol. 13, no. 2, pp. 482-495, 1965.

[19] C. R. Nespolo, A. D.Reggiardo, M.A.M.Canales, J.R.Wagner, E. M. Alvarez, A.Brandelli and P.H.Risso, "Effect of calcium on ovine caseinate functional properties," *Journal of Chemical & Engineering Data,* vol. 55, no. 11, pp. 4624-4631, 2010.

[20] M. Philippe, Y. L. Graet and F. Gaucheron, "The effect of different cations on the physicochemical characteristics of casein micelles," *Food Chemistry,* vol. 90, pp. 673-683, 2005.

[21] A. Pitkowski, T.Nicolai and D.Durand, "Stability of caseinate solutions in the presence of calcium.," *Food Hydrocolloids,* vol. 23, no. 4, pp. 1164-1168, 2009.

[22] D. Farrer and A. Lips, "On the self-assembly of sodium caseinate.," *International Dairy Journal*, vol. 9, no. 3-6, pp. 281-286, 1999.

[23] D. Raghavan, X.Gu, T.Nguyen, M. Landingham and A. Karim, "Mapping polymer heterogenity using atomic force microscopy phase imaging and nanoscale indentation.," *Macromolecules*, vol. 33, no. 7, pp. 2573-2583, 2000.

Chapter 3

ANTIOXIDANT CAPACITY OF GENISTEIN IN CASEINATE AND LIPOSOME SYSTEMS

3.1 INTRODUCTION

As we have already mentioned before that genistein fulfill many of structural requirements considered essential for radical scavenging by flavonoids and isoflavonoids. Genistein would primarily act as scavenger peroxyl radicals, a chain-breaking antioxidant, thus suppressing radical chain autoxidation. Genistein has been proposed to react with peroxyl radicals by a single electron transfer followed by deprotonation [1]. Therefore in this study, we used single electron transfer method, TEAC (trolox equivalent antioxidant capacity) to estimate genistein antioxidant activity. ABTS* cation was used as radical that would be scavenged by genistein alone or in encapsulated system.

In chapter 2, we have already discussed about the capability of caseins in dissolving genistein. Therefore we will discuss the effect of this encapsulation to the antioxidant capacity in this mixed system. Peptides generated from the digestion of milk proteins are reported to have anti-oxidative activities [2]. Then it is expected that genistein will has synergisms effect in antioxidant capacity with

caseinate, as it dissolved in the caseinate system.

Liposomes have been well applied as encapsulation materials for drugs and nutriceuticals regarding its stability and safety in foods and drugs application [3]. The main constituents of liposome are phospholipids, which are amphiphilic molecules containing water soluble phosphate hydrophilic head section, and a lipid-soluble hydrophobic tail section.

Amphiphilic lipids are poorly soluble in water as monomers, with low critical micelle concentration (CMC) typically between 10^{-8} – 10^{-12} M, depending on the hydrocarbon chain length. The double chain lipids usually tend to form bilayer and driven to be closed vesicles, i.e. liposome that can be distinguished in multilamellar vesicles (MLV, 0.1-10 μm) and small unilamellar vesicles (SUV, <100 nm), large unilamellar vesicles (LUV,100-500 nm) or giant unilamellar vesicles (GUV, ≥1 μm). We prepared small unilamellar liposome in order to get liposome with and without genistein in small size < 100 nm by disturbing MLV and MLV-genistein complex by sonication method. Sonication produces SUV with radii around 30-60 nm [4].

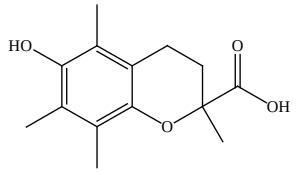
The possibly binding of genistein in liposome system has been studied before by Pawlikowska-Pawleka *et al.* who studied about localization and interaction of genistein in DPPC liposomes [5] structure DOPC and DPhyPC membrane and their elasticity in mixture with genistein and daidzein studied by Raghunatan *et al*. [6]. The binding of daidzein to liposome was studied by Lehtonen *et al*. [7]. Maniewska *et al.* studied the interaction of genistein benzyl derivatives with lipid bilayer [8]. Interaction of quercetin, genistein and its derivatives in lipid bilayers was studied by Cieslik-Boczula *et al*. [9]. However, the most study about antioxidant on membrane mostly point out of the membrane protection against

liposomal destruction. Antioxidant of genistein in liposomal system was studied by Arora *et al.* [10].

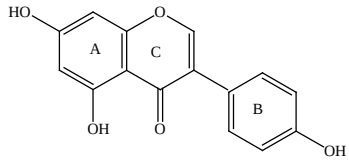
It is expected that after encapsulating process, genistein can retain and expectantly increase its primarily functional property as antioxidant in wide range medium. Hence in this chapter we will discuss our study about the influence protein base (caseinate) and lipid base (liposome) which are encapsulated materials to the antioxidant capacity of genistein compared to that of trolox. Each liposome was formed from saturated phospholipids (dipalmitoyl phosphatidylcholine/DPPC), and unsaturated phospholipids (dioleoyl phosphatidylcholine/ DOPC), respectively.

3.2 EXPERIMENTAL

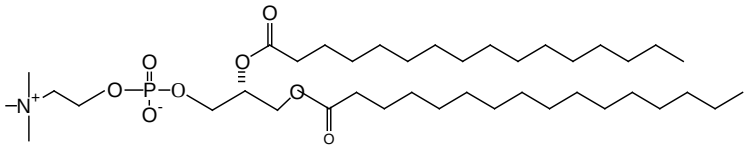
3.2.1 Materials



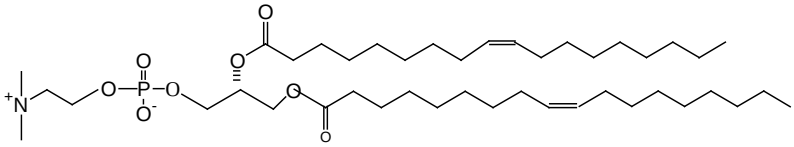
TROLOX
(6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid)



GENISTEIN (Gen, 4',5,7-trihydroxyisoflavone)



DPPC (Dipalmitoyl phosphatidylcholine)



DOPC (Dioleoyl phosphatidylcholine)

Fig.1 Chemical structures of materials

Sodium caseinate was obtained from Sigma-Aldrich (St. Louis, US). DOPC and DPPC were bought from NOF Corporation. Trolox was obtained from Sigma-Aldrich (St. Louis, US). ABTS was subscribed from Tokyo Chemical Industry (Tokyo, Japan). Potassium persulfate was purchased from Wako Pure Chemical Industry. 1 mM phosphate buffer (pH 7.4) was prepared by diluting phosphate

buffer stock (0.1 M, pH 7.4, 20 °C) in distilled water purchased from Nacalai Tesque (Kyoto, Japan). Genistein was purchased from the Tokyo Chemical Industry (Tokyo, Japan).

Tabel 1. Chemical information of caseinate (Sigma Aldrich)

Sub unit	MW/kDa	pI	Phosphates/moles	$E^{1\%}$ (280 nm)
α-$_{s1}$	22-23.7	4.2-4.7	8.0-10.0	10.0-10.1
α-$_{s2}$	25	----	10.0-13.0	---
β	24	4.6-5.1	4.0-5.0	4.5-4.7
κ	19	4.1-5.8	1	10.5

The structure of the chemicals were used in this study can be seen in **Fig. 1**. Cappelletti *et al.* mentioned about the possible site of antioxidant chemistry of genistein structure that is ring B [11]. Bors *et al.* also revealed that genistein considered fulfill the structural requirement as effective radical scavenging because it has a C-2,3 double bond in conjugation with a 4-oxo function in C-ring, which together can participate in electron delocalization from the B ring, additionally the positions of its phenolic hydroxyl groups favor a high antioxidant activity [12].

3.2.2 Sample preparation

3.2.1.1 Preparation of caseinate solution

The preparation of sample in this study is similar of it for solubility test. Sodium caseinate was dissolved in 1 mM phosphate buffer (pH 7.4). The genistein–casein complex was prepared by mixing genistein (0.1, 0.3, 0.5, and 1 mM of final concentrations) with various concentrations of caseinate (0.5 mM, 1 mM and 2 mM of final concentrations) in the buffer solution. The mixture was

stirred in a water bath at 25 °C until equilibrium was reached (approximately 48 h). Then the samples were filtrated by 1 μm filter.

3.2.1.2 Preparation of small unilamellar vesicles (SUV)

The encapsulation of genistein in liposomes system can be prepared as followed. Genistein in methanol (0.1, 0.3, 0.5, and 1 mM of final concentrations) and DPPC/DOPC in methanol (10, 14, 20, 25 and 30 mM of final concentration) were mixed in a round bottom flask. Solvent was removed in reduced pressure by rotary evaporator at 45 °C and the resulting film was kept under high vacuum condition for 1 hour. The liposome film was dissolved with 2 ml distilled water by vigorous shaking at temperature above the main phase transition of DPPC (45°C) and producing homogenous white suspension of multilamellar liposome. Transition temperature of DPPC is 42 °C, while DOPC is -15°C. The lipid suspension then was sonicated for 10 min with a 40 kHz sonicator to get small unilamellar liposome and filtrated by 0.45 μm filter to remove undissolved materials.

3.2.3 Antioxidant capacity (TEAC Assay)

The radical scavenging activity of genistein in the caseinate and liposome system (DPPC and DOPC) was evaluated according to Re *et al*. [13] in phosphate buffer. The measurement of this method is based on the ability of antioxidant to scavenge the stable ABTS radical cation in a blue/green chromophore, in comparison to that of trolox (a water soluble α-tocopherol analogue). To get ABTS* cation, ABTS in water (7 mM) was mixed with potassium persulfate (2.45 mM) **(Fig. 2)** and allowed in the dark condition at room temperature for 16 h before use. The ABTS radical cation solution was then diluted in 0.1 M phosphate

buffer solution at pH : 7.4 to get an absorbance of 0.70 (±0.02) at 732 nm and equilibrated at 30 °C. The 20 µl of trolox standard and samples were added to 1980 µl of this ABTS radical cation and read exactly after 1 min mixing for caseinate and 2 min for the liposome (DPPC and DOPC) until the next 5 minutes. The extent of decolorisation was expressed as percentage inhibition of the ABTS radical cation absorbance and plotted as a function of concentration of antioxidants.

Trolox equivalent antioxidant capacity (TEAC) of genistein in caseinate and liposome system were defined as IC50 (concentration is needed to reach the 50% inhibition against ABTS* cation) of trolox standard compared to IC50 of genistein in caseinate or liposome system. The final concentrations of trolox measured were 0-15 µM, and genistein in ethanol were 0-10 µM. Genistein in caseinate and liposome system were 1,3,5, and 10 µM, while caseinate were 0.05, 0.1 and 0.2 µM. Liposome (DPPC and DOPC) final concentrations were 0.10, 0.14, 0.20, 0.25 and 0.30 mM in ABTS* cationic solution. The antioxidant activity analysis was performed by U-2900 Hitachi Spectrophotometer.

The percent inhibition of antioxidant was calculated by equation as follow:

$$\%\ Inhibition : \frac{A-B}{A} \times 100\%$$

A : Abs ABTS* cation at 732 nm
B : Abs ABTS* cation + Antioxidant

While the trolox equivalent antioxidant capacity/TEAC can be calculated as:

$$TEAC : \frac{IC\ 50\ Trolox}{IC\ 50\ Antioxidant}$$

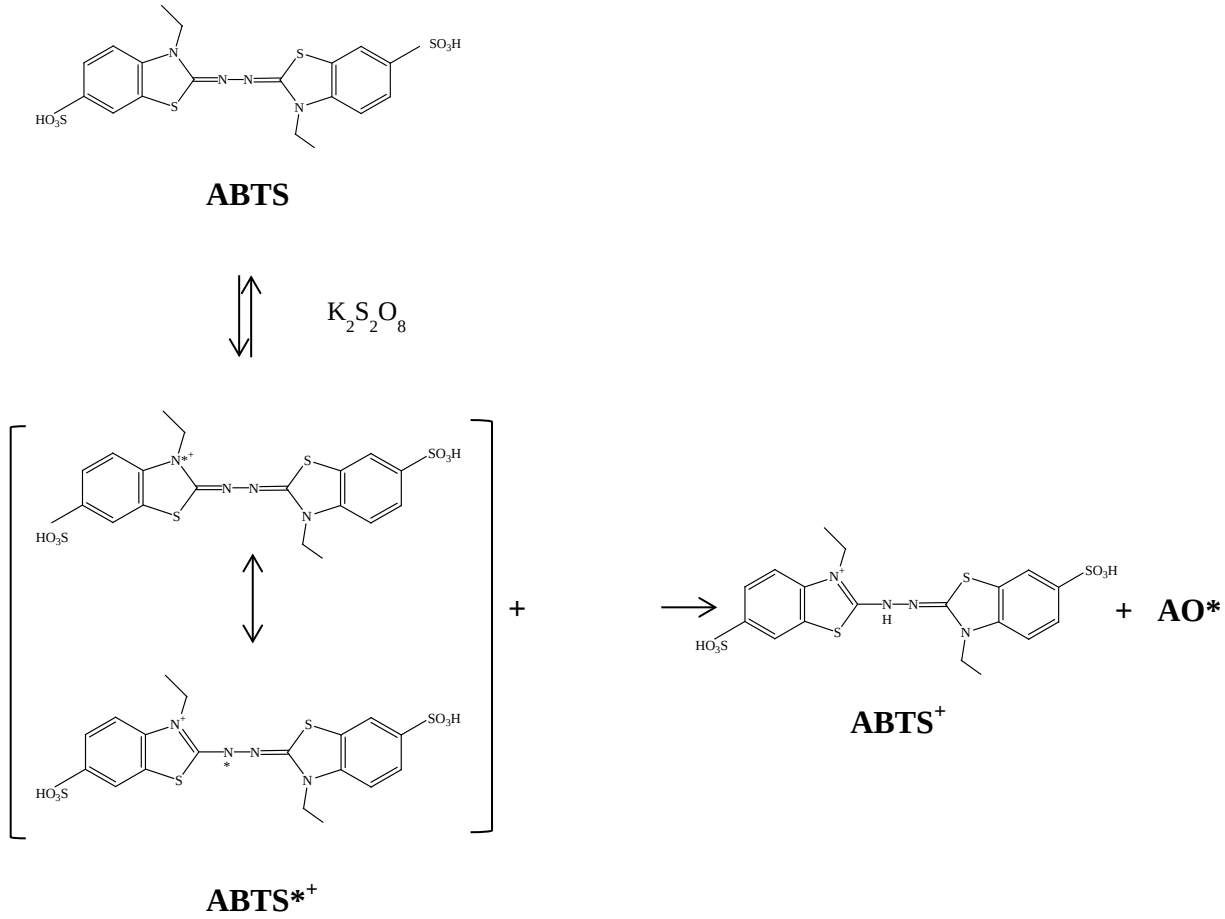


Fig.2 The oxidation of ABTS by potassium persulfate to generate ABTS* cation and its reaction with antioxidant /AOH [14]

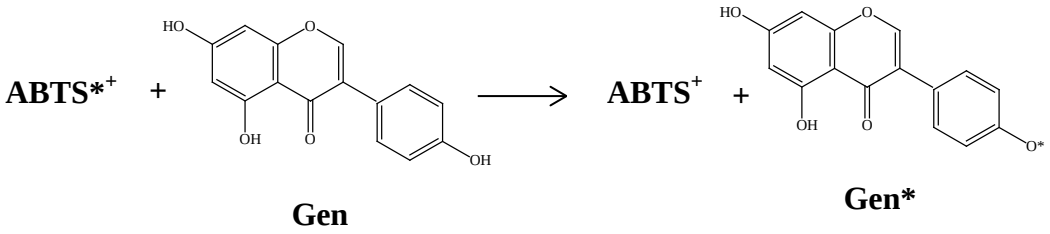


Fig. 3 The oxidation of genistein by ABTS* cation generates genistein radical. The electrons transfer from genistein to ABTS* cation maybe occurred in B ring.

3.3 RESULTS AND DISCUSSION

In this study, standard of trolox was prepared in ethanol stock solution and diluted until get the final concentrations of 0-15 μM in ABTS* cation solution. Trolox reaction against ABTS* cation in spectrophotometer was completed in 1 minute. From this assay, we get the IC50 of trolox standard as 9.8 μM.

3.3.1 CASEINATE SYSTEM

The antioxidant activity of genistein can be identified as absorbance decreasing in highest absorption spectrum, which is at 732 nm, that belong to wavelength of ABTS* absorpsion **(Fig. 4)**. This reduction was observed as the decolorisation ABTS* cation from blue/green to be more transparent color as consequence of binding of active sites in genistein molecule to the ABTS* cation. We would like to show by this figure that the more reduction color from its original ABTS* cation, describes as more decreasing peak at 732 nm. This picture also displays how genistein mixed to caseinate has lowest peak absorbance and caseinate`s peak absorbance is lower than genistein. Therefore we can expect that in this system, caseinate has its own antioxidant activity, and its activity is higher than genistein. However the quantitative calculation will be discuss more in our next discussion.

Figure 5 illustrates the duration of ABTS* cation interaction to genistein, caseinate and genistein that were dissolved in caseinate system. This result demonstrated that until 6 minute, the reaction of genistein in phosphate buffer alone almost complete as can be seen as plateau graphs compare to caseinate and the genistein mixing to caseinate.

The latest system (genistein mixed to caseinate) performs the tendency of sharp

decreasing of the graphs. That specifies how caseinate have more active sites that bind ABTS*cation compare to genistein. The amount of genistein and genistein mixed to caseinate in the system also influence the decreasing tendency of the graphs. That refers to more active binding sites of the chemicals to the ABTS* cation. In this study, we designed 6 minutes reaction as the end point to compare the antioxidant activity among genistein in caseinate and liposome. The mixture was analyzed 6 min after mixing, because this is the time usually used for assessing TEAC [15].

The inhibition concentration of 50% (IC50) of system can be simply acquired by plotting compounds concentration and % inhibition of antioxidant to ABTS* cation. The influence of genistein to the IC50 of it in caseinate system the system can be seen in **Fig. 6.** The IC50 of caseinate only is the highest of all system. By the increasing amount of genistein in caseinate system, the IC50 value is decreasing, shifting to the left. It is indicating the smaller amount of genistein in caseinate system needed to reach 50% inhibition against radical, means as stronger inhibitor. However, at 5 µM and 10 µM of genistein in the system have almost the same value of IC50, possibly because the number of genistein molecule that attached to caseinate reach saturation point. This figure also confirms the genistein capacity as radical scavenger against ABTS* cation.

In the contrary, **Fig.7** performs how caseinate influences the IC50 of genistein in the mixed system. The graph shows that genistein in phosphate buffer system has inhibition power against radical even though in a very weak value, therefore we have to make interpolation from linear equation in the graph. Our study before showed that about 25 µM genistein can be dissolved in phosphate buffer. The existence of caseinate in the system, increase dramatically the inhibition power

against ABTS* cation that shows by the shifting of the IC50 to the left side in the graph. Moreover, by the increasing caseinate concentration, the IC50 of the system decreases. Caseinate, which is an encapsulating material, has more powerful inhibition against radical scavengers in the system.

In this study, the antioxidant capacity of genistein in encapsulating system was determined in trolox equivalent. **Table 2** performs the comparison of IC50 and TEAC of various genistein in caseinate system. The higher concentration of genistein shows the more powerful antioxidant capacity of system. The increasing genistein concentration in the system increases the TEAC gradually, as can be confirmed by TEAC of genistein alone **(Table 3)**. This result also confirm that genistein alone has weak antioxidant capacity respect to its low solubility in phosphate buffer system. Moreover, the antioxidant capacities of genistein in caseinate system that are at 5 μM addition to 10 μM are only increasing slightly, as also mentioned before, toward saturated binding of genistein in caseinate.

The **Fig. 8** illustrates clearly the synergistic effect of antioxidant capacity of caseinate to genistein in the system, especially in the higher concentration. The sum of TEAC of genistein and caseinate alone are less that the TEAC of genistein in caseinate system. We suggest that caseins attach to ABTS* cation in various possibility, by electrostatic, hydrophilic and hydrophobic bindings. Caseins are unique protein as it has unfolded structure under native conditions, brought about their high net charge and low intrinsic hydrophobicity [16]. All casein proteins have different hydrophobicity and hydrophilic regions along the protein chains. α-Casein is major protein containing 8-10 seryl phosphate groups, while β-casein contains about 5 phosphoserine residues and is more hydrophobic compare to α-casein and κ-casein [16]. The caseinate that defined by Sigma Aldrich that we

used in this study have phosphates compound/moles as follows; α_{s1} casein, α_{s2} casein, β-casein and κ-casein are 8-10, 10-13, 4-5 and 1 respectively **(Tabel 1)**.

The negative charge of caseins as posphoserine plays an important role in attaching to the ABTS* cation via electrostatic binding. Caseins have polar domains that contain phosporylated serine residues and their characteristic sequences, -SerP-SerP-SerP-Glu-Glu, are effective cation chelators. Thus, phosphorylated casein and/ or their peptide in aqueous phase could be a source of natural chelators to control oxidation. However, free amino acids could not substitute for caseins as antioxidant respect to its primary structure of caseins [17].

Suetsuna *et al*. isolated and identified free radical scavenging activity from peptic digest of caseins [18]. The hexa-peptide, Tyr-Phe-Tyr-Pro-Glu-Leu, was found to possess a potent superoxide anion radical scavenging activity. The C-terminal dipeptide Glu-Leu sequence proved to be important for the activity.

On the other hand, Bourassa *et al*. showed that polyphenols bind casein via hydrophilic and hydrophobic sites. Genistein binds to α-casein at residues Arg-22 hydrophilic, while Gln-30, Phe-23, Phe-24, Phe-28, Phe-32, Pro-29 and Val-31 are hydrophobic. The attaching site of it to β-casein is at Gly-203, Ile-208, Leu-191, Leu-192, Leu-198, Phe-190, Tyr-180, and Tyr-193 are hydrophobic, while Val-197 and Val- 209 are hydrophilic [19]. Hence, we suggest that the binding of caseinate to genistein might alter the caseins conformation and provide more site to be attached by ABTS* cation.

The binding affinity of polyphenol to protein is size dependent and increases with their molecular size [20]. The binding can affect the electron donation capacity of the polyphenols by reducing the number of hydroxyl groups available

in solution and altering antioxidant activity of polyphenol [20]. However, our study has showed that sub-micelles are not appreciably larger than pure caseins because only few genistein attach to caseins after aggregation.

The data also confirmed how the existence of caseinate in the system will increase the antioxidant capacity sharply **(Table 4** and **Fig 9).** In this system, antioxidant capacity of genistein in ethanol that is 4.36 μM TE/μM gen comparable with TEAC of genistein in 0.1 to 0.2 μM caseinate at around 2.88-8.2 μM TE/μM gen. From this study, we understand that in respect to the low solubility of genistein, its antioxidant capacity also very weak. The caseinate encapsulation increasing slightly the solubility of genistein, however the antioxidant capacity increases sharply. The reason is because of its high antioxidant capacity of caseinate alone and the synergistic effect of genistein and caseinate in the mixing system.

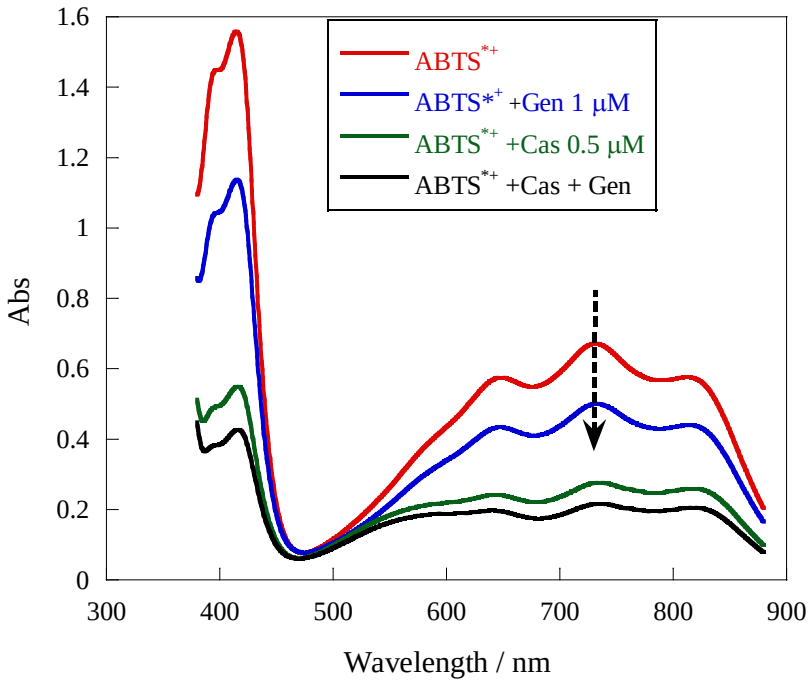


Fig. 4 The absorbance spectra of ABTS* cation mixed to genistein, caseinate and genistein in caseinate

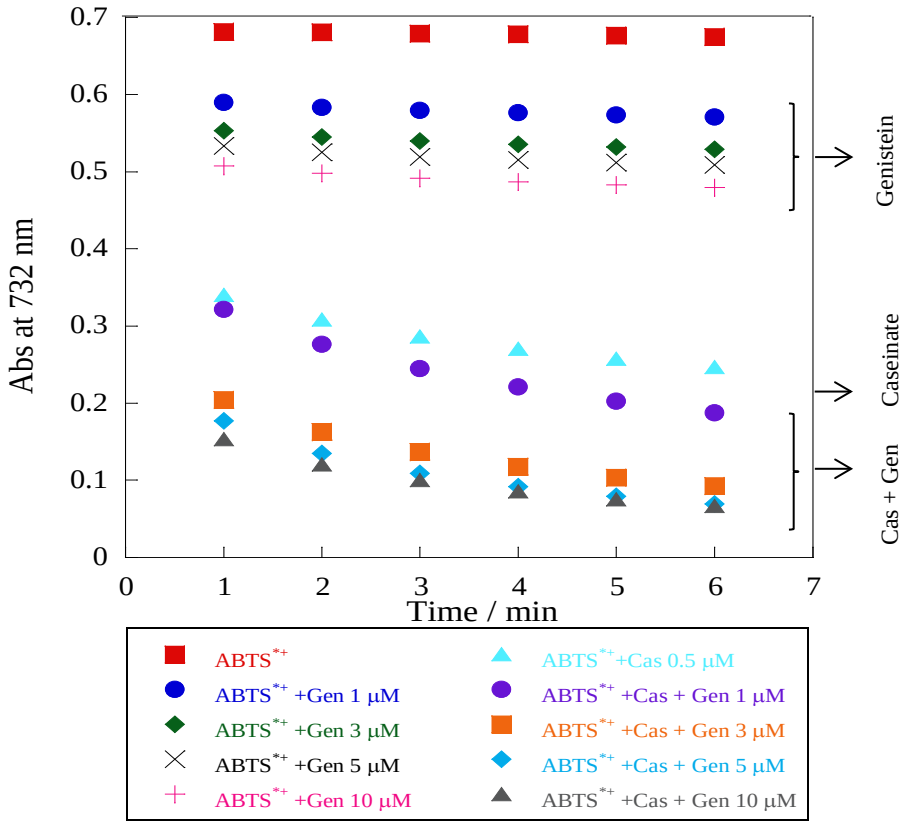


Fig. 5 The effect of time to the absorbance reduction of ABTS* cation mixed to caseinate with and without genistein

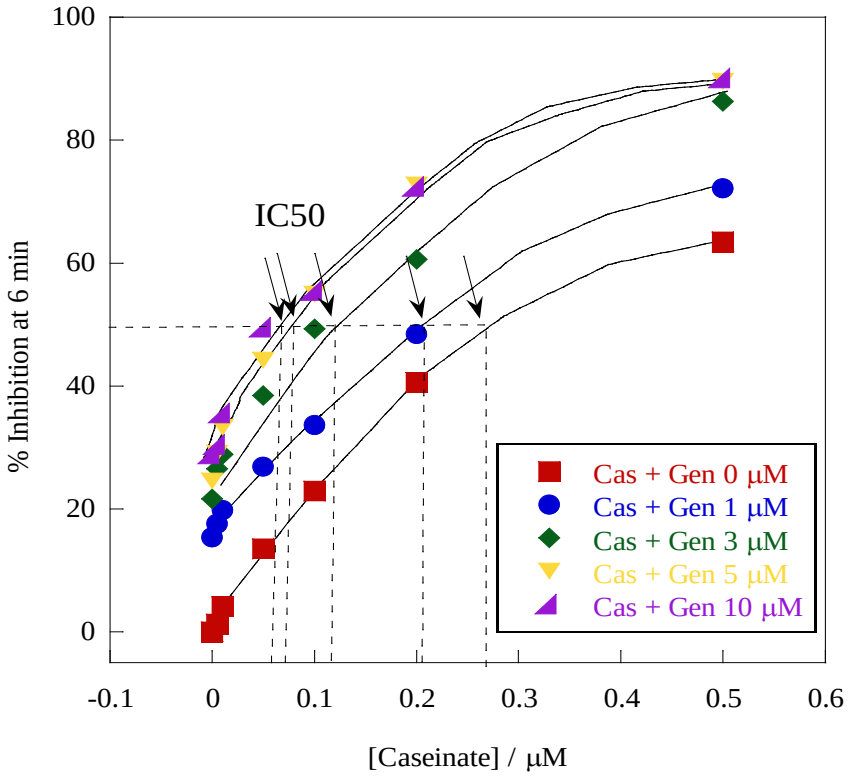


Fig. 6 Concentration that provide 50% inhibition against ABTS* cation (IC50) of various genistein in caseinate system (in µM caseinate).

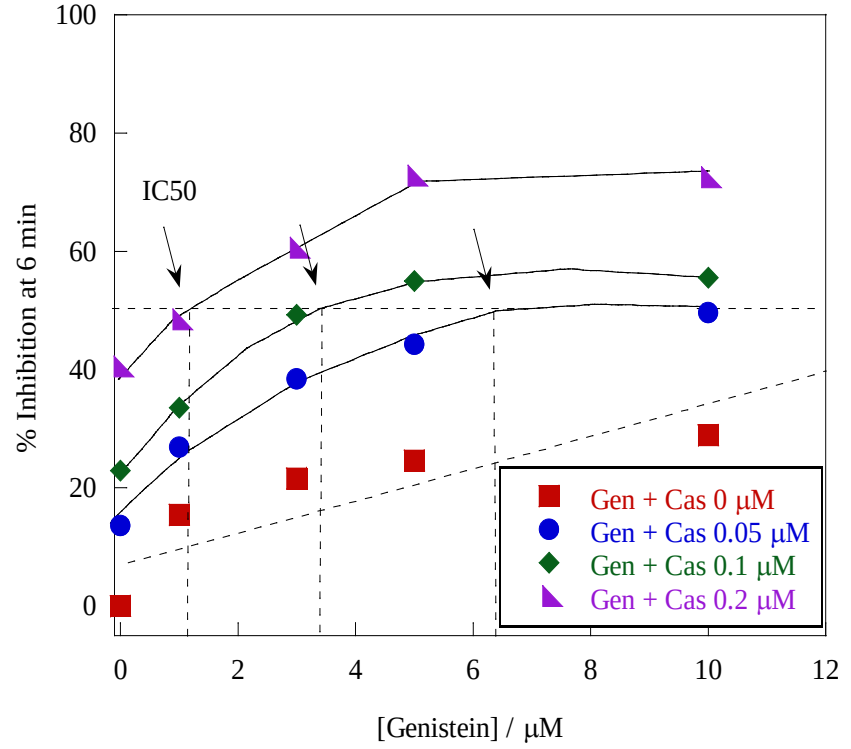


Fig. 7 Concentration that provide 50% inhibition against ABTS* cation (IC50) various caseinate that is bound to genistein (in µM genistein).

Tabel 2. IC50 and TEAC (Trolox equivalent of antioxidant capacity) of various genistein in caseinate in µmol TE/ mg caseinate

AOX / µM	IC 50 / µM cas	TEAC / µmol TE/mg caseinate
Cas / Gen 0	0.27	0.36
Cas + Gen 1	0.21	0.47
Cas + Gen 3	0.12	0.82
Cas + Gen 5	0.08	1.23
Cas + Gen 10	0.073	1.34

Tabel 3. IC50 and TEAC of genistein eq. to caseinate

Genistein / µM	IC 50 / µM cas	TEAC / µmol TE/mg caseinate*)
Gen 1	-	0.015
Gen 3	-	0.045
Gen 5	-	0.075
Gen 10	-	0.15

MW Caseinate : 100 kDa ; IC 50 Trolox : 9.8 µmol/L

*) : genistein eq. to caseinate

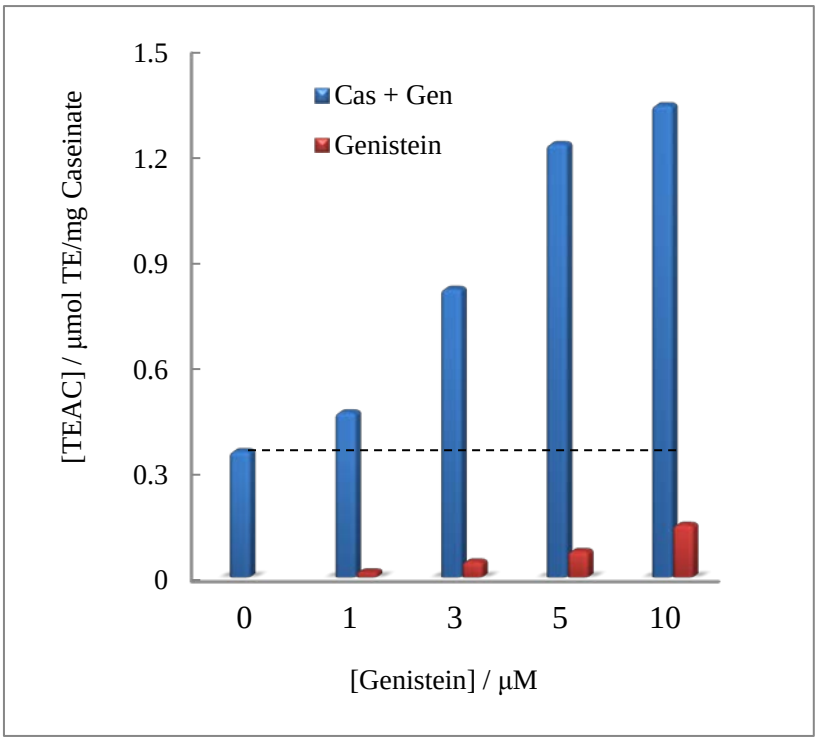


Fig. 8 The TEAC value of various genistein in caseinate system

Tabel 4. IC50 and TEAC (Trolox equivalent of antioxidant capacity) of various caseinate binding to genistein in μM TE/μM gen

AOX / μM	IC 50 / μM gen	TEAC / μM TE/μM gen
Gen in ethanol	2.25	4.36
Gen in PB/Cas 0	24.01	0.41
Gen + Cas 0.05	6.4	1.53
Gen + Cas 0.1	3.4	2.88
Gen + Cas 0.2	1.2	8.2

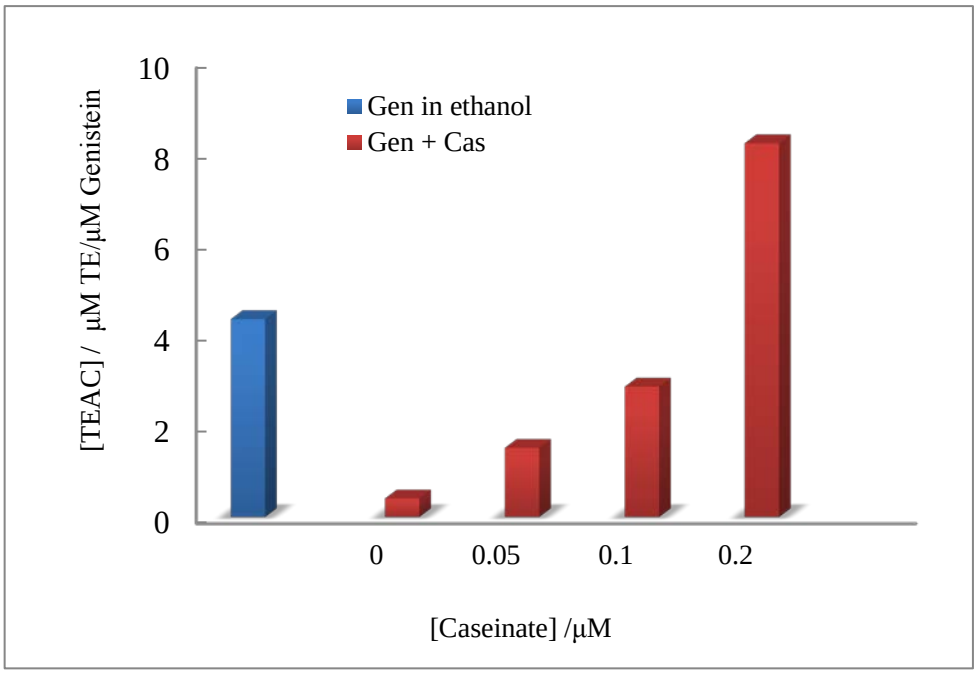


Fig. 9 The TEAC value of various caseinate binding to genistein

3.3.2 LIPOSOME SYSTEM

Phosphatidylcoline is the most common phospholipid employed in liposome manufacture. In this study, the preparation of genistein in liposome system was accomplished by directly mixing it to DPPC/DOPC in the methanol solvent altogether to get SUV of liposome. Moreover, the SUV with approximate size particle as <100 nm, was acquired by sonicating process of the MLV of DPPC and DOPC, that usually have size particle as 0.1-10 μm [4].

3.3.2.1 Antioxidant activity in DPPC

The encapsulation effect of liposome as SUV composed of rigid membrane, DPPC, to antioxidant of genistein was examined by spectrophotometer. The absorbance spectrum of ABTS* cation with various DPPC concentration can be seen in **Fig 12**. This picture shows clearly how the ABTS* radical absorbance does not change after mixed to various amount of DPPC. DPPC liposome which is belong to saturated phospholipid group does not have any active site that is attached to ABTS* cation as no decreasing graphs and decolorization detected. However, we see a little bit increasing absorbance of graph in DPPC 0.3 mM respect to its turbidity in higher concentration.

The antioxidant capacity of genistein in DPPC liposome system was measured in IC50 and TEAC. **Figure 13** shows the plotting percent inhibition of genistein in various DPPC concentrations. We cannot see the trend of IC50 in this picture as also confirm at **Fig. 14.** The IC50 of that DPPC encapsulation at 0.10 mM, 0.14 mM, 0.25 mM and at 0.30 mM to genistein are in the range of 3.0 μM to 3.6 μM. In consistent with this result, trolox equivalent antioxidant capacity /TEAC of this system does not perform any trend.

The antioxidant activity of genistein is very low in phosphate buffer

compared to it in ethanol which respect to very low solubility of genistein in water-base system, which is 0.41 compared to 4.36 μM TE/μM gen respectively. As can be seen in **Table 5**, DPPC encapsulation increases the antioxidant capacity of genistein sharply. This might be because of the increasing solubility of genistein as hydrophobic compound is the DPPC liposome.

The expected arrangement of genistein to DPPC was proposed by Pawlikowska-Pawlega *et al.* [5] as shown in **Fig 10.** They found the broad distribution of genistein in membranes with high tendency to polar head group zone and below the head group or lipid water interface. Because of its complex electrostatic and hydrogen-bonded structure, the membrane interface provides perfect environment for partially polar molecules as flavonoids. The FTIR analysis of their study showed that genistein incorporates into DPPC membranes via hydrogen bonding between the lipid polar head group in C-O-P-O-C segment and its hydroxyl groups [5].

Once attached to liposome of DPPC, the genistein changes the property of the membrane, genistein as isoflavone restrict the motility in all the regions with the greatest activity on the part of genistein in the polar head group regions [5] as also supported by Arora *et al.* [21] that mentioned about how intercalation of genistein into hydrophobic core take places causing a decrease in lipid fluidity in this region of the membrane.

The ABTS* cation introducing, probably attached in the free hydroxyl part of B ring, as purposed by Arora, *et al*. [1]. Their studied concluded that ring B of genistein is the principal site of antioxidant reaction of the flavonoids. [1]. As the binding site of genistein to DPPC and genistein to radical is different, most likely there was no competitive site in binding among the molecules **(Fig 10).**

However the antioxidant capacity of genistein in various DPPC is similar probably because of no interaction of DPPC liposome to the ABTS* cation, or the electron transfer from DPPC to ABTS* cation was not occurred. Then, we explain the similar result of antioxidant capacity of genistein in DPPC liposome as saturated solubility of genistein in DPPC liposome in the range of 0.10 mM until 0.30 mM.

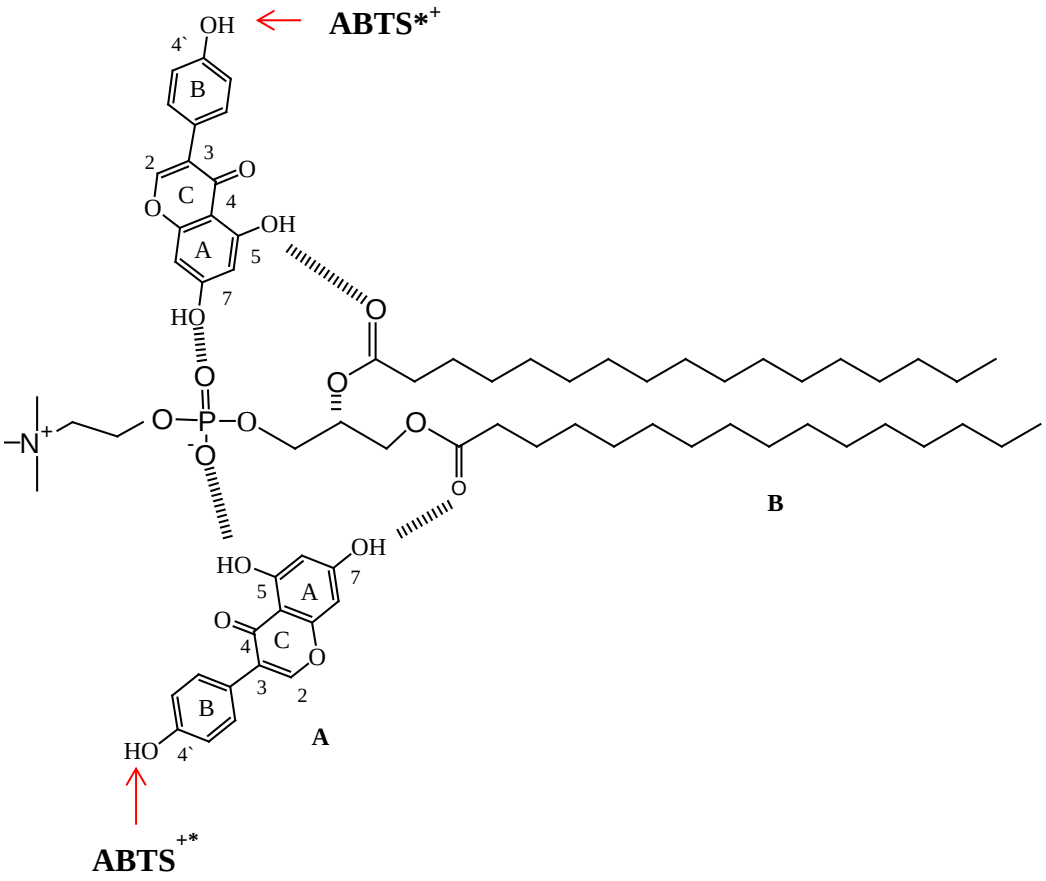


Fig. 10 The possibly binding of genistein to DPPC. A is genistein and B is DPPC. Genistein molecule creates hydrogen bonds between oxygen from positions 7 and 5 and segments of DPPC, the displayed hydrogen bond`s length is not in the real scale [5]. ABTS* cation possibly attach to OH in B-ring of genistein [1]

3.3.2.2 Antioxidant activity in DOPC

Contrasting to liposome composed by saturated DPPC, liposome of DOPC shows the slightly decreasing peak at 732 nm as resulted binding to ABTS* cation **(Fig. 15).** Liposome composed from DOPC is a neutral lipid contains unsaturated phospholipid, then it is expected that this unilamellar liposome will has radical scavenging power against ABTS* cation.

Figure 16 showed the % inhibition DOPC in various concentrations without genistein. We confirmed the capability of ABTS* cation in attaching DOPC. DOPC has one double bond in each tail fatty acid hydrocarbon area, as it is phospholipid bilayer, then it has 2 of double bond in total. It is suggested, therefore, that ABTS* cation attack the double bond in the tail part of DOPC. The IC 50 of DOPC liposome in this system was approximately 1.67 mM.

The plotting data of various genistein concentrations in DOPC to its % inhibition against ABTS* cation at 6 minutes can be seen in **Fig.17.** This result displays that, at every point of genitein, the DOPC mixing to it reaches the similar % inhibition to that ABTS* cation, as also can be confirmed in **Fig. 18**. As a consequence, the antioxidant capacity that estimated by trolox equivalent are also similar. The one possibility is because genistein reach its maximum solubility in DOPC liposome or its reach the saturated binding.

Genistein encapsulated by DOPC liposome performs a very good antioxidant activity as it has around 4.14 μM TE/μM gen compare to 4.36 μM TE/μM gen for genistein antioxidant activity in ethanol. We suggest that the DOPC binding site to genistein is similar to it of DPPC, as saturated analog phospholipid content of DOPC. That is between the lipid polar head group in C-O-P-O-C segment and its hydroxyl group via hydrogen bonding [5] as shown in **Fig. 11.** Tedeshi *et al*.

proposed that flavonoids insert in DOPC bilayer, positioning between the outer part of the hydrophobic core and the external hydrophilic layer [22]. Furthermore, Raghunatan *et al*. showed that the genistein inserted into hydrocarbon region in DOPC near carbonyls of lipids and decreased the bilayer thickness. The long axes of this flavonoid were oriented nearly parallel to the plane of the bilayer with their carbonyl group (C=O) pointed toward the proximal surface [6].

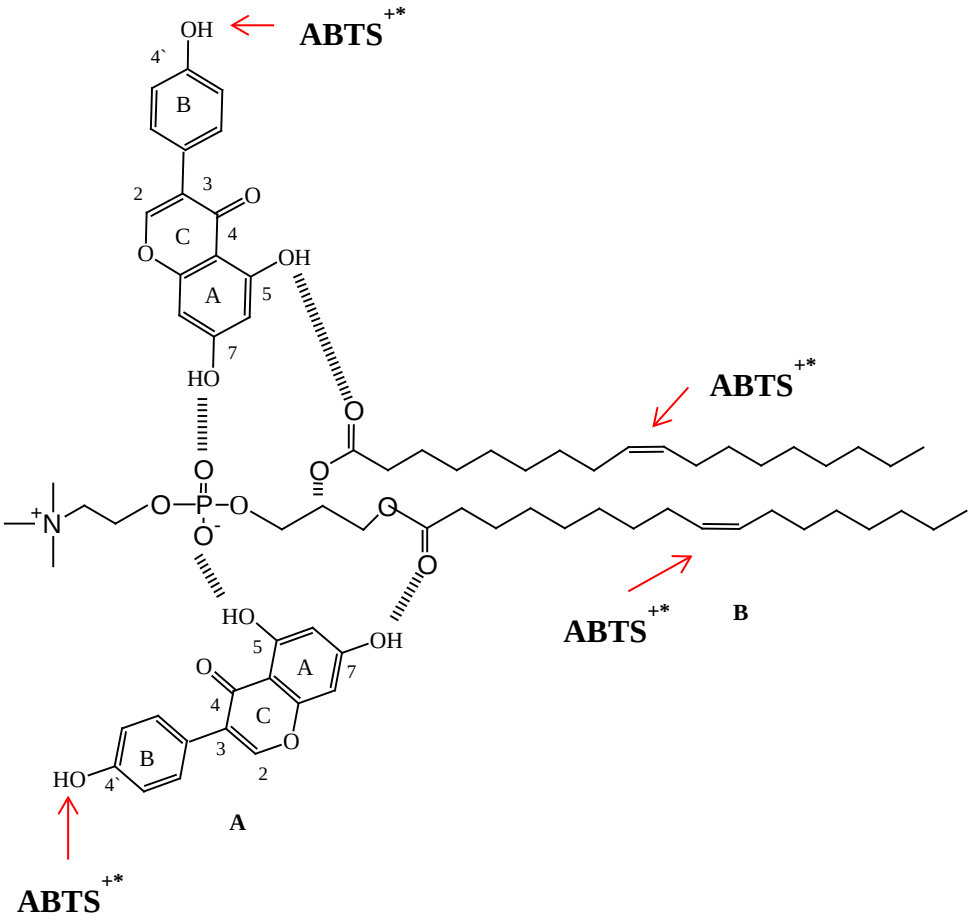


Fig. 11 The possibly binding of genistein to DOPC. A is genistein and B is DOPC. Genistein molecule creates hydrogen bonds between oxygen from positions 7 and 5 and segments of DPPC. The displayed hydrogen bond`s length is not in the real scale [5]. Since DOPC is analog structure of DPPC, we assumed that they have the similar binding site to genistein. ABTS* cation possibly attach to OH in B-ring of genistein [1] and also the double bound of DOPC.

3.3.2.3 Comparison of antioxidant activity of genistein in liposomes of DPPC and DOPC

This study shows that genistein encapsulated in liposome of DPPC and DOPC, both, have good activity against ABTS* cation, as they have comparable value with genistein in ethanol solution **(Table 5 and 6)**. However, the antioxidant capacity of it in DOPC liposome is higher than it in DPPC liposome. It might be because of the elasticity of acyl chain in DOPC to attach to active site of genistein in the system. Maherani *et al.* studies showed that fluidity values of liposome affected by double bound and degree of saturation in lipids. The presence of double bonds within acyl chain resulted in a decrease of packing density and chain ordering in lipid bilayers and consequently increasing the liposome fluidity and then elasticity [23]. The elasticity chain increase the capability to attach another molecule, then increasing the solubility of genistein in this DOPC liposome.

In addition, ABTS* cation has more attaching site to DOPC liposome than to DPPC liposome, as can be seen in **Figs. 10 and 11.** Since DOPC has double bond site that can be attack by ABTS* cation. Therefore, the antioxidant capacity total of genistein in DOPC is the sum of antioxidant of DOPC itself and antioxidant of genistein after dissolving to DOPC liposome, even though the antioxidant capacity of the DOPC liposome alone is very weak.

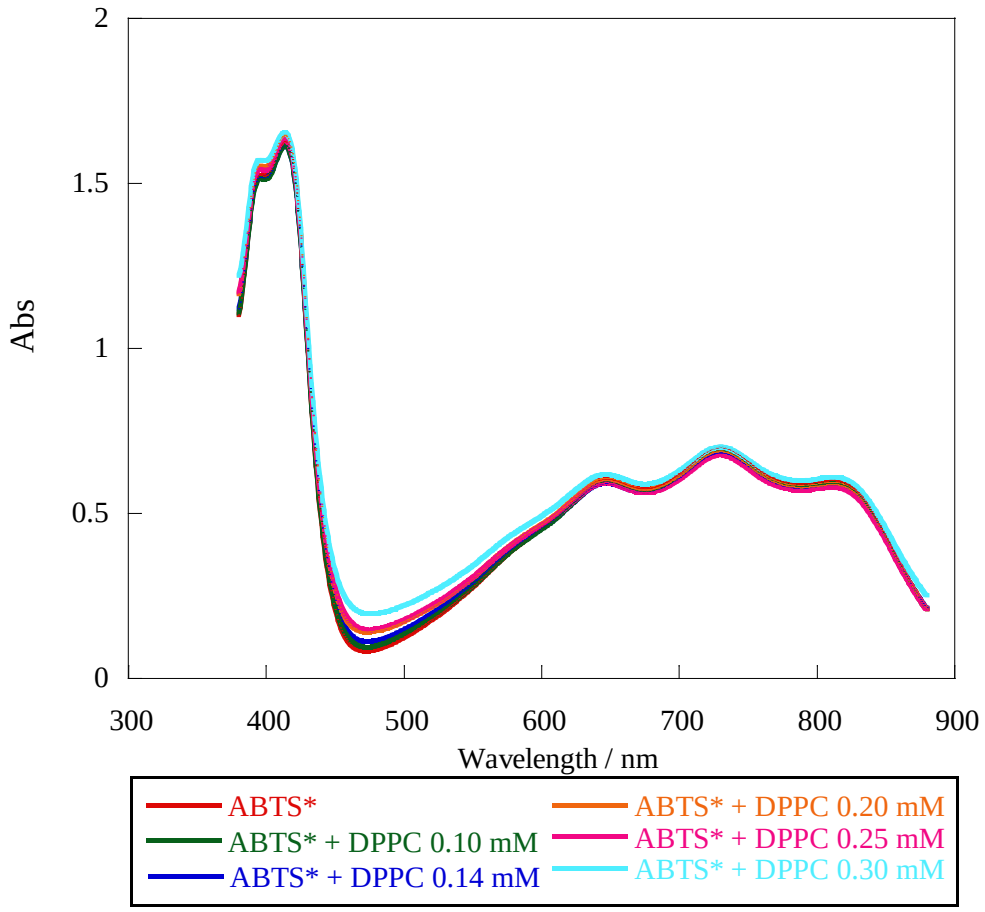


Fig. 12 The absorbance spectra of ABTS* cation with various DPPC concentration

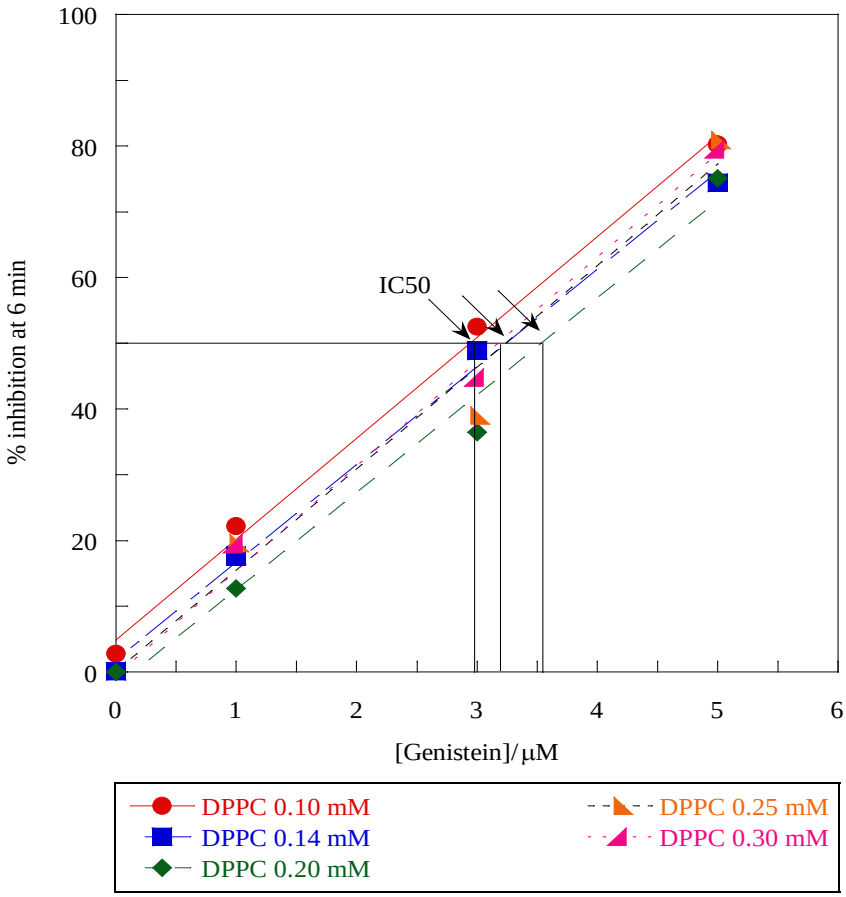


Fig. 13 % Inhibition of genistein in various DPPC liposomes

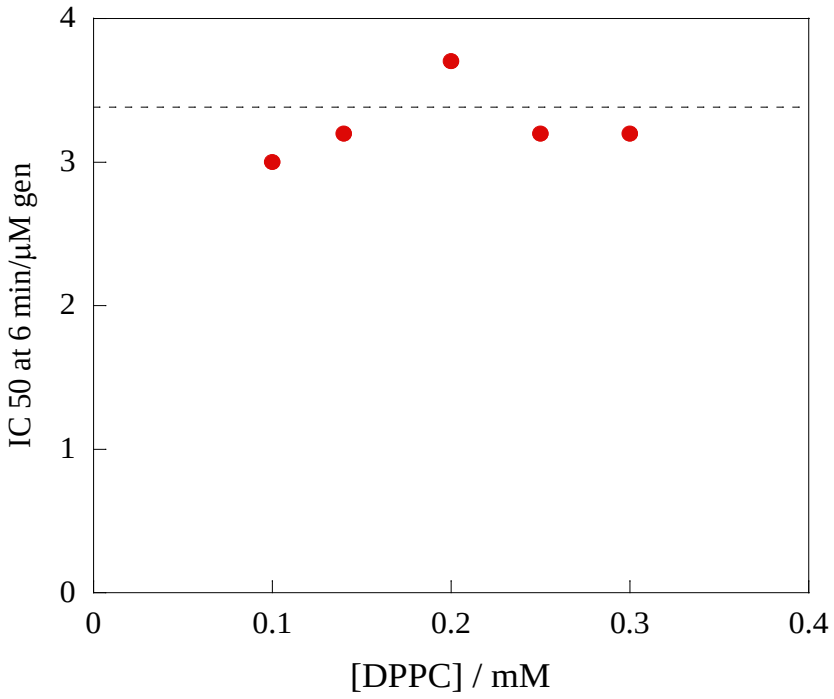


Fig. 14 IC 50 of genistein in various DPPC liposomes

Table 5. IC50 and TEAC (Trolox equivalent of antioxidant capacity) of genistein in DPPC

AOX / µM	IC 50 / µM gen	TEAC / µM TE/µM gen
Gen in ethanol	2.25	4.36
Gen in PB/Cas 0	24.01	0.41
Gen + DPPC 0.10 mM	3.0	3.27
Gen + DPPC 0.14 mM	3.2	3.06
Gen + DPPC 0.20 mM	3.6	2.72
Gen + DPPC 0.25 mM	3.2	3.06
Gen + DPPC 0.30 mM	3.2	3.06

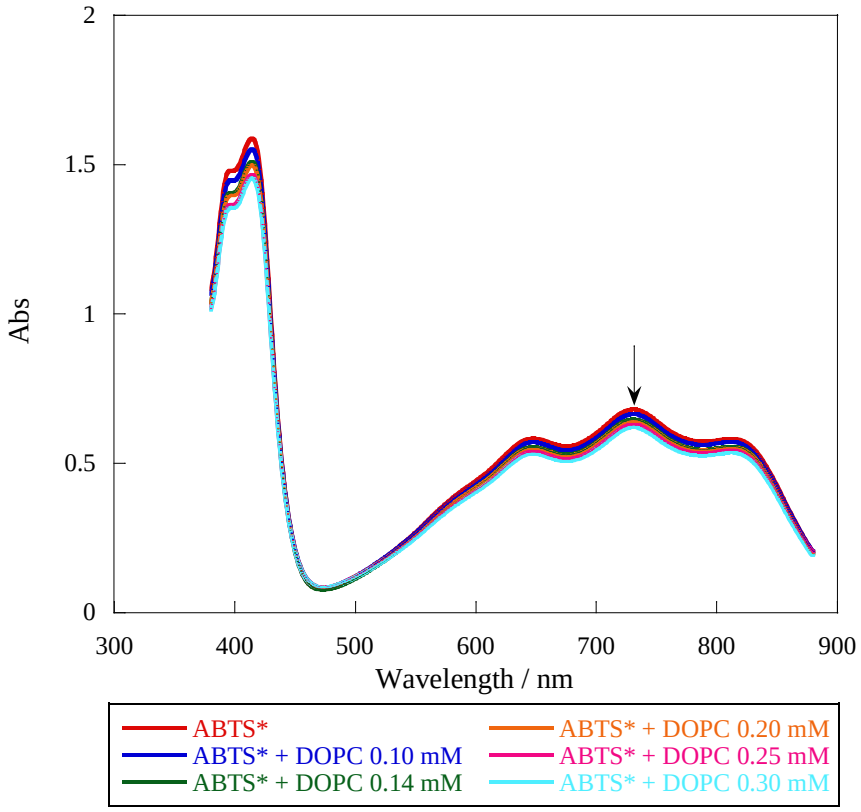


Fig. 15 The absorbance spectra of ABTS* cation with various DOPC concentration

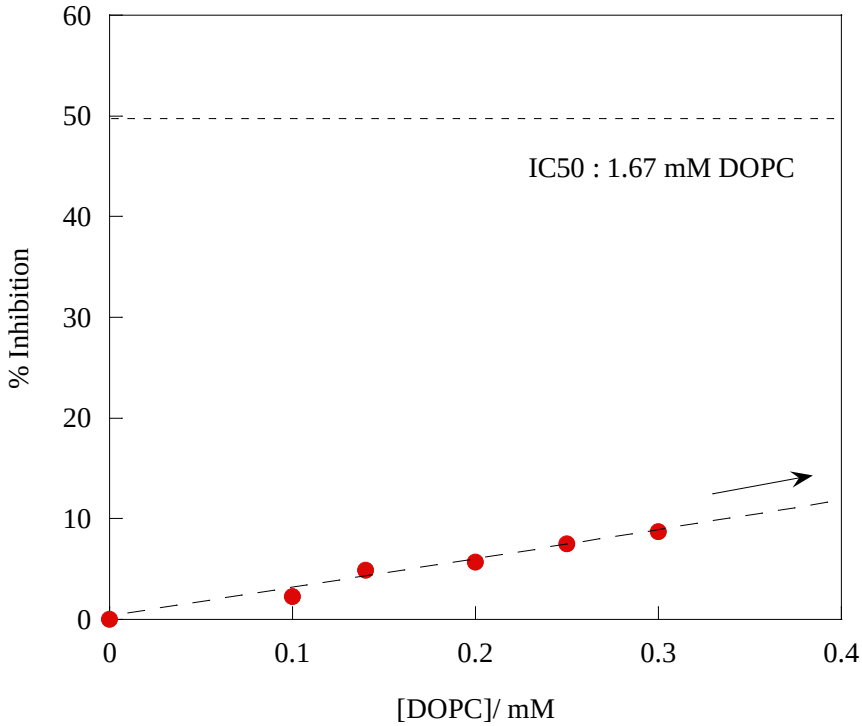


Fig. 16 The IC50 of DOPC against ABTS* cation

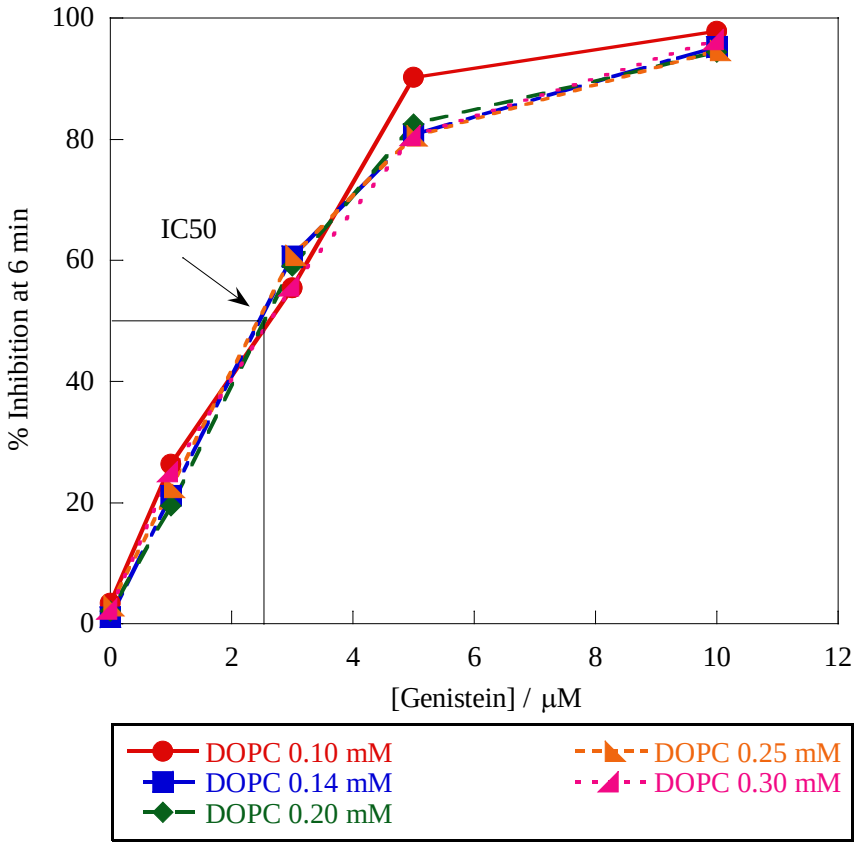


Fig. 17 % Inhibition of genistein in various DOPC liposomes

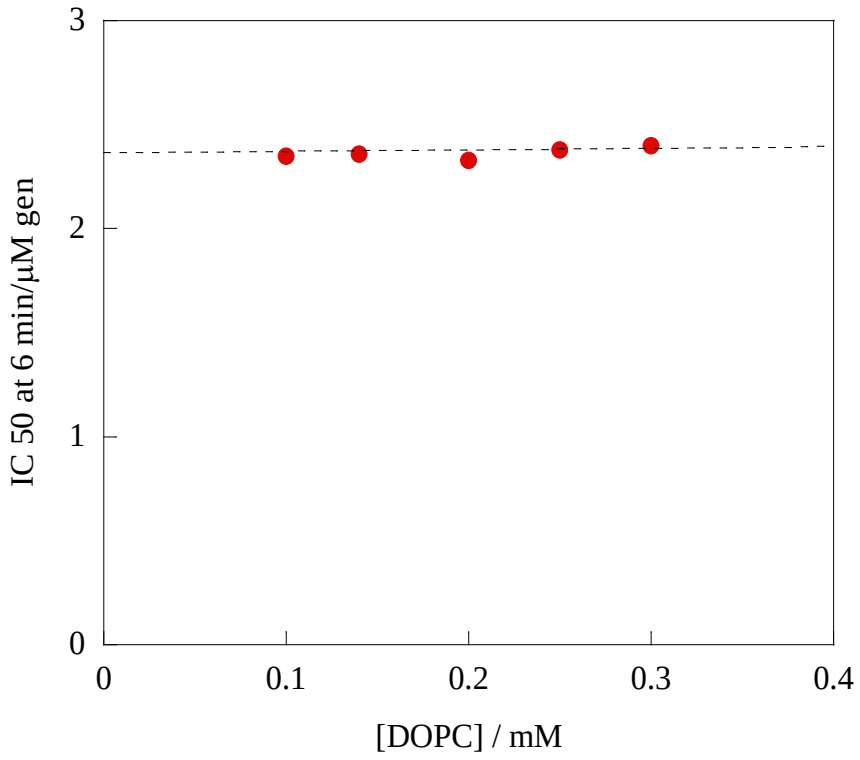


Fig. 18 IC 50 of genistein in various DOPC liposomes

Table 6. IC50 and TEAC (Trolox equivalent of antioxidant capacity) of genistein in DOPC

µM	IC 50 / µM gen	TEAC / µM TE/µM gen
Gen in ethanol	2.25	4.36
Gen in PB/Cas 0	24.01	0.41
Gen + DOPC 0.10 mM	2.35	4.17
Gen + DOPC 0.14 mM	2.36	4.15
Gen + DOPC 0.20 mM	2.33	4.20
Gen + DOPC 0.25 mM	2.38	4.12
Gen + DOPC 0.30 mM	2.40	4.08

3.4 REFERENCES

[1] A. Arora, S. Valcic, S. Cornejo and M. G. Nair, "Reactions of Genistein with Alkylperoxyl Radicals," *Chem. Res. Toxicol.*, vol. 13, no. 7, pp. 638-645, 2000.

[2] A. Pihlanto, "Antioxidative peptides derived from milk proteins," *International Dairy Journal*, vol. 16, no. 11, p. 1306–1314, 2006.

[3] L. Were, B.D.Bruce, P.M.Davidson and J.Weiss, "Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials," *Journal of agricultural and food chemistry,* vol. 51, no. 27, pp. 8073-8079, 2003.

[4] A. Ulrich, "Biophysical aspects of using liposomes as delivery vehicles," *Bioscience Reports,* vol. 22, no. 2, pp. 130-150, 2002.

[5] B. Pawlikowska-Pawlęgaa, L. E. Misiak, B. Zarzyka and R. Paduch, "Localization and interaction of genistein with model membranes formed with dipalmitoylphosphatidylcholine (DPPC)," *Biochimica et Biophysica Acta (BBA) - Biomembranes,* vol. 1818, no. 7, p. 1785–1793, 2012.

[6] M. Raghunatan, Y. Zubovski, R. Venable, R. Pastor, J. Nagle and S. Tristam-Nagle, "Structure and elasticity of lipid membranes with genistein and daidzein bioflavinoids using X-ray scattering and MD simulations," *The journal of physical chemistry,* vol. 116, no. 13, pp. 3918-3927, 2012.

[7] J. Lehtonen and P. K. K. H. Adlercreutz t, "Binding of daidzein to liposomes," *Biochimica et Biophysica Acta,* vol. 1285, no. 1, pp. 91-100, 1996.

[8] J. Maniewska, G. Grynkiewicz, W. Szeja and A. B. Hendrich, "Interaction of genistein benzyl derivatives with lipid bilayers—fluorescence," *Bioorganic & Medicinal Chemistry,* vol. 17, no. 6, p. 2592–2597, 2009.

[9] K. Cie´slik-Boczulaa, J. Maniewskab, G. Grynkiewiczc and W. Szejad, "Interaction of quercetin, genistein and its derivatives with lipid bilayers – An ATR IR-spectroscopy study," *Vibrational Spectroscopy,* vol. 62, pp. 64-69, 2012.

[10] A. Arora, M.G.nair and G.M.Strasburg, "Antioxidant activities of isoflavones and their biological metabolites in a liposomal system," *Archieves of biochemistry and biophysics,* vol. 356, no. 2, pp. 133-141, 1998.

[11] V. Cappelletti, L. F. P, Miodini and G. Di Fronzo, "Genistein Blocks Breast Cancer Cells in the G2M Phase of the Cell Cycle," *Journal of Cellular Biochemistry,* vol. 79, p. 594–600, 2000.

[12] W. Bors, W.Heller, C.Michel and M.Saran, "Flavonoids as antioxidants:Determination of radical schavenging activities," *Methods Enzymol.,* vol. 186, pp. 343-355, 1990.

[13] R. Re, N. Pellegrinia, A. Proteggente, A. Pannala, M.Yang and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine,* vol. 26, no. 9–10, p. 1231–1237, 1999.

[14] S. de Olievera, G. d. Souza and e. al., "Evaluation of antiradical assays used in determining the antioxidant capacity of pure compounds and plant extracts," *Quim.Nova,* vol. 37, no. 3, pp. 497-503, 2014.

[15] M. Arts, G. Haenan, H. Voss and A. Bast, "Antioxidant capacity of reaction products limits the applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) assay," *Food and Chemical Toxicology,* vol. 42, no. 1, p. 45–49, 2004.

[16] C. Phadungath, "Casein micelle structure:A concise review," *J.Scie.Technol.,* vol. 27, no. 1, pp. 201-212, 2005.

[17] S. Laakso, "Inhibition of lipid peroxidation by casein. Evidence of molecular encapsulation of 4,4-pentadiene fatty acids," *Biochimica et biophysica acta,* vol. 792, no. 1, pp. 11-15, 1984.

[18] K. Suetsuna, H. Ukeda and H.Ochi, "Isolation and characterization of free radical scavenging activities peptides derived from casein.," *Journal of Nutritional Biochemistry,* vol. 11, no. 3, p. 128–131, 2000.

[19] P. Bourassa, J.Bariyanga and H.A.Tajmir-Riahi, "Binding sites of resveratrol, genistein, and curcumin with milk α-and β-caseins.," *J. Phys. Chem. B.,* vol. 117, pp. 1287-1295, 2013.

[20] I. Hasni, P.Bourassa, S.Hamdani, G.Samson, R.Carpantier and H.A.tajmir-Riahi, "Interaction of milk α-and β-caseins with tea polyphenols," *Food chem.*, vol. 126, no. 2, pp. 630-639, 2011.

[21] A. Arora, T. Byrem, M. Nair and G. Strasburg, "Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids," *Arch. Biochem. Biophys,* vol. 373, no. 1, p. 102–109, 2000.

[22] A. Tedeschi, G. D'Errico, M. Lauroa, F. Sansonea, S. Marinoa, A. D'Ursia and R. Aquino, "Effect of flavonoids on the αβ(25-35)-phospholipid bilayers

interaction," *European Journal of Medicinal Chemistry,* vol. 45, no. 9, p. 3998–4003, 2010.

[23] B. Maherani, E. Arab-Tehrany, A. Kheirolomoom and F. Cleymand., "Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant L-carnosine," *Food Chemistry,* vol. 314, p. 632–640, 2012.

Chapter 4

GENERAL CONCLUSION

In this doctoral thesis, we discussed about how the genistein encapsulation by caseinate and liposome affecting its primarily property as antioxidant. This discussion will be started by explaining the background and purpose of this study in chapter 1.

Chapter 2 of this thesis focused on the measurement of genistein solubility in caseinate with and without calcium ion. The hydrophobicity measurement by ANS-fluorescence found out that the CAC of caseinate in phosphate buffer is 0.68 μM. Moreover, the solubility test revealed that caseins increase the solubility of genistein in CAC-dependent. Below the CAC, approximately 10 molecules of genistein attach to one caseinate, whereas above the CAC, approximately a single genistein molecule attaches to one casein in the sub-micelle aggregate. However, calcium ions existences do not influence the binding capacity of genistein in the caseinate system.

The structural observation of caseinate, with and without genistein by Cryo-TEM shows that solely caseins in buffer solution forms small sub-micelle aggregates of diameter 20 nm, and increasing to 30 nm when genistein molecules are bound. As a comparison, according to the AFM measurements, caseinate sub-micelles bound to genistein are doubled in diameter (from 12 nm to 24 nm),

even though no significant change in surface structure.

Furthermore, we proposed the probability of how genistein attach to this caseins surface from the AFM observation. The attaching of caseinate sub-micelles to genistein apparent as bright spots in AFM phase images, implying that the particles become harder, probably because genistein attaches to the surface or to the narrow part of the sub-micelle. This argument was supported with the result study of genistein attachment behavior to caseinate. The surface area of caseinate sub-micelles is larger before than after aggregation, implying that fewer genistein molecules can attach to aggregated casein sub-micelles.

In chapter 3 we discussed about how antioxidant capacity of genistein, as encapsulated materials to be attached, was affected by caseinate then compared to liposome system. The antioxidant capacities of genistein in caseinate and liposome were estimated by TEAC as μM TE/μM genistein. This result showed that the antioxidant capacity of genistein in caseinate increased with increasing caseinate concentration while that of genistein was independent to liposome concentration. On the other hand, DOPC shows a minor antioxidant capacity against ABTS* cation.

The study in chapter 2 explained that genistein slightly soluble in caseinate. However, caseinate alone has antioxidant activity in respect to its peptides active site to scavenge free radical, and also the high net charge on caseins surface. Therefore, it is expected that the antioxidant capacity of genistein in caseinate is the sum of antioxidant activity of genistein and caseinate. Moreover, this study discovered that antioxidant activity in caseinate system is synergic.

The last part of this chapter explored about the antioxidant capacity of genistein in liposome system. It was revealed that antioxidant capacity of

genistein in DPPC and DOPC liposome are comparable with it in ethanol system. Even though, the number of it DOPC liposome provide higher capacity than it in DPPC.

Hence, in this study we suggest that both caseinate and liposome are good encapsulation materials for genistein, in the case of antioxidant capacity in different mechanism. Caseinate offer the synergistic effect in increasing antioxidant capacity of genistein, while liposome is a good dissolving agent to genistein to optimize its property as antioxidant.

Finally, the discussion of this doctoral thesis was closed by summarizing it in chapter 4.

Acknowledgment

The author would like to express her biggest gratitude to Prof. Akio Ohta, as her chief supervisor for his kindly help, outstanding guidance, and advice in her study and also in daily life during her doctoral work in Kanazawa University. Furthermore, the author would like to thankful to Prof. Tsuyoshi Asakawa as the Chief of Surface Chemistry Laboratory for his support and guidance.

The author wishes to thank to Prof. Tomoki Ogoshi from Kanazawa University for AFM experiment and Prof Kazuma Yasuhara from NAIST for cryo-TEM measurement. She really appreciate and thankful for the kindly reviews, suggestions also advices from the board of the examiner in her final doctoral presentation who are Prof. Akio Ohta, Prof. Tsuyoshi Asakawa, Prof. Kuoki Kunimoto, Prof. Tomoki Ogoshi, and Prof. Teruya Maki.

She also express her thankful to her sponsorship, Indonesian Scholarship DIKTI under the KU-DIKTI Program Student 2011. During her study, the author also be supported and helped by International Affair and Graduate School of Natural Science and Technology office.

Special thanks also been expressed to all of the laboratory member, who give her really comfortable environment to study and `feels like home` feeling during her study in Japan. Thanks are due to Indonesian society PPI Ishikawa, the Berangs Group for best support and fun accompany. Finally, she wishes to thank sincerely to her lovely parents and brothers for outstanding loving and support.