



Short communication

Mechanisms of plastein formation influence the IgE-binding activity of egg white protein hydrolysates after simulated static digestion

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ABSTRACT

Egg is the second most common food allergen among infants and young children. This work investigated the influence of plastein reaction on immunoglobulin E (IgE)-binding activities of egg white protein hydrolysates after simulated gastrointestinal (GIT) digestion. Compared to hydrolysate precursors, the IgE-binding activity of Pepsin-Plastein significantly decreased from $35 \pm 7\%$ to $8 \pm 2\%$ ($P < 0.05$), and Papain-Plastein from $70 \pm 5\%$ to $59 \pm 4\%$. Further GIT hydrolysis of Pepsin-Plastein maintained the reduced IgE-binding activity ($7 \pm 3\%$) whereas Papain-Plastein digestion restored the IgE-binding reactivity to $66 \pm 7\%$. This discrepancy is related to the different mechanisms of plastein formation. Covalent modifications (decreased free amino nitrogen and sulfhydryl contents) provided biostability for Pepsin-Plastein, whereas hydrophobic interactions (increased surface hydrophobicity) mainly contributed to Papain-Plastein formation. The latter can be destroyed during GIT digestion leading to re-exposure of hidden IgE-binding epitopes. Taken together, plastein reaction is a promising strategy for inducing structural modifications that reduce the immune reactivity of allergenic proteins.

1. Introduction

Plastein is a less soluble aggregated macromolecular structure formed by concentrated hydrolyzed proteins or peptides ($\sim 30\text{--}50\%$, w/v) in the presence of appropriate proteases under optimum conditions (Gong, Mohan, Gibson, & Udenigwe, 2015; Udenigwe & Rajendran, 2016). Plastein was initially proposed as an efficient means of debittering food protein hydrolysates and providing essential amino acids (Gong et al., 2015). However, it has limited commercial potential due to the low yield and high cost of production. In the last decade, research efforts have been shifted towards the identification of the biological activities of plastein to explore its health benefits, such as antihypertensive, antioxidative, antithrombotic and metal-chelating activities (Sun & Zhao, 2012; Udenigwe, Wu, Drummond, & Gong, 2014; Zhang & Zhao, 2014).

Among different proteases, pepsin and papain are most widely used for plastein reaction (Edwards & Shipe, 1978; Yamashita, Arai, Tsai, &

Fujimaki, 1971). The precise mechanism of plastein formation has not been completely revealed. Some studies reported that peptide condensation and transpeptidation induced by covalent modifications are the major mechanisms responsible for plastein formation (Hajós, Elias, & Halasz, 1988; Stevenson, Ofman, Morgan, & Stanley, 1998; Yamashita et al., 1971). In contrast, others suggested that plastein is largely formed by the physical interactions of hydrophobic peptides (Andrews & Ali-chanidis, 1990). Due to the presence of covalent bonds or hydrophobic forces, plastein has been demonstrated to be stable at extreme conditions, such as acidic and alkaline pH ranging from 2 to 11 and at 100°C (Brownell, Williams, & Andrews, 2001; Udenigwe et al., 2014). Plastein was also reported to be resistant to gastric protease digestion, although conflicting results were reported (Sun & Zhao, 2012; Udenigwe et al., 2014). Overall, the mechanism of plastein reaction may play a role in determining the stability of plastein (e.g. stability to gastrointestinal and physical treatments) and retention of bioactive properties *in vivo*, for potential application as functional foods.

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Food allergy is the abnormal response of the body's immune system to certain food or food components, usually food proteins (Li, Offengenden, Fentabil, Gänzle, & Wu, 2013; Taylor & Hefle, 2001). Food allergy can cause urticaria, angioedema, atopic dermatitis, asthma, and even can be life threatening (Novembre et al., 1998). Egg is the second most common food allergen among infants and young children, and the estimated prevalence of egg allergy ranges from 1.6% to 3.2% of the total children population (Li et al., 2013). Therefore, reducing the allergenicity of egg protein would increase egg consumption, especially in the children population. The structural rearrangement of proteins/peptides during plastein formation may result in modifying epitopes and reducing immune-reactivity of allergenic proteins. IgE-mediated immune response, also known as type-I food hypersensitivity, is the dominant type of food allergy (Kagan, 2003; Li et al., 2013). Binding between IgE and food allergens results in the release of inflammatory mediators, such as histamine, and is directly responsible for the occurrence of clinical allergic symptoms (Bradding et al., 1992; Li et al., 2013). Thus, IgE-binding activity was applied in this study to evaluate potential allergenic property. The objectives of this study were to determine the effect of plastein reaction on the *in vitro* IgE-binding property of egg white protein (EWP) hydrolysates before and after simulated digestion, and to elucidate the possible chemical mechanisms of the peptide modification.

2. Materials and methods

2.1. Materials and chemicals

Egg white (7 g protein/63 g egg white, Naturegg simply egg whites, Burnbrae farms, Brockville, ON) was purchased from a local store in Ottawa (ON, Canada). Calcium chloride, α -amylase from porcine pancreas, pancreatin from porcine pancreas, pepsin from porcine gastric mucosa, papain from papaya latex, sodium dodecyl sulfate (SDS), o-phthalaldehyde (OPA), dithiothreitol, L-serine, hydrochloric acid (37%), glycine, urea, 5,5'-dithiobis-[2-nitrobenzoic acid], and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Millipore Sigma (Burlington, MA, USA). Milli-Q water was prepared using the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).

2.2. Protein hydrolysis and plastein formation

The egg white solution was diluted with deionized water to 0.1% (w/v, protein basis) and hydrolyzed for 5 h with pepsin (pH 2, 37 °C) or papain (pH 7, 65 °C) at E/S ratio of 1:100 (w/w, protein basis). The optimum pH of pepsin and papain were maintained by adding 0.1 N HCl and 0.1 N NaOH, respectively. The reaction was terminated by heating the mixtures at 90 °C for 15 min, adjusted to pH 7, followed by centrifugation at 10,000 $\times$ g for 10 min, and the supernatant freeze-dried and used as the hydrolysate powder. To produce the plastein, hydrolysate powders were suspended in deionized water (40%, w/v) either pepsin or papain added to their respective hydrolysates at E/S 1:100 (w/w, protein basis). The mixtures were stirred gently at 37 °C for 16 h, frozen at -20 °C and freeze dried to recover the pepsin-induced (Pepsin-Plastein) and papain-induced (Papain-Plastein) plastein products (Brownsell et al., 2001; Udenigwe et al., 2014).

2.3. Characterization of EWP hydrolysates and plasteins

2.3.1. Turbidity measurement

The turbidity of samples in water was determined as reported by Udenigwe et al. (2014), with modifications. The EWP hydrolysates and plasteins freeze-dried powders were suspended in deionized water at 3 mg/mL and stood for 15 min at room temperature before vortex mixing. The absorbance of the solution or suspension was then measured at 460 nm.

2.3.2. Free amino nitrogen content determination

Free amino nitrogen of the EWP hydrolysates and plasteins was determined using the OPA method (Nielsen et al., 2001). Briefly, 0.4 mL of sample (1 mg/mL) was mixed with 3 mL of OPA reagent containing sodium dodecyl sulfate (SDS) and dithiothreitol. The absorbance of the mixture was measured at 340 nm after 2 min incubation at room temperature. Serine, at the concentration of 0.1 mg/mL, was used as the standard and the free amino nitrogen was calculated as milliequiv of serine NH_2/g protein. All samples were solubilized in deionized water.

2.3.3. Surface hydrophobicity determination

Surface hydrophobicity of the EWP hydrolysates and plasteins was assayed by the fluorescence method using ANS as a hydrophobic probe (Hayakawa & Nakai, 1985). The samples were analyzed at concentrations of 0.0009–0.015% (w/v) with excitation and emission wavelengths of 390 and 470 nm, respectively. Surface hydrophobicity was determined as the initial slope (S_0) of the fluorescence intensity versus concentration (%) plot.

2.3.4. Particle size and polydispersity index (PDI)

Particle size and PDI of the hydrolysates and plasteins were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., UK) at 25 °C. The measured samples were suspended in Milli-Q water (1 mg/mL) and each measurement was performed after 120 s equilibration (Sun, Ohanenye, Ahmed, & Udenigwe, 2020).

2.3.5. Determination of sulfhydryl group content

The free sulfhydryl group content of the hydrolysates and plasteins was measured to determine peptide aggregation (Hong et al., 2012). First, samples (0.5 mL, 10 mg/mL) were mixed with 2 mL of 0.1 M Tris-glycine buffer containing 10 M urea (pH 8.0), and incubated at 40 °C for 30 min. Then, 62.5 μL of 5,5'-dithiobis(2-nitrobenzoic acid) (4 mg/mL 0.1 M Tris-glycine buffer, pH 8) was added to the mixture. The resulting solution was incubated at room temperature for 10 min, and the absorbance was measured at 412 nm. The total sulfhydryl group concentration (μM SH/g of protein) was calculated using the following Eq. (1):

$$\text{SH}(\mu\text{mol/g}) = \frac{73.53 \times (\text{A}_{\text{sample}} - \text{A}_{\text{blank}}) \times D}{C} \quad (1)$$

here, D is the dilution factor and C is the sample concentration (mg/mL).

2.4. *In vitro* simulated gastrointestinal (GIT) digestion

Simulated GIT digestion was carried out according to the standardised static *in vitro* digestion model with oral, gastric and intestinal phases at 37 °C (Sun et al., 2020). In the oral phase, suspension of hydrolysates or plasteins was mixed with a solution comprising simulated salivary fluid and salivary α -amylase (1500 U/mL) for 2 min. In the gastric phase, the resulting bolus from the oral phase was incubated with the solution of simulated gastric fluid and porcine pepsin (25000 U/mL) at pH 3.0 for 2 h. The gastric digesta was further subjected to intestinal digestion (150 rpm, pH 7.0, 2 h) by adding a solution containing simulated intestinal fluid, bile extract (10 mM) and pancreatin (100 trypsin U/mL). The resulting digested samples were frozen at -20 °C until further analysis. The degree of hydrolysis (DH) of the digesta samples was determined by the OPA method (Nielsen et al., 2001).

2.5. Immunoglobulin (Ig) E binding assay

The immune-reactivity of the EWP hydrolysates, plasteins and their digests was measured by a sandwich ELISA kit using egg white protein as a positive control (Maplewood, MA, USA) (Gazme, Rezaei, & Udenigwe, 2020). A concentration of 100 ng/mL of each sample was used for the analysis. The immune-reactivity was expressed as percentage compared

Table 1
Physicochemical properties of egg white protein (EWP) hydrolysates and their plasteins.

Sample	Free amino nitrogen (mg/g protein)	Turbidity in water	Size (nm)	PDI	Sulfhydryl group ($\mu\text{M/g}$ protein)	Surface hydrophobicity (S_0)
PepHyd	63.2 ± 2.1^b	0.045 ± 0.003^a	234 ± 129^b	0.61 ± 0.09^{ab}	3.50 ± 0.09^c	11999 ± 344^c
PepPlast	46.8 ± 3.1^a	0.045 ± 0.000^a	244 ± 96^b	0.41 ± 0.07^a	2.38 ± 0.06^b	14052 ± 390^c
PapHyd	42.9 ± 3.9^a	0.046 ± 0.000^a	268 ± 93^b	0.83 ± 0.06^b	9.90 ± 0.73^a	16436 ± 152^b
PapPlast	40.3 ± 4.2^a	0.048 ± 0.001^a	557 ± 209^a	0.69 ± 0.15^{ab}	9.53 ± 0.23^a	19310 ± 752^a

PDI, polydispersity index; PepHyd and PapHyd, EWP hydrolysates produced with pepsin and papain, respectively; PepPlast and PapPlast, plastein produced from EWP hydrolysates using pepsin and papain, respectively.

Different letters in the same column indicate significantly different mean values ($P < 0.05$).

to the positive control, which was set to be 100%.

2.6. Statistical analysis

All assays were performed in triplicate and the results were expressed as mean $\pm$ standard deviation (SD). All statistical analysis was conducted by one-way ANOVA (Analysis of variance) using IBM SPSS statistics 19 (SPSS Inc, USA). Significant differences were defined at a 5% level ($P < 0.05$).

3. Results and discussion

3.1. Mechanisms of pepsin- and papain-induced plastein formation from egg white protein hydrolysates are different

In this study, pepsin and papain were used to prepare plasteins from EWP hydrolysates. To elucidate the possible mechanisms of the plastein formation, free amino nitrogen, turbidity, particle size, PDI, sulfhydryl group content and surface hydrophobicity were evaluated (Table 1). Decreased free amino nitrogen after plastein formation can be used to support the peptide condensation or transpeptidation mechanism (Gong et al., 2015). Compared to its protein hydrolysate precursor, the free amino nitrogen of pepsin-induced EWP plastein (Pepsin-Plastein) decreased significantly by 35% ($P < 0.05$). However, there was no significant decrease in the free amino nitrogen content of papain-induced EWP plastein (Papain-Plastein) (Table 1). The turbidity of the EWP hydrolysate and plastein solutions was measured to monitor if peptide aggregation occurred during plastein reaction (Udenigwe et al., 2014). Neither Pepsin-Plastein nor Papain-Plastein significantly changed the turbidity in water ($P > 0.05$); although not statistically significant, the turbidity of Papain-Plastein increased by 6% compared to its

hydrolysate precursor.

Furthermore, increased particle size of hydrolysates indicates peptide aggregation during plastein reaction (Gong et al., 2015). As shown in Table 1, the plastein reaction induced by papain significantly increased the particle size by two folds whereas there was no change for the plastein reaction with pepsin. The molecular weight ranges were not significantly changed by both plastein reactions, based on the PDI ($P < 0.05$), but the lower values obtained for the plasteins indicate a tendency towards more uniform particles compared to the hydrolysates. A decrease in sulfhydryl group content suggests the formation of covalent disulfide bonds, which may be involved in the stabilization of plastein structures (Udenigwe, Mohan, & Wu, 2015). Only Pepsin-Plastein had a significantly (32%) decreased sulfhydryl group content ($P < 0.05$) compared to the hydrolysate precursor (Table 1). The increased surface hydrophobicity (S_0) was also used to support the hydrophobic interaction mechanism (Udenigwe et al., 2015). Compared to their hydrolysate precursors, significantly increased S_0 was observed only in Papain-Plastein.

Taken together, condensation and transpeptidation induced by covalent modifications appear to be the major mechanisms of the pepsin-mediated plastein synthesis from EWP hydrolysates due to the decreased free amino nitrogen and SH contents, and no significant changes in turbidity, particle size and surface hydrophobicity. On the other hand, the increased turbidity, particle size and surface hydrophobicity values associated with the papain-induced plastein formation suggested that hydrophobic interaction is mostly involved in peptide aggregation. The different mechanisms of plastein formation is expected to influence the property of the peptide aggregate product. Thus, the EWP-derived Pepsin-Plastein and Papain-Plastein would likely behave differently in terms of their IgE reactivity and behaviour in simulated GIT digestion system.

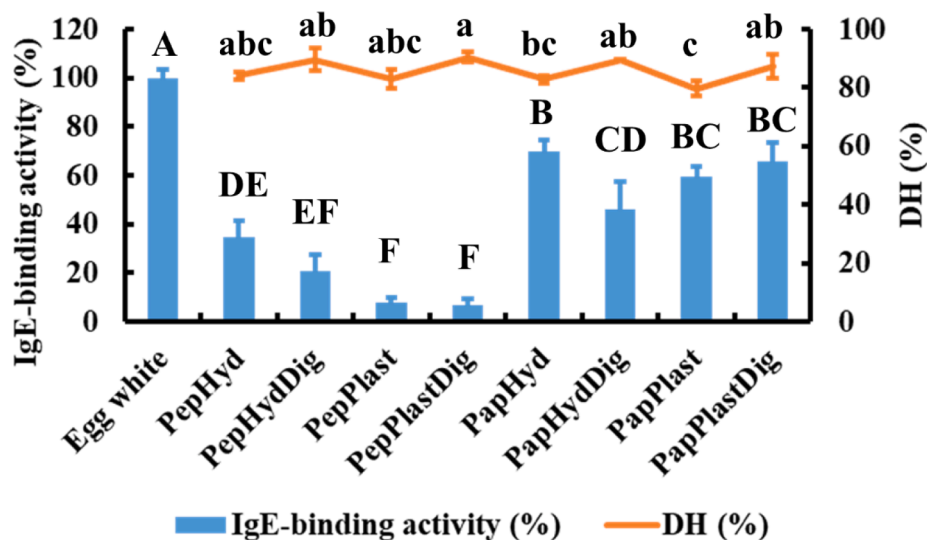


Fig. 1. The IgE-binding activity and degree of hydrolysis (DH) of egg white protein hydrolysates, their plasteins and simulated gastrointestinal digesta. Different letters indicate significantly different mean values ($P < 0.05$).

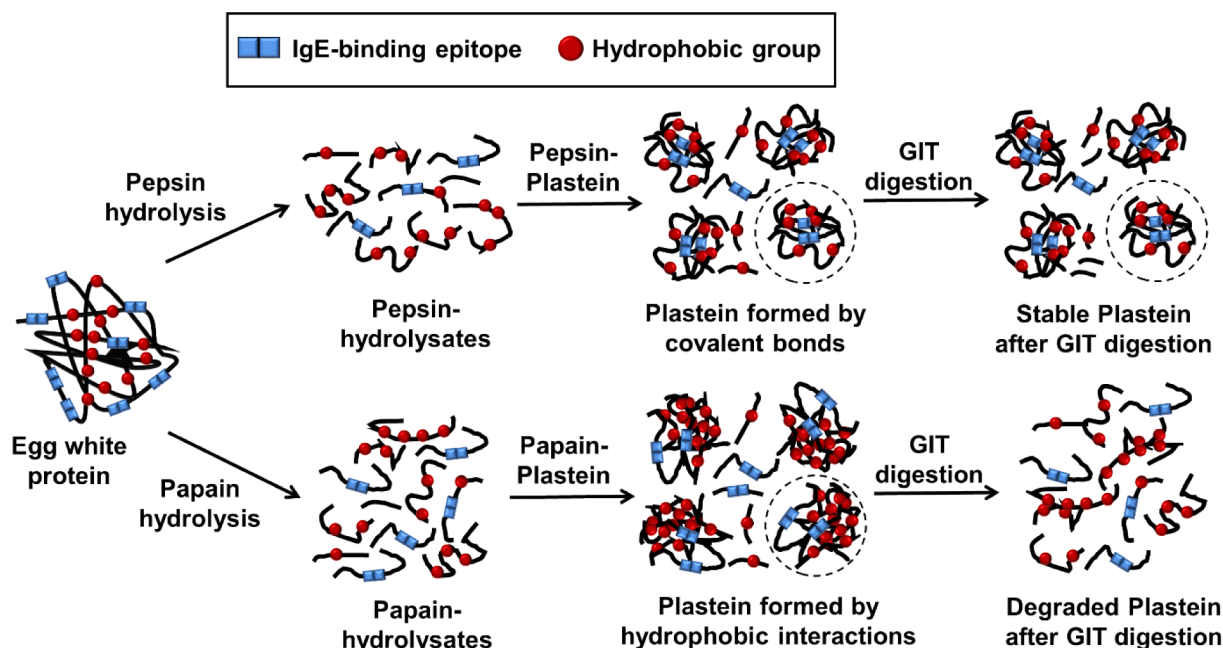


Fig. 2. Schematic representation of the influence of plastein formation mechanisms induced by pepsin or papain on IgE-binding activities of egg white protein hydrolysates after simulated static digestion.

3.2. IgE-binding activity of egg white protein hydrolysates is reduced by plastein reaction: functional biostability depends on formation mechanism

Enzymatic hydrolysis is the most commonly used approach to reduce the IgE-binding activity of food allergens possibly due to the degradation of food proteins and destruction of IgE-binding immunodominant epitopes (Li et al., 2013; Sathe, Teuber, & Roux, 2005; Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005). As shown in Fig. 1, IgE-binding activities of EWP hydrolyzed by pepsin and papain were significantly decreased from 100% to $35 \pm 7\%$ and $70 \pm 5\%$, respectively. The cleavage specificity of pepsin is influenced by the amino acid residues at position P1; for example, Phe and Leu are favored residues for pepsin cleavage, but His, Lys, Arg, or Pro residues prevent cleavage when occupying the P1 position (Hamuro, Coales, Molnar, Tuske, & Morrow, 2008). Papain shows a preference for the amino acid residues bearing a large hydrophobic side chain at the position P2 while does not accept Val at the position P1 (Kamphuis, Drenth, & Baker, 1985). These distinct recognition and cleavage specificities of pepsin and papain may lead to different effects in EWP epitope hydrolysis and thus responsible for the different IgE-binding activities of the resulting hydrolysates. After *in vitro* simulated GIT digestion, the IgE-binding activities further reduced to $21 \pm 7\%$ and $46 \pm 11\%$, respectively. However, the DH values of hydrolysates prepared with pepsin or papain and their respective *in vitro* GIT digesta were not different ($P > 0.05$).

As shown in Fig. 1, plastein reaction caused further decreases in IgE-binding activity but no significant decreases in DH compared to the hydrolysate precursors. Specifically, the IgE-binding activity of Pepsin-Plastein significantly decreased from $35 \pm 7\%$ in the hydrolysate precursor to $8 \pm 2\%$ ($P < 0.05$), and Papain-Plastein reduced the IgE-binding activity from $70 \pm 5\%$ in the hydrolysate to $59 \pm 4\%$. These results confirmed one part of our hypothesis that plastein reaction causes peptide rearrangement that can modify the biomolecular interactions with IgE and thus reduce their immune reactivity *in vitro*. Intriguingly, further GIT hydrolysis of Pepsin-Plastein did not change the IgE-binding activity ($7 \pm 3\%$) compared to the undigested Pepsin-Plastein ($8 \pm 2\%$). However, GIT digestion of Papain-Plastein caused a restoration of the IgE-binding reactivity ($66 \pm 7\%$), which is similar to the initial value for its hydrolysate precursor ($70 \pm 5\%$). Furthermore, the DH of Papain-Plastein after simulated GIT digestion increased ($P <$

0.05) from $80 \pm 3\%$ to $87 \pm 4\%$, whereas that of Pepsin-Plastein did not change significantly after digestion (Fig. 1). These discrepancies may be attributed to the different mechanisms of the pepsin- and papain-induced plastein formation. It was demonstrated that disulfide bonds significantly contribute to protecting the intact structure and stability of IgE-binding epitopes, making them biostable and resistant to GIT digestion (Sen, Kopper, Pons, Abraham, Burks, & Bannion, 2002). Therefore, the retained IgE-binding activity of Pepsin-Plastein after GIT digestion confirms the possibility that covalent modifications are the major mechanism of the pepsin-induced EWP plastein synthesis. On the other hand, Papain-Plastein is possibly held together by hydrophobic interactions, which would be destroyed during GIT hydrolysis (as indicated by the increased DH) mainly due to the presence of bile extract. Thus, the hidden IgE-binding epitopes in Papain-Plastein may re-expose and regain their IgE-binding activity after GIT digestion. The influence of the different mechanisms of EWP plastein formation on IgE-binding activities of the products after simulated static digestion are illustrated in Fig. 2.

4. Conclusion

This study demonstrated that plastein reaction resulted in further decreases in IgE-binding activity due to the peptide rearrangement compared to the EWP hydrolysate precursors. However, the different mechanisms of plastein formation played a critical role in the IgE-binding activities after simulated static digestion. Covalent modification was the major mechanism of the Pepsin-Plastein synthesis, which irreversibly maintained the low IgE-binding activity of Pepsin-Plastein after GIT digestion. On the other hand, hydrophobic interactions were mainly responsible for Papain-Plastein formation, which were not stable and reversible during GIT digestion and resulted in the restoration of IgE-binding activity. Future studies would further characterize the proposed covalent modification by pepsin during EWP plastein reaction as well as cellular and *in vivo* validation of the biostability using IgE-mediated food allergy models. Also, plastein reaction can be explored for reducing the IgE-binding activity of allergenic proteins from other food sources.

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CRediT authorship contribution statement

Xiaohong Sun: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, revision. **Caleb Acquah:** Formal analysis, Investigation, Methodology, Validation. **Behzad Gazme:** Investigation, Methodology, Validation. **Ruth T. Boachie:** Investigation, Methodology. **Ifeanyi D. Nwachukwu:** Investigation, Methodology. **Chibuike C. Udenigwe:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing - original draft, revision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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