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ENHANCING PROTEIN FUNCTIONALITY THROUGH DUAL CONJUGATION WITH GALLIC ACID AND RUTIN

Hyo Gyeong Lee[a], Jiseon Lee[b], Mi-Jung Choi[b,*]

[a]Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Republic of Korea
[b]Department of Department of Animal, Food Science and Marketing, Konkuk University, Seoul 05029, Republic of Korea
*Corresponding author email: choimj@konkuk.ac.kr

I. INTRODUCTION

Proteins are fundamental macromolecules in food systems, contributing to essential functional properties such as solubility, emulsification, gelation, and water-holding capacity. However, under processing conditions involving heat, pH fluctuations, and mechanical stress, many proteins exhibit limited solubility and conformational rigidity, which can impair their performance in complex food matrices [1]. To overcome these limitations, covalent conjugation of proteins with polyphenolic compounds has emerged as a promising strategy. Polyphenols possess multiple hydroxyl groups and intrinsic antioxidant capacity, which can enhance the physicochemical and bioactive properties of proteins. Under alkaline conditions, phenolic compounds are oxidized to quinone intermediates that can react with nucleophilic side chains on proteins, including lysine, cysteine, and tyrosine residues, forming Schiff bases or Michael addition products. These covalent modifications have been shown to improve protein solubility, thermal stability, and resistance to oxidative degradation [2]. Recent studies have demonstrated the effectiveness of polyphenol conjugation in various protein systems. For instance, the conjugation of chicken myofibrillar proteins with (-)-epigallocatechin gallate (EGCG) or tannic acid significantly improved antioxidant properties, water-holding capacity, and emulsifying behavior [3]. Similarly, conjugation of ovalbumin with polyphenols enhanced radical-scavenging activity and thermal stability. Studies on whey protein–polyphenol conjugates further support their ability to enhance protein functionality and oxidative resistance [1]. These findings suggest that protein–polyphenol conjugation can be used to tailor protein functionality based on polyphenol structure and reaction conditions. Moreover, pretreatment methods such as thermal denaturation and ultrasonication can increase the accessibility of reactive amino acid residues by unfolding the protein structure and disrupting aggregates, thereby enhancing polyphenol binding efficiency. These combined strategies offer a versatile approach to improve protein performance in food formulations. In this study, proteins pretreated with heat and ultrasonication were conjugated with gallic acid (GA) and rutin (RU) using a pH-driven method. The effects of different RU-to-GA ratios on polyphenol binding, antioxidant capacity, and structural modification were evaluated, providing insight into the design of functional protein–polyphenol ingredients with enhanced processing stability.

II. MATERIALS AND METHODS

A protein dispersion (4 wt%) was prepared and subjected to heat and ultrasonication treatment. The treated sample was adjusted to pH 10.0 and stirred at 500 rpm for 30 minutes. Polyphenol solutions containing different rutin-to-gallic acid (RU-to-GA) ratios were also adjusted to pH 10.0 and stirred for 10 minutes. The protein and polyphenol solutions were then mixed in a 1:1 volume ratio, pH was readjusted to 10.0 using 1 M NaOH, and the mixture was stirred at 500 rpm for 30 minutes. The reaction was terminated by adjusting the final pH to 7.0 using 1 M HCl.

Table 1. Formulation ratios of protein-GA/RU conjugates

Samples (GA : RU)	Total polyphenol concentration (%)					
	GA 0.05 % (w/v)		GA 0.10 % (w/v)		GA 0.20 % (w/v)	
	GA	RU	GA	RU	GA	RU
1.0 : 0.0	0.05	0.00	0.10	0.00	0.20	0.00
1.0 : 0.2		0.01		0.02		0.04
1.0 : 0.4		0.02		0.04		0.08
1.0 : 0.8		0.04		0.08		0.16
1.0 : 1.0		0.05		0.10		0.20

GA, gallic acid; RU, rutin.

III. RESULTS AND DISCUSSION

The particle size of the protein–polyphenol conjugates decreased with increasing polyphenol content. This reduction is likely attributed to the inhibition of protein aggregation through polyphenol binding, which shields hydrophobic

regions and minimizes protein–protein interactions. ζ-potential values showed a slight decrease in absolute magnitude with increasing polyphenol concentration, suggesting surface charge neutralization by phenolic compounds and reduced electrostatic repulsion. Structural characterization revealed an increase in both free amino group and sulfhydryl (–SH) content. This suggests that polyphenols, particularly through their hydroxyl groups, formed covalent bonds with amino groups via C–N linkage. Simultaneously, disruption of disulfide (S–S) bonds likely occurred, exposing additional SH groups and reflecting partial unfolding of the protein structure. These structural changes enhanced the conformational flexibility and reactivity of the protein. Notably, sulfhydryl (–SH) content increased progressively with higher RU-to-GA ratios, suggesting more effective disulfide bond cleavage and partial protein unfolding. RU's higher hydroxyl group density and bulkier structure likely promoted stronger interactions with protein backbones, enhancing conjugation efficiency and structural reactivity. These findings highlight that RU incorporation not only improves polyphenol binding and structural flexibility but also contributes to reduced particle size and improved dispersion behavior in protein–polyphenol conjugate systems.

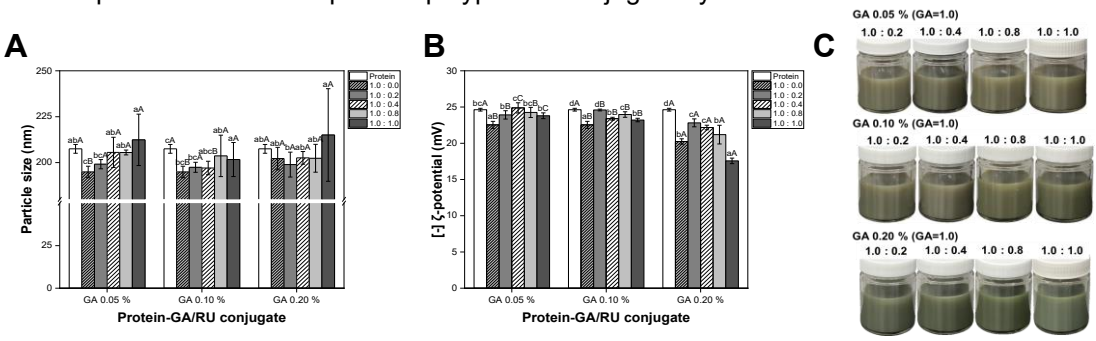


Fig. 1. Particle size (A) and [-] ζ-potential (B), and visual appearance (C) of protein-GA/RU conjugates with various GA concentrations. RU content adjusted based on RU-to-GA ratios ranging from 0.2 to 1.0. GA, gallic acid; RU, rutin.
[a–d] Data labeled with different letters indicate statistically significant differences for same RU-to-GA ratios at $P < 0.05$.
[A–C] Data labeled with different letters indicate statistically significant differences for same GA concentration ratio at $P < 0.05$.

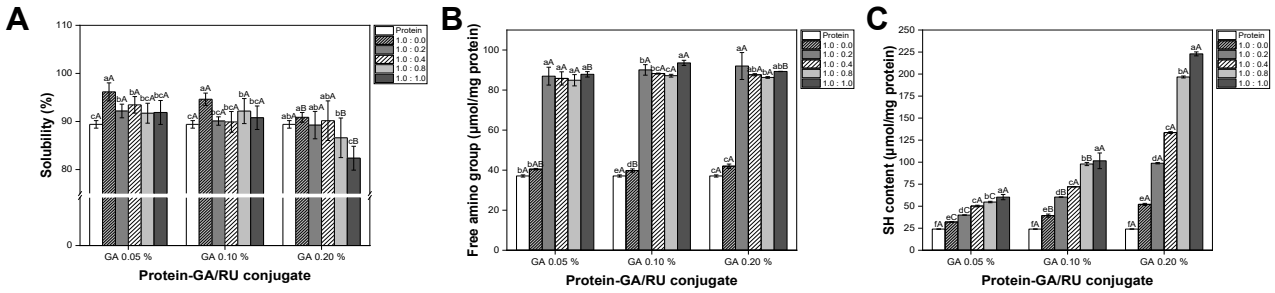


Fig. 2. Characterization of protein-polyphenol conjugate nanoparticles; solubility (A), free amino group content (B), SH content (C), polyphenol conjugate equivalent (D), and SDS-PAGE results (E, F) of protein-GA/RU conjugates with various GA concentrations. RU content adjusted based on RU-to-GA ratios ranging from 0.2 to 1.0. GA, gallic acid; RU, rutin.
[a–e] Data labeled with different letters indicate statistically significant differences for same RU-to-GA ratios at $P < 0.05$.
[A–C] Data labeled with different letters indicate statistically significant differences for same GA concentration ratio at $P < 0.05$

IV. CONCLUSION

The covalent conjugation of protein with gallic acid (GA) and rutin (RU) effectively improved its physicochemical and functional properties. Rutin incorporation led to significantly higher polyphenol binding efficiency and sulfhydryl content than gallic acid alone, indicating stronger structural modifications such as disulfide bond cleavage and protein unfolding. These changes contributed to enhanced solubility and reduced particle size, promoting better dispersion stability. The dual-polyphenol conjugation approach, particularly with higher RU ratios, demonstrated synergistic effects that enhance protein reactivity and stability. These findings support the potential application of protein–polyphenol conjugates as functional carriers for bioactive compounds in food and nutraceutical systems.

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