

Computational Simulation for Antioxidant Activities: Identification of Physical Properties of Peptide from Mare Milk

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Abstract

Peptide characterisation is essential for finding the functionalities of the synthesised peptides. The peptides were recently isolated from the fraction < 3 kDa of Sumbawa mare milk's protein through hydrolysis by *B. thuringiensis* protease, with profiling by RP-HPLC and sequence analysis by LC-MS/MS. However, screening of peptide activities is typically avoided due to the required cost and time expense. Herein, the peptides were analysed using molecular mechanics and docking software to find the physicochemical properties and reactivity with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The dissociation constants (pK_d) of peptides with ABTS and DPPH were calculated and found to correlate with the antioxidant activity between peptide and radical ABTS or DPPH. The peptide HPYFYAPELLEYANK ($pK_d = 4.16$) was found to have the highest antioxidant activity with ABTS, while peptide VPQVSTPTLVEVSR ($pK_d = 5.99$) the highest antioxidant activity with DPPH. The influence of the peptide's stereochemistry and the correlation between antioxidant activity and dissociation constant were then investigated by statistical analysis.

Keywords

Peptide, Molecular modelling, Antioxidant, Dissociation constant

Introduction

Free radicals are commonly produced during metabolism in living organisms. Antioxidants are necessary molecules for maintaining oxidative and anti-oxidative balances in biological system to prevent oxidative damages and degenerative diseases [1, 2]. Antioxidants are produced naturally by organisms, but in certain condition their production are insufficient. Antioxidants from external sources are commonly used to supplement those naturally produced in organism when insufficient [3]. For these reasons, many researchers are keen to explore potential antioxidants from natural sources, such as peptides derived from readily available proteins.

Mare milk has a high protein content compared to that of other species, notably buffalo, camel, cow, ewe, goat, and human [4]. The composition of true whey protein from mare's milk is higher than cow's and human's milk [5]. The mare milk therefore has a higher potential as a bioactive peptide resource. Additionally, the protein from mare milk induces fewer allergenic effect than those from cow milk [6]. Of specific interest in this study was the mare milk from Sumbawa mare, a horse native to Nusa Tenggara Barat province, Indonesia, and likely descendent

from Mongolian and Arabian horses [7].

Sumbawa mare milk has been utilised as a traditional medicine [8] but few studies on the scientific value of these treatments have been performed. From previous studies, Sumbawa mare milk has shown potential in increasing immunity of mice against hepatitis A [9], antimicrobial activities [10], and probiotic function [11]. For these reasons, Sumbawa mare milk could be a potential source of exploitable peptides.

The peptides of Sumbawa mare milk have recently been investigated [12]. However, exploring physio-chemical properties or bioactivities, such as antioxidant potential, was limited due to the cost and time constraint associated with single peptide synthesis. To overcome this issue, we employed the use of computational simulation, which is becoming a potential method of seeking characteristics and bioactivities predictions of peptides. Herein, the peptides derived from Sumbawa mare milk were analysed for their potential antioxidant activities against 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals using molecular modelling, which were then confirmed by antioxidant activity testing.

Materials and methods

Materials

The peptide sequences (13 peptides) were taken from a previous report (Table S1) [12]. The peptides were generated from hydrolysis of Sumbawa mare's milk protein using *B. thuringiensis* protease, fractionated using 3 kDa membrane (AMICON Ultra centrifugal units, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co) and sequenced and identified by LCMS/MS.

2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, and potassium persulphate ($K_2S_2O_8$) were obtained from Sigma-Aldrich, USA. The peptides HPYFYAPELLYYANK and LVNELTEFAK were synthesized by First Base Laboratories, Malaysia. Double-distilled ultrapure water (Millipore, UK) was used for all experiments. All chemicals and solvents were analytical or HPLC grade and used without any purification.

Computational simulation

Bovine serum albumin (BSA) was initially acquired from the RCSB Protein Data Bank (4F5S) as a dimer crystal. By opening the protein pdb file as text, the 13 peptide sequences previously published by Kusumaningtyas et al., were found to correspond to amino acid sequences in each protein monomer (Table S1) [12]. These peptide sequences were then isolated from the protein without structural modification, giving peptides A and B for each BSA monomer in the configuration found in the protein crystal. Peptides A and B were then subjected to an automated screening technique ('Leapfrog', available within the Sybyl 7.3 software package) in which ABTS and DPPH were sequentially placed around the peptide and the interaction energy recorded. Analysis of the

position of maximum binding energy as determined by the screening for each then gave approximations of the positions of highest interactions between each peptide (A and B) and ligand (DPPH and ABTS). The Surflex docking software was then used to predict values for the dissociation constant, K_d , for each peptide and ligand, using the positions determined from Leapfrog screenings as the site for more precise docking analysis.

Table S1: Position of peptide in Bovine serum albumin (BSA), RCSB Protein Data Bank (4F5S).

No	Peptide	Position in BSA
1	LVNELTEFAK	66 – 75
2	YLVEIAR	161 – 167
3	HPYFYAPELLYYANK	169 – 183
4	AEFVEVTK	249 – 256
5	DAIPENLPPLTADFAEDK	319 – 336
6	DAFLGSFLYEYSR	347 – 359
7	RHPEYAVSVLLR	360 – 371
8	HPEYAVSVLLR	361 – 371
9	HLVDEPQNLK	402 – 412
10	LGEYGFQNALIVR	421 – 433
11	KVPQVSTPTLVEVSR	437 – 451
12	VPQVSTPTLVEVSR	438 – 451
13	KQTALVELLK	548 – 557

Antioxidant activity testing

The antioxidant activity test was the same as the previous study [12, 13] and proceeds as follows: a solution of ABTS 7.4 mM and $K_2S_2O_8$ 2.6 mM (1:1, v/v) was incubated at room temperature in the dark for 18 hours, diluted with deionized water, and measured by microplate reader at $\lambda = 405$ nm until reaching absorbance of 1.1 ± 0.05 . 100 μ L HPYFYAPELLYYANK (1 mg/mL) was then added to 200 μ L ABTS mix solution, incubated at room temperature for 15 minutes, and its absorbance measured at $\lambda = 405$ nm by microplate reader. The scavenging activity of peptide HPYFYAPELLYYANK to ABTS radicals was expressed using equation 1. This procedure was also tested for peptide LVNELTEFAK.

$$\text{Radical scavenging activity} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100\%$$

Equation 1: Where A_{control} was absorbance of blank sample treated with no added peptide and A_{sample} is the absorbance in the presence of peptide. The assays were performed in three replications and the results were presented as the mean of each.

The analytical procedure of antioxidant activity for DPPH is slightly different with those for ABTS. The DPPH solution was diluted with ethanol until reaching absorbance of 1.1 ± 0.05 at $\lambda = 540$ nm. 100 μ L HPYFYAPELLYYANK (1 mg/mL) was then added to 200 μ L DPPH solution, incubated at room temperature for 30 minutes, and its absorbance measured at $\lambda = 540$ nm by microplate reader. The scavenging activity of peptide HPYFYAPELLYYANK to DPPH radicals was expressed using equation 1. This procedure was re-

peated for peptide LVNELTEFAK.

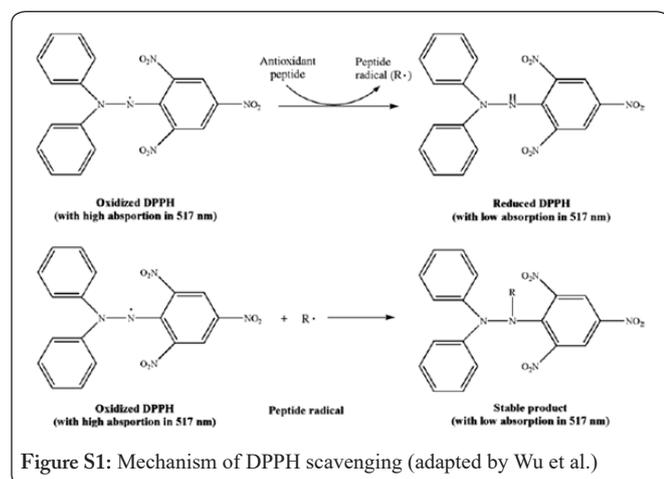
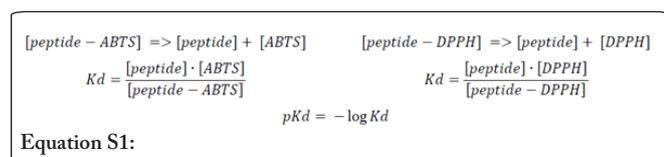
Statistical analysis

Data analysis was performed by IBM SPSS Statistic 25 Software. The Levene's test and independent t-test were applied for determining the significance between calculated dissociation constants in 13 peptide sequences. Pearson correlation was used to find significant correlations between peptides in individual and group positions regarding to interact with ABTS and DPPH.

Results and discussion

Dissociation constant and radical scavenging activity

In this study, the dissociation constant was calculated and investigated for possible correlation with the antioxidant activity of a series of peptides. The antioxidant activity was examined by calculating radical scavenging activity (Equation 1), by observing the reaction between the peptide and DPPH shown figure S1 [14]. The dissociation constant was found to be related to the antioxidant activity via equation S1. It seems that the higher pK_d of peptide complexes correlate with higher antioxidant activity of the peptide. Hence, determining the pK_d could present an alternative approach to finding the antioxidant activity of peptides without any synthesis required.

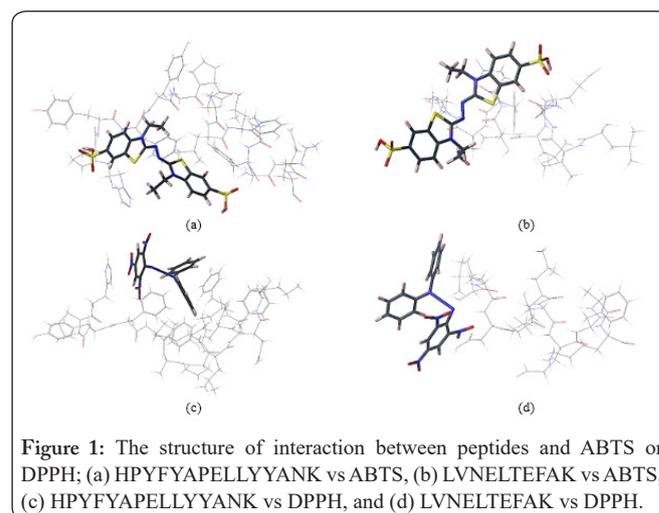


The empirical studies showed the same results as the computational simulation studies when the peptides HPYFYAPEL-LYYANK and LVNELTEFAK were compared. The antioxidant activity and pK_d of HPYFYAPEL-LYYANK are $12.04 \pm 5.60\%$ and 0.93 ± 0.10 respectively, which was higher than those of LVNELTEFAK (7.66 ± 5.09 and 0.08 ± 0.03) to ABTS (Table 1). Similarly, the antioxidant activity was linearly correlated with pK_d for interaction between peptides and DPPH; the peptide HPYFYAPEL-LYYANK showed higher antioxidant activity and pK_d than LVNELTEFAK as shown in table 1.

Table 1: Comparison between dissociation constant and antioxidant activities of peptides HPYFYAPEL-LYYANK and LVNELTEFAK

Single peptide	PKd			Antioxidant activity (%)		
	Min - Max	Mean	SD	Min - Max	Mean	SD
ABTS interaction						
HPYFYAPEL-LYYANK	0.85 - 1.00	0.93	0.1	6.74 - 14.81	12.04	5.6
LVNELTEFAK	0.06 - 0.10	0.08	0.03	3.97 - 13.46	7.66	5.09
DPPH interaction						
HPYFYAPEL-LYYANK	2.75 - 2.84	2.8	0.06	9.76 - 10.72	10.34	0.51
LVNELTEFAK	2.17 - 2.91	2.54	0.52	4.24 - 7.28	5.28	1.73

These results can be rationalized by acknowledging that the pK_d and antioxidant activity of the peptide HPYFYAPEL-LYYANK would both be expected to be higher than that those observed with LVNELTEFAK because of the presence of methionine, cysteine and aromatic amino acid such as tyrosine, histidine, phenylalanine and tryptophan as shown figure 1 [15].



The stereochemistry effect to antioxidant activity

The peptide sequences were discovered to be common to both bovine serum albumin (BSA) and the isolated horse milk protein. While there are undoubtedly differences in the structure the conservation of these sequences in both suggests strong overall similarity, allowing BSA to function as a suitable model for the horse serum albumin protein. Peptides were therefore isolated from the crystal structure of BSA for the calculation of their respective pK_d values. After identification of the sequences within the BSA protein, these peptides could then be extracted and isolated from the protein (referred to as 'single') or analysed while part of the greater protein structure ('part of BSA'). Docking software was then used to calculate the pK_d values of ABTS and DPPH with each of these peptides in isolation and as a part of the BSA protein. The results are given in figure 2. Strong variation can be observed between each peptide, and between each peptide in isolation

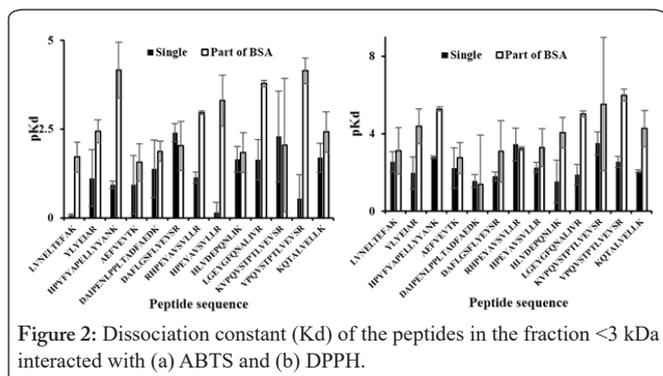


Figure 2: Dissociation constant (Kd) of the peptides in the fraction <3 kDa interacted with (a) ABTS and (b) DPPH.

Correlation the pKd between two different stereochemistry

The role of stereochemistry in the interaction between the two ligands ABTS and DPPH can be observed in table 2. A broad range of dissociation constants was observed for the different peptides with both ligands, with the greatest difference being observed in analysis of the peptide within the intact protein. In these conditions the surrounding protein can act to sterically prevent comfortable binding of the ligands or can inversely support binding by providing additional interactions in regions of the ligand not involved in binding to the specific peptide.

Table 2: pKd of peptide position with ABTS and DPPH interaction.

Peptide position	pKd from ABTS interaction			pKd from DPPH interaction		
	Min - Max	Mean	SD	Min - Max	Mean	SD
Single	-0.06 – 3.20	1.22	0.83	0.75 – 4.05	2.32	0.77
Part of BSA	0.72 – 4.72	2.63	1.05	-0.44 – 7.96	3.95	1.64

The Levene's test for equality of variances was investigated for all interaction occurred in this study; those occurring between isolated single peptides and ABTS and DPPH, and interaction between peptide as part of BSA with ABTS and DPPH. The results showed that there were two position combinations resulting in $p < 0.05$, and that the difference in stereochemical effect of peptide between isolated single peptides or the equivalent sequences as part of BSA is negligible. However, the Pearson correlation provided contradictory results, in which a correlation is demonstrated between the observed interaction of the sequences (as part of BSA) with ABTS relative to DPPH, as illustrated in Figure 3. Single peptides did not show this correlation and showed generally lower interaction with ABTS or DPPH than the peptides as part of BSA. These results demonstrate the importance of the supporting protein to accommodating radical ligands, and the likely necessity of the whole protein for effective antioxidant activity.

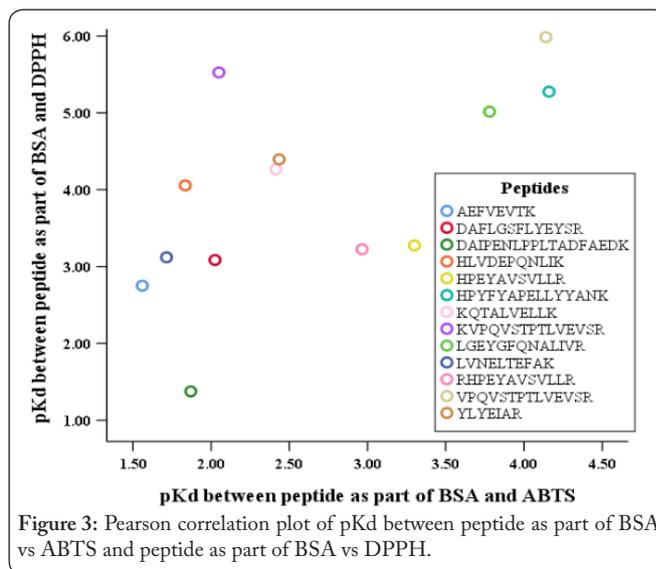


Figure 3: Pearson correlation plot of pKd between peptide as part of BSA vs ABTS and peptide as part of BSA vs DPPH.

Conclusion

Molecular modelling was successfully used to identify antioxidant activity of peptides isolated from the protein of Sumbawa mare milk without any peptide synthesis process. The antioxidant activity was found to correlate with the dissociation constant, which can be explained as resulting from the stability of peptide complexes with ABTS and DPPH. The comparison of pK_d and antioxidant activity gave a positive correlation when observed for peptides HPYFYAPELLYYANK and LVNELTEFAK. The stereochemistry and other peptide effects apparently result in this difference of pK_d , but full validation will be demonstrated by experimental studies in the future.

Conflict of Interest

There are no conflicts to declare.

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References

- Liu Z, Li G, Long C, Xu J, Cen J, et al. 2018. The antioxidant activity and genotoxicity of isogarcinol. *Food Chem* 253: 5-12. <https://doi.org/10.1016/j.foodchem.2018.01.074>
- Rani K. 2019. Clinical role of antioxidants in the treatment of diseases. In: *Nutraceutical and Functional Foods in Disease Prevention*. IGI Global, pp 392-401. <https://doi.org/10.4018/978-1-5225-3267-5.ch013>
- Ye N, Hu P, Xu S, Chen M, Wang S, et al. 2018. Preparation and characterization of antioxidant peptides

- from carrot seed protein. *Journal of Food Quality* 2018: 8579094. <https://doi.org/10.1155/2018/8579094>
4. Holmes AD, Spelman AF, Smith CT, Kuzmeski JW. 1947. Composition of mares' milk as compared with that of other species. *J Dairy Sci* 30: 385-395. [https://doi.org/10.3168/jds.S0022-0302\(47\)92363-1](https://doi.org/10.3168/jds.S0022-0302(47)92363-1)
 5. Malacarne M, Martuzzi F, Summer A, Mariani P. 2002. Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. *Int Dairy J* 12: 869-877. [https://doi.org/10.1016/S0958-6946\(02\)00120-6](https://doi.org/10.1016/S0958-6946(02)00120-6)
 6. Businco L, Giampietro PG, Lucenti P, Lucaroni F, Pini C, et al. 2000. Allergenicity of mare's milk in children with cow's milk allergy. *J Allergy Clin Immunol* 105: 1031-1034. <https://doi.org/10.1067/mai.2000.106377>
 7. Turk A (2017) A scientific and historical investigation on mongolian horses. *Sci. Appl. Hist. Mag.* <https://historic-journal.ru/scientific-and-historical-investigation-on-mongolian-horses>
 8. Sujaya IN, Putu N, Aryantini N, Wayan N, Cakrawati D, et al. 2012. exopolysaccharides from *Lactobacillus Sp.* isolated from sumbawa mare's milk and its potential application as prebiotics. *J Vet* 13: 136-144. <https://ojs.unud.ac.id/index.php/jvet/article/view/5995>
 9. Sasmito E, Rumiayati, Rahayu WS, Andriyani E, Istikharah R. 2006. Influence of fermented horse milk supplementation on immunity against hepatitis A vaccine in Balb / c mice. *Maj Farm Indones* 17(1): 13-18.
 10. Aryantini NPD, Yamasaki E, Kurazono H, Sujaya IN, Urashima T, et al. 2017. In vitro safety assessments and antimicrobial activities of *Lactobacillus rhamnosus* strains isolated from a fermented mare's milk. *Anim Sci J* 88(3): 517-525. <https://doi.org/10.1111/asj.12668>
 11. Murtini D, Aryantini NPD, Sujaya IN, Urashima T, Fukuda K. 2016. Effects of prebiotic oligosaccharides consumption on the growth and expression profile of cell surface-associated proteins of a potential probiotic *Lactobacillus rhamnosus* FSMM15. *Biosci Microbiota Food Health* 35(1): 41-49. <https://doi.org/10.12938/bmfh.2015-014>
 12. Kusumaningtyas E, Widiastuti R, Kusumaningrum HD, Suhartono MT. 2016. Bioactivities and analysis of peptides of sumbawa horse milk generated by *Bacillus thuringiensis* protease. *J Ilmu Ternak dan Vet* 21: 244-254. <http://doi.org/10.14334/jitv.v21i4.1627>
 13. Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Compos Anal* 19(6-7): 669-675. <https://doi.org/10.1016/j.jfca.2006.01.003>
 14. Wu RB, Wu CL, Liu D, Yang X, Huang J, et al. 2015. Overview of antioxidant peptides derived from marine resources: the sources, characteristic, purification, and evaluation methods. *Appl Biochem Biotechnol* 176: 1815-1833. <https://doi.org/10.1007/s12010-015-1689-9>
 15. Aluko RE. 2015. Amino acids, peptides, and proteins as antioxidants for food preservation. In: Shahidi F (ed) *Handbook of Antioxidants for Food Preservation*. Woodhead Publishing, Cambridge, pp 105-140. <https://doi.org/10.1016/B978-1-78242-089-7.00005-1>