

# Covalent versus noncovalent chlorogenic acid binding: Divergent effects on the gastrointestinal fate and allergenic potential of soybean 7S globulin

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## Article

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# Abstract

Soybean 7S globulin, a major allergen, poses challenges due to its resilience to gastrointestinal digestion and the persistence of its antigenic fragments. This study elucidates how the mode (covalent vs. noncovalent) and concentration of chlorogenic acid (CHA) interaction coordinately govern the digestibility and IgE-binding antigenicity of 7S globulin. Through molecular docking, simulated digestion, and immunochemical assays, we demonstrate that CHA engages with 7S globulin via two fundamentally distinct paradigms. Noncovalent binding, particularly at a low concentration (0.3 mM), promotes a more flexible protein conformation, significantly enhancing the degree of hydrolysis (25.12%) and facilitating the proteolytic clearance of the immunoreactive ~ 55 kDa core fragment. In stark contrast, covalent conjugation, even at equivalent concentrations, imposes stable steric and conformational constraints, effectively masking linear IgE epitopes and reducing the retention of ThT-responsive amyloid-like aggregates. Notably, higher CHA concentrations (1.0 mM) diminish the hydrolysis-promoting effect for both modes, yet maintain a low IgE-binding capacity, suggesting a shift towards direct epitope masking over degradation-dependent clearance. Collectively, our findings reveal that noncovalent CHA interactions primarily act as "proteolysis enhancers," while covalent interactions function as "epitope stabilizers/maskers." This work provides a mechanistic framework for rationally designing hypoallergenic soybean ingredients by selecting appropriate polyphenol interaction strategies.

## 1. Introduction

Soybean protein is widely used in food products owing to its high nutritional value and favorable processing properties <sup>1</sup>. However, soybean is also an important source of food allergens, and  $\beta$ -conglycinin, also known as soybean 7S globulin, is one of its major IgE-reactive allergens <sup>2, 3</sup>. Its  $\alpha$ ,  $\alpha'$ , and  $\beta$  subunits contain multiple IgE-reactive regions, and its compact oligomeric structure may contribute to the persistence of antigenic fragments during gastrointestinal digestion <sup>4</sup>. Therefore, the IgE-binding antigenicity of soybean 7S globulin depends not only on the epitopes present in the native protein but also on their exposure, degradation, and structural rearrangement during digestion <sup>5, 6</sup>.

Gastrointestinal digestion is a critical process that determines the quantity and structural state of food allergens reaching the intestinal immune system <sup>7</sup>. Previous studies have shown that soybean 7S globulin can generate digestion-resistant fragments, particularly those of approximately 50–55 kDa, that may retain IgE-reactive epitopes <sup>8, 9</sup>. In addition, native soybean 7S globulin contains amyloid-like aggregates with characteristic cross- $\beta$ -sheet structures <sup>4</sup>. The compact organization and digestion resistance of these aggregates may promote the persistence and presentation of IgE-reactive structures after digestion <sup>3</sup>. Consequently, assessing protein modification solely in the undigested state may overlook digestion-induced changes in epitope accessibility and the retention of antigenic fragments <sup>5, 6</sup>.

Protein–polyphenol interactions are increasingly recognized as a potential strategy for modulating protein structure, digestibility, and IgE recognition <sup>10, 11</sup>. Chlorogenic acid is a naturally occurring

phenolic acid containing multiple hydroxyl groups and a catechol moiety, which enable both noncovalent and covalent interactions with proteins 12. Noncovalent interactions are primarily mediated by hydrogen bonding, hydrophobic interactions, van der Waals forces, and  $\pi$ -related interactions, whereas oxidized CHA can form stable covalent bonds with nucleophilic amino acid residues 13. These interaction modes may exert different effects on protein conformation, proteolytic-site accessibility, aggregate stability, and epitope recognition 10, 14, 15. These effects may also vary with CHA concentration and may not follow a simple dose–response relationship 16, 17.

Previous studies have shown that polyphenol modification can reduce antibody binding to several food allergens by inducing conformational rearrangement, directly modifying or masking epitopes, and altering digestibility 11, 15. CHA has likewise been reported to reduce IgE binding to soybean 7S globulin through both covalent and noncovalent interactions 2. Nevertheless, the effects of CHA interaction mode and concentration on the dynamic structural changes and IgE-binding antigenicity of soybean 7S globulin during gastrointestinal digestion remain unclear 2, 4. Moreover, whether covalent and noncovalent interactions reduce IgE binding through similar or distinct digestion- and epitope-related mechanisms has not been fully resolved.

Therefore, the present study systematically compared covalent and noncovalent soybean 7S globulin–CHA complexes prepared with different CHA concentrations. Molecular docking was used to characterize the potential binding modes of CHA and their spatial relationships with IgE-reactive regions. Changes in digestion-resistant fragments and the degree of hydrolysis were monitored throughout simulated gastrointestinal digestion, while secondary structure and amyloid-like aggregate retention were assessed at key digestion stages. These digestion-related changes were then linked to overall IgE-binding capacity and the molecular-weight distribution of IgE-reactive fragments. The study aimed to clarify how CHA interaction mode and concentration jointly influence the digestion process and IgE-binding antigenicity of soybean 7S globulin, thereby providing a mechanistic basis for developing soybean protein ingredients with reduced IgE binding.

## 2. Results

### 2.1. Predicted binding modes and intermolecular interactions of soybean 7S globulin–CHA complexes

Molecular docking was performed to identify potential binding sites and characterize the binding conformations and major intermolecular interactions of soybean 7S globulin–CHA complexes. As shown in Fig. 1, CHA displayed distinct predicted binding patterns in the noncovalent and covalent complexes. In the noncovalent complex (Fig. 1A), CHA adopted an extended conformation within a surface groove of the protein. The polyhydroxylated moiety of CHA formed a hydrogen bond with Glu333 and weak polar contacts with Ser331 and Tyr313. Its aromatic moiety formed conventional hydrogen bonds with Thr384 and Arg329, as well as an amide– $\pi$  interaction with Ser385 and a  $\pi$ –alkyl interaction

with Arg329. Ala312, Ser315, Ile330, Gly332, Asp334, Asn369, Ser383, and Ser386 also formed van der Waals contacts with CHA, contributing to its stabilization within the binding pocket. Collectively, the predicted noncovalent association between CHA and soybean 7S globulin was stabilized by hydrogen bonding,  $\pi$ -related interactions, and van der Waals forces. Consistent with these findings, Ma et al. (2024) reported that CHA interacted noncovalently with soybean 7S and 11S globulins through hydrophobic interactions, hydrogen bonding, and van der Waals forces, which were associated with a more compact and stable protein conformation 18.

In the covalent complex (Fig. 1B), oxidized CHA fit within the predicted binding pocket of the protein, with a CDOCK affinity score of  $-3.676$ . The docking model predicted a C–N covalent bond between oxidized CHA and Lys393, consistent with nucleophilic attack by the  $\epsilon$ -amino group of Lys393 on the quinone moiety through Michael addition 18. In addition to the predicted covalent linkage, Tyr189 formed a  $\pi$ – $\pi$  stacking interaction with the quinone ring, while Lys393 participated in a  $\pi$ –alkyl interaction. The carboxyl group of CHA formed a salt bridge with Lys193, and hydrogen bonds stabilized the polyhydroxylated moiety. Ala395 and Ser192 also formed local hydrogen-bonding or weak polar contacts. Thr191, Asp396, Phe398, Asn399, Phe400, and Ala401 formed a network of van der Waals contacts around the ligand. Thus, the predicted covalent complex was stabilized by the covalent linkage together with a network of salt-bridge, hydrogen-bonding, aromatic, and van der Waals interactions. This binding pattern was consistent with the findings of Yun et al. (2024), who reported covalent attachment of CHA to Lys residues in soybean protein, accompanied by hydrogen bonding between the remaining hydroxyl groups and Ser or Lys residues 2.

The predicted noncovalent interaction residues were mainly located in  $\alpha$ -helical and  $\beta$ -sheet regions (Fig. 1A1), whereas those involved in covalent binding were primarily located in  $\alpha$ -helical and random-coil regions (Fig. 1B1). This distribution suggested that the two interaction modes could affect different structural regions of soybean 7S globulin. Previous studies have shown that the three-dimensional conformation of soybean 7S globulin contributes to the integrity and accessibility of IgE epitopes. Some IgE-reactive regions are located in exposed  $\alpha$ -helical or random-coil regions and may therefore be readily accessible to antibodies 19–20. Accordingly, CHA binding may perturb the local organization and accessibility of IgE-reactive regions in soybean 7S globulin, thereby contributing to changes in its IgE-binding antigenicity.

## 2.2. Potential overlap between CHA-binding residues and linear IgE epitopes

To assess whether CHA-binding residues were associated with known IgE-reactive regions of soybean 7S globulin, the interaction residues identified by molecular docking were positionally mapped to experimentally identified human linear IgE epitope records for Gly m 5 retrieved from IEDB.

As shown in Table 1, the predicted interaction residues of the noncovalent complex clustered mainly within three regions, residues 329–334, residue 369, and residues 383–386, and overlapped with nine IEDB linear IgE epitope records, several of which contained overlapping sequences. Specifically, Arg329,

Ile330, Ser331, Gly332, Glu333, and Asp334 mapped to several overlapping epitope sequences, including KEQIRALSKRAKSSSRKTISSED, EQIRALSKRAKSSSRKTISSEDKPFN, FFLSSTEAQ, and FLSSTEAQQSYLQGFSRNIL. Asn369 mapped to the epitope sequences DPIYSNKLKGFEITPEKNPQLRDLD, VLFSREEGQQQGEQR, FLSSTQAQQSYLQGFS, and QRLQESVIVE, whereas Ser383, Thr384, Ser385, and Ser386 overlapped with ISKEQIRA. The predicted interaction residues of the covalent complex were concentrated within residues 189–193 and 393–399 and overlapped with seven IEDB linear IgE epitope records (Table 1). Tyr189, Thr191, Ser192, and Lys193 overlapped with five epitope sequences: LQNLRDYRILEFNSKPNTLLLPNHAD, LETSFHSEFEEINR, DSELRR, ESQESEGSESQREP, and NRVLFG. Notably, the predicted C–N linkage at Lys393 fell within the epitope AKSSSRKT, while the adjacent interaction residues Ala395, Asp396, Phe398, and Asn399 overlapped with GREEGQ.

Table 1

Positional overlap between predicted CHA-binding residues and experimentally identified linear IgE epitopes of soybean 7S globulin.

| Complex type          | Interaction residues                           | Linear IgE epitope sequence | Position |
|-----------------------|------------------------------------------------|-----------------------------|----------|
| Noncovalent complexes | Arg329; Ile330; Ser331; Gly332; Glu333; Asp334 | KEQIRALSKRAKSSSRKTISSED     | 319–341  |
|                       | Arg329; Ile330; Ser331; Gly332; Glu333; Asp334 | EQIRALSKRAKSSSRKTISSEDKPFN  | 320–345  |
|                       | Arg329; Ile330                                 | FFLSSTEAQ                   | 322–330  |
|                       | Arg329; Ile330; Ser331; Gly332; Glu333; Asp334 | FLSSTEAQQSYLQGFSRNIL        | 323–342  |
|                       | Asn369                                         | DPIYSNKLKGKFFEITPEKNPQLRDLD | 350–375  |
|                       | Asn369                                         | VLFSREEGQQQGEQR             | 356–370  |
|                       | Asn369                                         | FLSSTQAQQSYLQGFS            | 358–373  |
|                       | Asn369                                         | QRLQESVIVE                  | 369–378  |
|                       | Ser383; Thr384; Ser385; Ser386                 | ISKEQIRA                    | 379–386  |
| Covalent complexes    | Tyr189                                         | LQNLRDYRILEFNSKPNTLLLPNHAD  | 165–190  |
|                       | Tyr189; Thr191; Ser192; Lys193                 | LETSEFHSEFEEINR             | 181–194  |
|                       | Tyr189                                         | DSELRR                      | 184–189  |
|                       | Thr191; Ser192; Lys193                         | ESQESEGSESQREP              | 190–203  |
|                       | Lys193                                         | NRVLFG                      | 193–198  |
|                       | Lys393; Ala395; Asp396; Phe398                 | AKSSSRKT                    | 391–398  |
|                       | Ala395; Asp396; Phe398; Asn399                 | GREEGQ                      | 394–399  |

These positional overlaps suggested that noncovalent CHA binding could contact multiple continuous and overlapping linear epitope regions, whereas covalent attachment at Lys393, together with interactions involving adjacent residues, could provide more persistent local modification or masking.

Polyphenols may also reduce the accessibility of key IgE-binding sites through epitope masking and local structural perturbation, thereby lowering IgE recognition 21–23. Accordingly, CHA binding may alter the local conformation of soybean 7S globulin and mask or reduce the accessibility of some IgE-recognition sites, thereby providing a potential structural basis for reduced recognition of linear epitopes. However, molecular docking represents only static predicted binding states and cannot capture the structural and proteolytic changes occurring during gastrointestinal digestion. Therefore, simulated gastrointestinal digestion was subsequently performed to examine the digestion behavior, structural changes, and IgE-binding antigenicity of the complexes.

## **2.3. Gastrointestinal degradation profiles of soybean 7S globulin–CHA complexes**

To compare the effects of CHA interaction mode and concentration on the gastrointestinal degradation of soybean 7S globulin, SDS–PAGE was used to monitor changes in intact subunits and digestion products of different molecular weights throughout gastric and intestinal digestion.

As shown in Fig. 2A1–A7, gastric digestion produced three major molecular-weight regions at approximately 70 kDa, 50–55 kDa, and 10–40 kDa. Based on their apparent molecular weights and the known subunit architecture of soybean 7S globulin, the bands at approximately 70 kDa were assigned primarily to incompletely degraded  $\alpha$  and  $\alpha'$  subunits. The 50–55 kDa region likely contained the  $\beta$  subunit and core fragments generated by partial hydrolysis of the  $\alpha$  and  $\alpha'$  subunits, whereas the 10–40 kDa region mainly comprised lower-molecular-weight peptic products 20. After 120 min of gastric digestion, distinct  $\alpha$  and  $\alpha'$  subunit bands remained in the control but were markedly weaker in all CHA complexes, indicating increased susceptibility of these intact subunits to peptic hydrolysis following CHA binding. Shi et al. (2024) similarly reported that covalent and noncovalent binding of phenolic acids to rice protein improved gastric digestibility, possibly by inducing conformational changes that increased the accessibility of proteolytic cleavage sites 24. Notably, at 1.0 mM CHA, the approximately 70 kDa band was weaker in N-1 than in C-1 (Fig. 2A4&A7), suggesting a stronger promotion of intact-subunit degradation by noncovalent than covalent binding under this condition.

In the control, bands within the 50–55 kDa region progressively intensified during gastric digestion. This increase may have resulted from preferential hydrolysis of the N-terminal extension regions of the intact  $\alpha$  and  $\alpha'$  subunits, leading to the accumulation of relatively stable core fragments, together with retention of the pepsin-resistant  $\beta$  subunit. Previous studies have shown that  $\beta$ -conglycinin and its approximately 50 kDa digestion-resistant fragments exhibit considerable proteolytic resistance and may retain IgE-reactive epitopes. Their persistence may therefore contribute to the retention of IgE-binding antigenicity after digestion 25. Compared with the control, N-0.3, N-0.5, C-0.3, and C-0.5 showed weaker 50–55 kDa bands, consistent with reduced accumulation and enhanced degradation of these digestion-resistant fragments. At the same CHA concentration, the noncovalent complexes generally exhibited weaker bands than the corresponding covalent complexes, suggesting differences in proteolytic cleavage-site accessibility between the two interaction modes.

In parallel, all CHA complexes exhibited stronger bands in the 10–25 kDa region and new products below 15 kDa that were barely detectable in the control. Given that the  $\alpha$  and  $\alpha'$  subunits contain N-terminal extensions protruding from the protein core, these low-molecular-weight products may have originated from the extension regions or from subsequent hydrolysis of larger fragments 26. Together, the decreased accumulation of 50–55 kDa fragments and increased formation of lower-molecular-weight products indicate enhanced conversion of digestion-resistant material at low and moderate CHA concentrations. By contrast, neither N-1 nor C-1 showed a clear enhancement in the degradation of 50–55 kDa fragments, indicating a nonmonotonic concentration response. A similar pattern was reported for pea protein complexes with rosmarinic or caffeic acid, in which moderate polyphenol concentrations promoted protein unfolding, whereas higher concentrations favored interactions that restricted proteolysis 27.

Upon transition to intestinal digestion, the residual approximately 70 kDa  $\alpha$  and  $\alpha'$  subunit bands disappeared in all samples, while the approximately 55 kDa bands became markedly weaker than those observed after gastric digestion (Fig. 3B1–B7). These changes indicate continued pancreatin-mediated degradation of both residual intact subunits and gastric digestion-resistant fragments. The approximately 55 kDa bands remained weaker in N-0.3, N-0.5, C-0.3, and C-0.5 than in the control, indicating that the enhanced degradation observed at low and moderate CHA concentrations persisted into the intestinal phase. Increased intestinal digestibility has similarly been reported for CHA-modified whey protein and was associated with structural changes that increased the accessibility of pancreatic cleavage sites 28. In contrast, N-1 and C-1 showed no clear reduction in the approximately 55 kDa region relative to the control, consistent with their gastric profiles and suggesting that greater occupation or steric shielding of proteolytic regions at high CHA concentrations may restrict further enzymatic access.

Further intestinal hydrolysis was accompanied by the appearance of new bands at approximately 40 kDa and the weakening of the approximately 55 kDa region, consistent with conversion of gastric digestion-resistant fragments into intermediate products. N-0.3, N-0.5, C-0.3, and C-0.5 also exhibited weaker bands within the 10–40 kDa region than the control, suggesting more extensive conversion of both digestion-resistant and intermediate fragments into lower-molecular-weight products. By contrast, N-1 and C-1 retained more intense bands within the 10–40 kDa region and showed accumulation of products at approximately 15 kDa, further indicating restricted downstream hydrolysis at the highest CHA concentration. Notably, although differences between covalent and noncovalent complexes were more evident during gastric digestion, their intestinal band patterns became broadly similar at corresponding CHA concentrations. This convergence suggests that sequential gastric and intestinal hydrolysis progressively attenuated the initial degradation differences associated with CHA interaction mode.

## **2.4. Degree of hydrolysis during gastrointestinal digestion**

The OPA assay was used to quantify free amino groups released during digestion and calculate the DH, thereby providing a quantitative measure of protein hydrolysis. As shown in Fig. 4A1–A3, the DH of all samples increased slowly during gastric digestion but rose sharply between 120 and 150 min, immediately after the intestinal phase began. This pattern suggested that pancreatin further hydrolyzed

the high-molecular-weight fragments remaining after gastric digestion and that the intestinal phase accounted for most of the increase in total DH. All soybean 7S globulin–CHA complexes showed higher DH values than the control throughout digestion, consistent with increased susceptibility to digestive proteases following CHA binding. CHA-induced conformational changes may have exposed previously buried proteolytic cleavage sites and thereby facilitated hydrolysis. Similar improvements in digestibility have been reported for covalent CHA–whey protein complexes and noncovalent CHA–pea protein complexes 28–29.

The effect of CHA concentration on soybean 7S globulin hydrolysis was nonmonotonic. After 240 min of digestion, the DH was 12.23% for the control, 25.12% for N-0.3, and 18.18% for C-0.3, whereas N-0.5, N-1, C-0.5, and C-1 showed DH values of approximately 15–18%. Thus, 0.3 mM CHA produced the greatest enhancement of hydrolysis, with N-0.3 exhibiting the highest final DH among all samples. This result was consistent with the pronounced weakening of the digestion-resistant bands in the 0.3 mM groups described in Section 3.3. As the CHA concentration increased, however, the hydrolysis-promoting effect diminished, possibly because extensive CHA binding increased steric hindrance, masked proteolytic cleavage sites, or altered protein–polyphenol interaction patterns. A similar nonmonotonic response has been observed in covalent soy protein isolate–epigallocatechin-3-gallate (EGCG) complexes, where low EGCG concentrations promoted network formation, whereas higher concentrations inhibited it through competition among binding sites 30. Other studies have likewise reported reduced protein digestibility at higher polyphenol levels, suggesting that extensive polyphenol binding may hinder enzyme access to protein substrates 31–32.

At 0.3 mM CHA, N-0.3 showed a markedly higher final DH than C-0.3, indicating that noncovalent binding promoted proteolysis more effectively under this condition (Fig. 4A1). This pattern is consistent with greater conformational flexibility or relaxation under relatively weak noncovalent constraints, which may increase the accessibility of proteolytic cleavage sites to digestive enzymes. By contrast, stable covalent anchoring may impose stronger local conformational constraints and steric restrictions, thereby limiting access to specific cleavage sites. Consistent with this interpretation, noncovalent anthocyanin–milk protein complexes have been reported to retain digestibility comparable to that of the control, whereas covalent complexes were less digestible because covalent modification restricted cleavage-site accessibility 33. Covalent complexation with EGCG has also been associated with greater structural order, whereas noncovalent interactions more readily promote protein unfolding and conformational relaxation 34.

## **2.5. Retention of amyloid-like aggregate during gastrointestinal digestion**

Native soybean 7S globulin has been reported to contain both globular oligomers and amyloid-like aggregates characterized by cross- $\beta$  structures. Compared with globular oligomers, amyloid-like aggregates exhibit greater resistance to gastrointestinal digestion and may persist as IgE-reactive structures. Their persistence may therefore contribute to the post-digestion IgE-binding antigenicity of



soybean 7S globulin 3<sup>4</sup>. ThT binds preferentially to ordered cross- $\beta$  structures in amyloid-like aggregates, resulting in enhanced fluorescence. Accordingly, ThT fluorescence was used to assess relative changes in ThT-responsive cross- $\beta$  aggregate structures before and after digestion.

As shown in Fig. 4B1–B3, the undigested control exhibited relatively high ThT fluorescence, consistent with the presence of ThT-responsive cross- $\beta$  structures in soybean 7S globulin. After gastric digestion, the ThT fluorescence of the control decreased but remained higher than that of all CHA complexes. This pattern suggested that peptic digestion partially disrupted the ThT-responsive structures, while some digestion-resistant aggregates remained. During the intestinal phase, the ThT fluorescence of the control decreased further, consistent with continued disassembly or degradation of the residual aggregate structures during pancreatic digestion. Similar digestion patterns have been reported for amyloid-like aggregates from other food proteins, with partial disassembly or degradation during gastric digestion followed by further disruption during the intestinal phase 35<sup>36</sup>. All undigested CHA complexes exhibited lower ThT fluorescence than CK and generally maintained lower fluorescence throughout gastrointestinal digestion. These findings suggest that CHA binding reduces the formation or stability of ThT-responsive cross- $\beta$  structures. The inhibitory effect of CHA on amyloid aggregation has also been supported by kinetic modeling of A $\beta$  fibrillation 37.

After gastric digestion, N-0.3 and N-0.5 retained relatively high ThT fluorescence, whereas N-1 exhibited a markedly lower signal. By contrast, C-0.3, C-0.5, and C-1 all exhibited relatively low ThT fluorescence, with no clear dose-dependent trend (Fig. 4B2). After intestinal digestion, ThT fluorescence decreased further in all samples, while the covalent complexes generally retained lower ThT-responsive signals than the corresponding noncovalent complexes (Fig. 4B3). This contrast suggests that stable covalent anchoring may interfere with the continued assembly and extension of intermolecular cross- $\beta$  structures even at relatively low CHA concentrations, thereby limiting amyloid-like aggregate formation and stabilization. In contrast, the concentration-dependent decrease in ThT fluorescence observed for the noncovalent complexes suggests that a greater extent of CHA association may be required to effectively compete with the intermolecular interactions that sustain cross- $\beta$  assembly. Consistent with this interpretation, covalent CHA binding to pea 7S and 11S globulins has been reported to reduce fibrillation capacity by decreasing surface hydrophobicity and limiting fibril elongation 38.

## 2.6. Secondary structure changes during gastrointestinal digestion

CD spectroscopy was used to characterize secondary-structure changes in undigested samples and samples collected after 120 min of gastric or intestinal digestion. As shown in Fig. 5A1–A7, all samples exhibited a broad negative band between 200 and 220 nm, reflecting overlapping contributions from  $\alpha$ -helical and  $\beta$ -sheet structures. Digestion produced pronounced changes in spectral shape and signal intensity, consistent with substantial rearrangement of the protein secondary structure. As shown in Table S2, the undigested control contained 16.97%  $\alpha$ -helix and 29.39%  $\beta$ -sheet. In the CHA complexes, the  $\alpha$ -helix content ranged from 12.63% to 15.63%, whereas the  $\beta$ -sheet content ranged from 30.98% to

33.86%. Only minor changes occurred in the  $\beta$ -turn and random-coil contents. These differences suggested that CHA binding altered the secondary-structure distribution of soybean 7S globulin before digestion. This redistribution may arise from hydrogen bonding between polyphenolic hydroxyl groups and protein backbone or side-chain groups, which could perturb the native intramolecular hydrogen-bonding network 13.

The effects of CHA on the undigested protein structure also varied with interaction mode and concentration. Among the complexes, C-1 exhibited the lowest  $\alpha$ -helix content and the highest  $\beta$ -sheet content, suggesting that covalent binding at 1.0 mM CHA produced the greatest secondary-structure redistribution (Table S2). Ke et al. (2024) reported that covalent soy protein isolate–EGCG complexes exhibited a relatively ordered structure, whereas noncovalent interactions more readily promoted protein unfolding and structural disorder 34. These observations support distinct conformational effects of covalent and noncovalent interactions. In the soybean 7S globulin–CHA system, covalent interactions have also been reported to predominantly reduce  $\alpha$ -helix content, whereas noncovalent interactions more readily increased random-coil content, further supporting interaction-mode-dependent structural changes 2.

After 120 min of gastric digestion, the negative signals between 210 and 220 nm weakened markedly, and the spectral minima generally shifted toward approximately 200 nm. These changes were consistent with pepsin-induced disruption and rearrangement of ordered secondary structures. The relative  $\alpha$ -helix content decreased substantially, whereas the relative  $\beta$ -sheet content increased and the random-coil content decreased slightly. This pattern was consistent with the preferential loss or rearrangement of  $\alpha$ -helical structures during peptic digestion. This preferential change may reflect differences in the accessibility of secondary-structure regions to pepsin. The extent of gastric digestion-induced structural change varied among the complexes. N-0.3 and N-0.5 showed relatively pronounced decreases in  $\alpha$ -helix content and increases in relative  $\beta$ -sheet content, whereas N-1 and C-1 exhibited smaller changes. Notably, the  $\alpha$ -helix content of C-1 remained nearly unchanged after gastric digestion. These structural trends were consistent with the greater hydrolysis observed at low and moderate CHA concentrations and the weaker hydrolysis-promoting effect at 1.0 mM. The more pronounced secondary-structure rearrangement at low and moderate CHA concentrations was consistent with greater cleavage-site accessibility and enhanced hydrolysis.

After intestinal digestion, the negative signals between 200 and 220 nm weakened further and shifted slightly toward shorter wavelengths, consistent with continued structural disruption during pancreatic digestion. Relative to the gastric digesta, most samples showed a slight further decrease in  $\alpha$ -helix content and a modest increase in relative  $\beta$ -sheet content, while the  $\beta$ -turn and random-coil contents remained largely unchanged. After 120 min of intestinal digestion, the  $\alpha$ -helix contents of all groups converged to 11.19–11.81%, while their  $\beta$ -sheet contents converged to 35.90–36.96%. The reduced variation among groups suggested that sequential gastric and intestinal hydrolysis progressively diminished the structural differences initially associated with CHA concentration and interaction mode.

## 2.7. Changes in IgE-binding antigenicity during gastrointestinal digestion

An ic-ELISA was used to assess the IgE-binding antigenicity of samples collected after 120 min of gastric or intestinal digestion. Higher inhibition rates indicated greater residual IgE-binding capacity, whereas lower values indicated reduced IgE-binding antigenicity 39.

As shown in Fig. 6A, the IgE-binding inhibition rate of CK after 120 min of gastric digestion was 77.46% relative to that of the undigested allergen, indicating that soybean 7S globulin retained considerable IgE-binding capacity after peptic digestion. After intestinal digestion, the inhibition rate of CK decreased further to 57.07%, suggesting that pancreatin promoted further degradation of the residual antigenic fragments or impaired the recognition of some IgE epitopes (Fig. 6B). Except for C-0.3, all complexes showed lower inhibition rates after intestinal digestion than after gastric digestion, consistent with further loss or alteration of residual IgE-reactive structures during the intestinal phase. Compared with CK, all CHA complexes showed lower IgE-binding inhibition rates after both gastric and intestinal digestion, indicating that both covalent and noncovalent CHA interactions reduced the post-digestion IgE-binding antigenicity of soybean 7S globulin. Previous studies have similarly reported that covalent CHA modification reduces IgE binding to whey proteins and shrimp tropomyosin by altering conformational epitope integrity or accessibility and by modifying or masking linear epitopes 28, 40.

The noncovalent complexes showed an overall concentration-related decline in IgE-binding capacity, although the gastric-phase response was not strictly monotonic. After gastric digestion, the inhibition rates of N-0.3, N-0.5, and N-1 were 51.60%, 52.31%, and 30.93%, respectively (Fig. 6A). After intestinal digestion, the corresponding values decreased to 46.22%, 38.50%, and 24.42% (Fig. 6B). By contrast, the covalent complexes maintained relatively low IgE-binding inhibition rates across the tested CHA concentrations, with intestinal inhibition rates remaining within a narrow range of approximately 23–27% (Fig. 6B). At 0.3 and 0.5 mM CHA, the covalent complexes exhibited lower inhibition rates than their corresponding noncovalent counterparts, indicating a stronger reduction in IgE recognition at low and moderate CHA concentrations.

Importantly, C-0.3 showed a lower DH than N-0.3 but a lower IgE-binding capacity. This dissociation between hydrolysis and IgE binding indicates that enhanced proteolysis alone cannot account for the reduced IgE recognition of the covalent complexes. At 1.0 mM CHA, N-1 and C-1 exhibited similarly low IgE-binding capacities, whereas the hydrolysis-promoting effect of both interaction modes was weaker than that observed at 0.3 mM. Together, these observations indicate that the magnitude of IgE-binding reduction was not directly proportional to the extent of protein hydrolysis across CHA concentrations and interaction modes.

## 2.8. IgE-reactive fragment profiles during gastrointestinal digestion

To further resolve the molecular-weight distribution of IgE-reactive digestion products, WB was used to examine protein and peptide bands that remained recognizable by serum IgE after denaturation. Band intensity therefore reflected the relative IgE reactivity of the corresponding component.

As shown in Fig. 7A, CK mainly exhibited IgE-reactive bands at approximately 55 kDa and within the 25–35 kDa region during gastric digestion. The approximately 55 kDa band showed the strongest signal and remained detectable from 10 to 120 min. Based on the SDS–PAGE profiles described in Section 3.3, this band was assigned mainly to digestion-resistant core fragments of the  $\alpha$  and  $\alpha'$  subunits and the  $\beta$  subunit. Notably, the approximately 55 kDa band was more intense after gastric digestion than in the undigested sample. This pattern suggested that peptic cleavage may have exposed or generated IgE-reactive regions while leaving the digestion-resistant fragments largely intact. Thus, the  $\alpha$  and  $\alpha'$  core fragments and the  $\beta$  subunit may serve as major carriers of linear or denaturation-resistant IgE-reactive epitopes after gastric digestion. During intestinal digestion, the approximately 55 kDa band of CK weakened markedly but remained detectable from 10 to 120 min, while new IgE-reactive bands appeared within the 35–55 kDa region. These changes were consistent with further pancreatin-mediated cleavage of the gastric digestion-resistant fragments. Some resulting products nevertheless retained IgE reactivity, and the newly detected bands may have originated from cleavage of the approximately 55 kDa fragments.

Compared with CK, all soybean 7S globulin–CHA complexes exhibited weaker IgE-reactive bands after both gastric and intestinal digestion, with the clearest differences observed after 120 min of intestinal digestion (Fig. 7A&B). In all complexes, the approximately 55 kDa bands were markedly weaker, while new IgE-reactive bands appeared at approximately 25 kDa. These patterns were consistent with further conversion of high-molecular-weight IgE-reactive fragments into lower-molecular-weight products following CHA binding. CHA and other polyphenols have been reported to alter protein conformation and cleavage-site accessibility, which may facilitate the hydrolysis of IgE-reactive fragments 28· 41· 42.

The IgE-reactive bands were generally weaker at low CHA concentrations, particularly in the noncovalent complexes. After gastric digestion, the approximately 55 kDa band was weaker in N-0.3 than in N-0.5 and N-1. A similar pattern was observed for C-0.3 relative to C-0.5 and C-1. During intestinal digestion, the residual bands at approximately 55 kDa and within the 25–55 kDa region also remained relatively weak in the 0.3 mM groups. This pattern suggested that low CHA concentrations favored the degradation of digestion-resistant fragments carrying linear or denaturation-resistant epitopes. Greater retention of the approximately 55 kDa fragment was observed at higher CHA concentrations, possibly because increased local compaction or steric shielding restricted digestive-enzyme access 27.

At 0.3 mM CHA, the approximately 55 kDa band was generally weaker in N-0.3 than in C-0.3, particularly after 90 min of intestinal digestion. This pattern, together with the higher DH of N-0.3 and the more rapid weakening of its SDS–PAGE bands, suggested that low-concentration noncovalent binding reduced IgE reactivity partly by promoting the degradation of epitope-carrying fragments. Although C-0.3 retained more visible protein bands than N-0.3, it showed a lower IgE-binding inhibition rate in the ELISA. These

contrasting profiles further indicated that the extent of IgE-reactive fragment degradation alone could not account for the differences in IgE recognition between the two interaction modes.

### 3. Discussion

The present findings indicate that the effects of chlorogenic acid (CHA) on the gastrointestinal fate and IgE-binding antigenicity of soybean 7S globulin depend on both interaction mode and concentration, rather than following a simple dose-dependent relationship between CHA level, protein hydrolysis, and IgE recognition. At low CHA concentration, N-0.3 exhibited the highest degree of hydrolysis, reduced accumulation of the approximately 55 kDa digestion-resistant fragments, and weaker IgE-reactive bands, supporting a predominantly proteolysis-facilitating effect. The relatively weak and reversible hydrogen bonding, hydrophobic interactions, and van der Waals forces involved in noncovalent association may permit greater structural responsiveness and facilitate protease access to susceptible regions. By contrast, covalent anchoring may impose stronger local conformational constraints or steric restrictions that limit cleavage-site accessibility, consistent with previous observations for noncovalent and covalent polyphenol–protein complexes 33. However, this hydrolysis-promoting effect was not proportional to CHA concentration. At higher CHA levels, greater steric shielding of proteolytic regions, and possibly direct polyphenol–enzyme interactions, may restrict hydrolysis 43. Thus, the proteolytic advantage of noncovalent CHA appears to be most pronounced at relatively low concentrations.

The comparison between N-0.3 and C-0.3 further indicates that proteolysis alone cannot explain the reduction in IgE recognition. Although C-0.3 showed a lower degree of hydrolysis and retained more visible protein fragments, it exhibited a lower IgE-binding capacity than N-0.3. Molecular docking predicted covalent attachment of oxidized CHA to Lys393, while epitope mapping placed this residue within the linear IgE epitope AKSSSRKT and identified neighboring interaction residues within other IgE-reactive sequences. Because oxidized CHA can form stable covalent linkages with nucleophilic protein residues, covalent modification may maintain CHA attachment at or near IgE-reactive regions more persistently than reversible noncovalent binding, thereby altering local epitope structure or reducing antibody accessibility 2. This interpretation is supported by reports of stronger protein–polyphenol association in covalent complexes 44 and lower post-digestion IgE binding in covalent than in noncovalent polyphenol–protein complexes 33. Together, the molecular docking and CD results suggest that CHA binding may perturb structural regions associated with antibody recognition, thereby providing an additional mechanism for reducing IgE recognition beyond enhanced proteolytic degradation.

Amyloid-like aggregate retention may provide an additional contribution to the covalent effect. Covalent complexes generally retained lower ThT-responsive signals than the corresponding noncovalent complexes, including at relatively low CHA concentrations, suggesting that stable CHA attachment may interfere with the assembly, extension, or stabilization of intermolecular cross- $\beta$  structures. Residual amyloid-like aggregates of soybean 7S globulin have been reported to resist gastrointestinal digestion and may serve as carriers or presentation structures for IgE-reactive epitopes 3, 45. Similar relationships between amyloid organization and enhanced IgE recognition have been reported for Gad m 1, where

repetitive epitope presentation contributes to IgE binding and cross-linking 46–47. Covalent CHA may therefore influence IgE recognition through two complementary processes: persistent modification or masking of IgE-reactive regions and reduced retention of amyloid-like aggregate structures.

The concentration dependence of these effects further suggests a shift in the relative contribution of proteolytic and epitope-related mechanisms. At 0.3 mM CHA, noncovalent binding was associated primarily with pronounced hydrolysis and degradation of IgE-reactive fragments, whereas covalent binding produced lower IgE recognition despite less extensive proteolysis. At 1.0 mM CHA, however, N-1 and C-1 maintained similarly low IgE-binding capacities even though their hydrolysis-promoting effects were weaker than those observed at 0.3 mM. This dissociation suggests that increasing CHA concentration reduces the relative importance of accelerated proteolysis while increasing the contribution of epitope coverage, local structural perturbation, and reduced antibody accessibility.

Taken together, the data support a working model in which low-concentration noncovalent CHA predominantly follows a proteolysis-facilitating route, whereas covalent CHA is more strongly associated with a stable-anchoring route involving persistent epitope modification and reduced retention of ThT-responsive amyloid-like aggregates. As CHA concentration increases, the balance shifts away from proteolysis-associated regulation toward epitope-related effects. Collectively, our findings move beyond phenomenological observation by providing a mechanistic framework indicating that the IgE-binding fate of soybean 7S globulin is governed not simply by polyphenol concentration, but critically by the intrinsic nature of the protein–polyphenol linkage. This work highlights strategic selection of interaction mode, rather than dose escalation alone, as a pivotal lever for the rational design of next-generation soy ingredients with more predictable IgE-binding and immunological profiles.

## 4. Methods

### 4.1. Chemicals and materials

Soybeans were purchased from Wumei Supermarket (Beijing, China). Human sera from patients with soybean allergy were obtained from PlasmaLab (Everett, USA). CHA (purity  $\geq 98\%$ ), ThT, NaOH, NaHSO<sub>3</sub>, NaCl, HCl, CaCl<sub>2</sub>, and L-serine were purchased from Macklin Bio-Technology Co., Ltd. (Shanghai, China). PBS, Tris-Gly buffer, TBST, bile salts, SDS–PAGE sample loading buffer (5×), SolarFast Coomassie Brilliant Blue rapid staining solution, transfer buffer (10×), BSA, TMB substrate solution, and PVDF membranes with a pore size of 0.45  $\mu\text{m}$  were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Pepsin (P7012) and pancreatin (P7545) were purchased from Sigma–Aldrich, USA. ECL substrate was purchased from HuaXin Biotechnology Co., Ltd. (Shanghai, China). Spectroscopic-grade potassium bromide was purchased from Aladdin Co., Ltd. (Shanghai, China). Dialysis tubing (8–14 kDa) was purchased from Beijing Bomeibio Technology Co., Ltd. (Beijing, China). All other analytical-grade chemicals were purchased from Solarbio Technology Co., Ltd. (Beijing, China).

### 4.2. Preparation of soybean 7S globulin

Defatted soybean powder (100 g) was dispersed in 1.5 L of distilled water, and the pH was adjusted to 8.5 with 2 M NaOH. The suspension was stirred at room temperature for 1 h and centrifuged at  $13,000 \times g$  and  $4^{\circ}\text{C}$  for 15 min.  $\text{NaHSO}_3$  (1.05 g) was added to the supernatant, and the pH was adjusted to 6.4 with 2 M HCl. The mixture was then stored at  $4^{\circ}\text{C}$  overnight and centrifuged at  $13,000 \times g$  and  $4^{\circ}\text{C}$  for 15 min. The supernatant was collected, NaCl (16.08 g) was added, and the pH was adjusted to 5.0 with 2 M HCl. The solution was then stirred at room temperature for 30 min and centrifuged at  $13,000 \times g$  and  $4^{\circ}\text{C}$  for 15 min. The resulting supernatant was mixed with an equal volume of prechilled distilled water ( $4^{\circ}\text{C}$ ) and stirred for 30 min. The pH was subsequently adjusted to 4.8 with 2 M HCl, and the mixture was centrifuged at  $13,000 \times g$  and  $4^{\circ}\text{C}$  for 15 min. The resulting precipitate was washed three times with distilled water, redissolved in Tris-Gly buffer (pH 8.5), and dialyzed against distilled water at  $4^{\circ}\text{C}$  for 48 h using dialysis tubing with an MWCO of 8–14 kDa. The dialyzed sample was lyophilized and ground into a fine powder, which was stored at  $-20^{\circ}\text{C}$  until further use 2.

### 4.3. Preparation of soybean 7S globulin–CHA complexes

Soybean 7S globulin–CHA complexes were prepared according to the method of Yun et al. (2024) with minor modifications 2. Soybean 7S globulin powder was dispersed in distilled water at a final protein concentration of 6 mg/mL and stirred overnight to allow complete hydration. A soybean 7S globulin solution prepared without CHA was processed under otherwise identical conditions and designated as the control (CK). To prepare the noncovalent complexes, CHA was dissolved in distilled water and added directly to the soybean 7S globulin solution. After thorough mixing, the solution was purged with nitrogen to remove dissolved oxygen. The resulting noncovalent complexes prepared with 0.3, 0.5, and 1.0 mM CHA were designated N-0.3, N-0.5, and N-1, respectively. To prepare the covalent complexes, CHA was dissolved in distilled water and oxidized with periodate resin for 1 min, during which the solution turned yellow. The oxidized CHA solution was then added to the soybean 7S globulin solution. After thorough mixing, the solution was purged with nitrogen to remove dissolved oxygen. The resulting covalent complexes prepared with 0.3, 0.5, and 1.0 mM CHA were designated C-0.3, C-0.5, and C-1, respectively. The CK and all complex samples were stirred at 500 rpm in the dark for 4 h and then dialyzed against distilled water at  $4^{\circ}\text{C}$  for 48 h using dialysis tubing with an MWCO of 8–14 kDa. Dialysis was performed to remove unbound CHA from the complex samples. After dialysis, all samples were lyophilized, ground into a fine powder, and stored at  $-20^{\circ}\text{C}$  until further analysis.

### 4.4. Molecular docking and IgE epitope mapping

The crystal structure of soybean 7S globulin (PDB ID: 3AUP) was retrieved from the RCSB Protein Data Bank, whereas the three-dimensional structures of CHA (PubChem CID: 1794427) and CHA quinone (PubChem CID: 73759913) were obtained from PubChem. For noncovalent docking, CHA was used as the ligand, and the search space was defined to encompass the entire protein. For covalent docking, CHA quinone, the oxidized form of CHA, was used as the ligand. All ligand atoms were included in the docking calculation, and the selected lysine residue (Lys393) was defined as the protein-side reactive site, with Michael addition specified as the reaction type. Before docking, the protein and ligand structures were prepared using the Protein Preparation Wizard and LigPrep modules in Schrödinger

2022. Water molecules were removed, hydrogen atoms were added, and the structures were energy-minimized. Following docking and pose filtering, the protein–ligand complexes with the lowest predicted binding energies were selected for subsequent analysis. The docking poses and intermolecular interactions were visualized and analyzed using PyMOL and Discovery Studio 2025.

## 4.5. In vitro gastrointestinal digestion

The digestion procedure followed the INFOGEST 2.0 protocol with minor modifications 48. Throughout digestion, the temperature and stirring speed were maintained at 37°C and 300 rpm, respectively, using a temperature-controlled magnetic stirrer (RCT digital 2.07, IKA, Germany). The electrolyte stock solutions for SGF and SIF were prepared as specified in Table S1. Each sample was dispersed in the SGF electrolyte stock solution, and the pH was adjusted to 3.0. Pepsin and CaCl<sub>2</sub> solutions were then added to give final concentrations of 2,000 U/mL and 0.15 mM, respectively, in the gastric digestion mixture. A sample-to-pepsin mass ratio of 50:1 (w/w) was used. The gastric phase was conducted for 2 h in a temperature-controlled water-bath shaker (SYC-10WD, TUOHE, Shanghai, China). At the end of the gastric phase, the digesta were adjusted to pH 7.0. To initiate the intestinal phase, an equal volume of SIF electrolyte stock solution (pH 7.0) was added, followed by pancreatin, bile salts, and CaCl<sub>2</sub> solutions. The intestinal phase was then conducted for 2 h. The final concentrations of pancreatin, bile salts, and CaCl<sub>2</sub> in the intestinal digestion mixture were 100 U/mL, 10 mM, and 0.6 mM, respectively. A sample-to-pancreatin mass ratio of 20:1 (w/w) was used. During each digestion phase, aliquots were collected at 10, 30, 60, 90, and 120 min and immediately stored at – 80°C until further analysis.

## 4.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS–PAGE was performed to characterize the molecular-weight profiles of the digestion products. The gastrointestinal digesta were diluted with PBS to a final protein concentration of 1 mg/mL and mixed 1:1 (v/v) with 1× SDS–PAGE loading buffer containing DTT. After vortex mixing, the samples were heated in boiling water for 10 min and cooled to room temperature. A 10 µL aliquot of each sample was loaded onto a 4–20% gradient SDS–PAGE gel. Electrophoresis was initially performed at 80 V for 40 min. Once the bromophenol blue dye front became level, the voltage was increased to 120 V, and electrophoresis was continued for a further 80 min. The gels were stained with SolarFast Coomassie Brilliant Blue rapid staining solution for 2 h and destained in deionized water for 6 h to reduce background staining. The gels were subsequently imaged and analyzed using a gel imaging system (JY04S-3C, Junda Dongfang, Beijing, China).

## 4.7. Determination of the degree of hydrolysis (DH)

The OPA method was used to quantify free amino groups released during protein hydrolysis and to compare the extent of hydrolysis among samples. All samples were diluted with PBS to a final protein concentration of 1 mg/mL. The diluted samples were further diluted 10-fold in OPA reagent and incubated at 37°C for 10 min. The reaction mixtures were transferred to 96-well plates, and the absorbance was measured at 340 nm using a microplate reader.



For the digested complex samples, matched aliquots were diluted 10-fold with PBS instead of OPA reagent, and their absorbance at 340 nm was measured to correct for CHA-derived background absorbance. The free amino group content was determined using L-serine calibration curve. The calibration curve was generated using a 2.0 mM L-serine stock solution diluted to 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 mM (Fig. S1). The DH was calculated according to Eq. (1):

$$DH(\%) = \frac{NH_{2,digested} - NH_{2,secretions} - NH_{2,undigested}}{NH_{2,total} - NH_{2,undigested}} \times 100$$

1

where  $NH_{2,undigested}$  and  $NH_{2,digested}$  represent the free amino group contents of the samples before and after digestion, respectively.  $NH_{2,secretions}$  represents the background free amino groups contributed by the digestion reagents. During the gastric phase, this contribution was derived mainly from pepsin, whereas during the intestinal phase, it was derived from pepsin, pancreatin, and bile salts.  $NH_{2,total}$  represents the total free amino group content of each control or complex sample after complete acid hydrolysis with 6 M HCl at 100°C for 24 h. The samples were mixed with 6 M HCl at an HCl-to-sample volume ratio of 10:1. After acid hydrolysis, the resulting hydrolysates were neutralized with an equal volume of 6 M NaOH solution.

## 4.8. Thioflavin T (ThT) Fluorescence Assay

ThT fluorescence was measured in samples collected after 120 min of gastric or intestinal digestion to evaluate the relative retention of ThT-responsive aggregate structures. A ThT stock solution was prepared by dissolving 8 mg of ThT in 10 mL of PBS. Before analysis, the stock solution was diluted 50-fold in PBS to prepare the ThT working solution. The samples were diluted with PBS to a final protein concentration of 0.5 mg/mL and mixed with the ThT working solution at a sample-to-ThT working solution volume ratio of 1:100. After incubation for 1 min, fluorescence spectra were recorded using a fluorescence spectrophotometer (FS5, Edinburgh Instruments, Edinburgh, UK). The spectra were corrected by subtracting the background signals from SGF or SIF containing the corresponding digestive enzymes. The excitation wavelength was set to 440 nm, and the emission spectra were recorded from 450 to 600 nm. The excitation and emission slit widths were both set to 5 nm. Each sample was scanned in triplicate.

## 4.9. Circular dichroism (CD) spectrum

CD spectra of samples collected after 120 min of gastric digestion and 120 min of intestinal digestion were measured to evaluate differences in protein secondary structure during digestion. Prior to analysis, the gastric digesta were diluted with distilled water adjusted to pH 3.0 to a protein concentration of 0.2 mg/mL, whereas the intestinal digesta were diluted with distilled water adjusted to pH 7.0 to the same protein concentration. The undigested samples were prepared at a protein concentration of 0.2 mg/mL using distilled water. The samples were transferred to a quartz cuvette with a path length of 1 mm, and their far-UV CD spectra were recorded. SGF and SIF containing the corresponding digestive enzymes

were diluted with distilled water adjusted to pH 3.0 and 7.0, respectively, and used as the corresponding blanks. The final spectra are presented after subtraction of the respective background signals. Spectra were recorded over the wavelength range of 195–260 nm, and each sample was scanned three times.

## **4.10. Indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) analysis**

An ic-ELISA was performed according to the method of Li et al. (2021) with minor modifications to assess the IgE-binding capacity and IgE-binding antigenicity of the samples 49. Soybean 7S globulin was dissolved in coating buffer (pH 9.6) at 10 µg/mL, and 100 µL was added to each well of a 96-well microplate. The plate was incubated overnight at 4°C. The digested samples were heated in boiling water for 5 min to inactivate the digestive enzymes and then diluted to 20 µg/mL with PBST (10 mM PBS containing 0.1% Tween-20). Serum from patients with soybean allergy, serving as the source of primary IgE antibodies, was diluted 1:75 with PBST and mixed 1:1 (v/v) with the diluted digested samples. For the control without competing antigen, the diluted serum was mixed 1:1 (v/v) with PBST. The mixtures were incubated overnight at 4°C to allow antigen–IgE binding. The coated plate was blocked with 2% BSA (w/v) in PBST for 1 h with gentle shaking and washed five times with PBST for 5 min each. The preincubated serum–sample mixtures were added to the wells and incubated at 37°C for 2 h, after which the plate was washed five times with PBST. HRP-conjugated mouse anti-human IgE antibody diluted 1:3,000 in PBST containing 0.5% BSA was added and incubated at 37°C for 1 h. The plate was then washed five times with TBST. TMB substrate solution (100 µL) was added to each well, and the plate was incubated at 37°C for 30 min. The reaction was stopped with 50 µL of 2 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance was measured at 450 nm using a microplate reader.

## **4.11. Western blotting (WB) analysis**

WB was performed to evaluate changes in IgE-reactive components among the samples during gastrointestinal digestion. SDS–PAGE was performed as described in Section 2.6. After electrophoresis, the separated proteins were transferred from the gels onto PVDF membranes. The transfer assembly consisted of two sponge pads, six sheets of filter paper, the SDS–PAGE gel, and a PVDF membrane with a pore size of 0.45 µm. The PVDF membrane was cut to match the dimensions of the gel. The sponge pads and filter papers were pre-equilibrated in transfer buffer, and the gel was kept hydrated during assembly. The PVDF membrane was activated by sequential immersion in methanol, deionized water, and transfer buffer for 30 s, 2 min, and 5 min, respectively. The activated membrane and gel were assembled in the transfer cassette, with care taken to remove air bubbles between the layers. Protein transfer was performed at 80 V for 120 min in an ice bath, with the current limited to 200 mA. After transfer, the membrane was blocked with 5% BSA (w/v) in TBST at room temperature for 1 h with gentle shaking. The membrane was washed three times with TBST for 5 min each and incubated overnight at 4°C with serum from patients with soybean allergy, diluted 1:30 in TBST containing 5% BSA. After incubation with the serum, the membrane was washed five times with TBST and incubated for 1 h at room temperature with HRP-conjugated mouse anti-human IgE antibody diluted 1:5,000 in TBST containing 5% BSA. The membrane was then washed five times with TBST. For chemiluminescent

detection, the membrane was evenly covered with ECL substrate and incubated in the dark for 60 s. The IgE-reactive bands were visualized using a chemiluminescence imaging system. Images were acquired using the automatic exposure mode with the high-sensitivity setting.

## 4.12. Data analysis

All experiments were performed with at least three replicates, and the data are presented as the mean  $\pm$  standard deviation. Figures were prepared using GraphPad Prism 9.0 and Origin 2021. Statistical analyses were conducted using SPSS (v16.0), significant differences among means were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Significant differences between treatments were determined at  $p < 0.05$ .

## Declarations

## Competing interests

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.

## Author Contribution

P.Z.: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. Z.Y.: Writing – original draft, Methodology, Investigation. J.L.: Investigation, Methodology, Data curation. X.Y.: Investigation, Methodology. J.M.: Investigation, Data curation. L.M.: Visualization, Supervision, Investigation. F.C.: Visualization, Supervision, Conceptualization. X.H.: Visualization, Supervision, Conceptualization. J.J.: Writing – review & editing, Supervision, Conceptualization.

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## Data availability

Data will be made available on request.

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## Figures

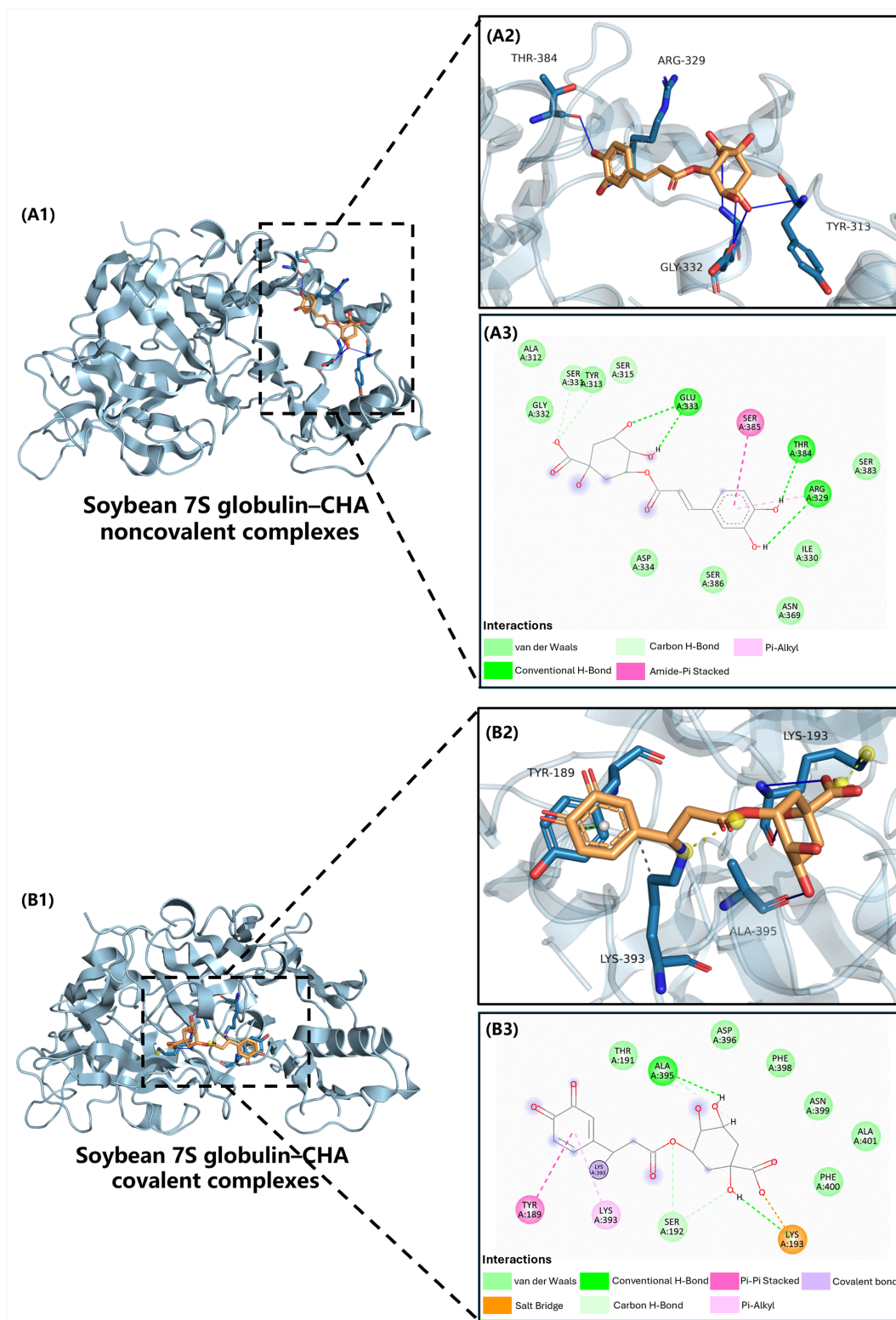


**Figure 1**

Predicted binding modes and intermolecular interactions of CHA with soybean 7S globulin. (A1–A3) Overall binding conformation, enlarged binding pocket, and two-dimensional interaction map of the noncovalent complex, respectively. (B1–B3) Corresponding representations of the covalent complex.



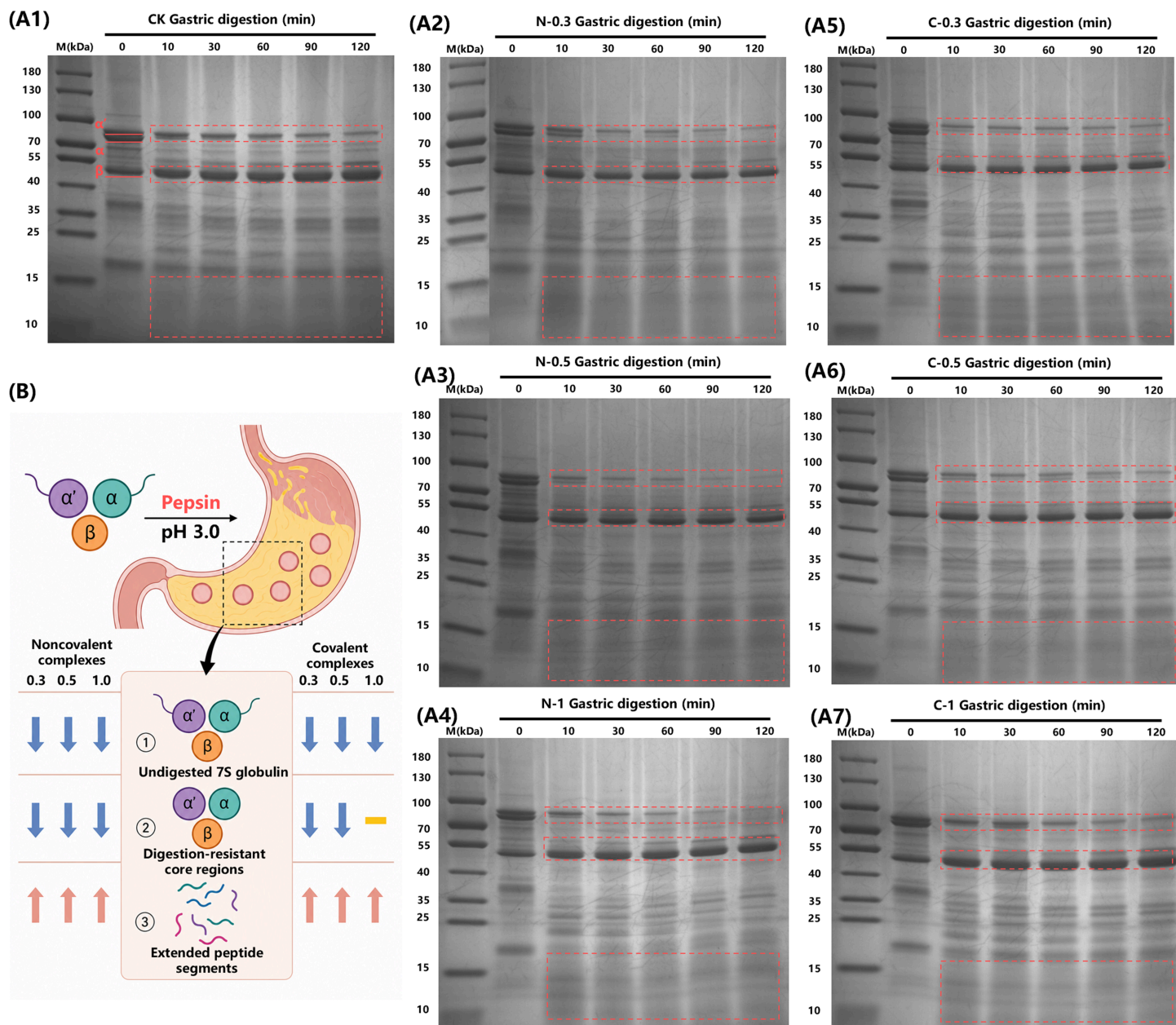


**Figure 2**

SDS-PAGE profiles and schematic representation of the gastric degradation of soybean 7S globulin-CHA complexes. (A1–A7) SDS-PAGE profiles of CK, N-0.3, N-0.5, N-1, C-0.3, C-0.5, and C-1 during gastric digestion. (B) Schematic summary of the effects of CHA interaction mode and concentration on the gastric degradation of soybean 7S globulin.

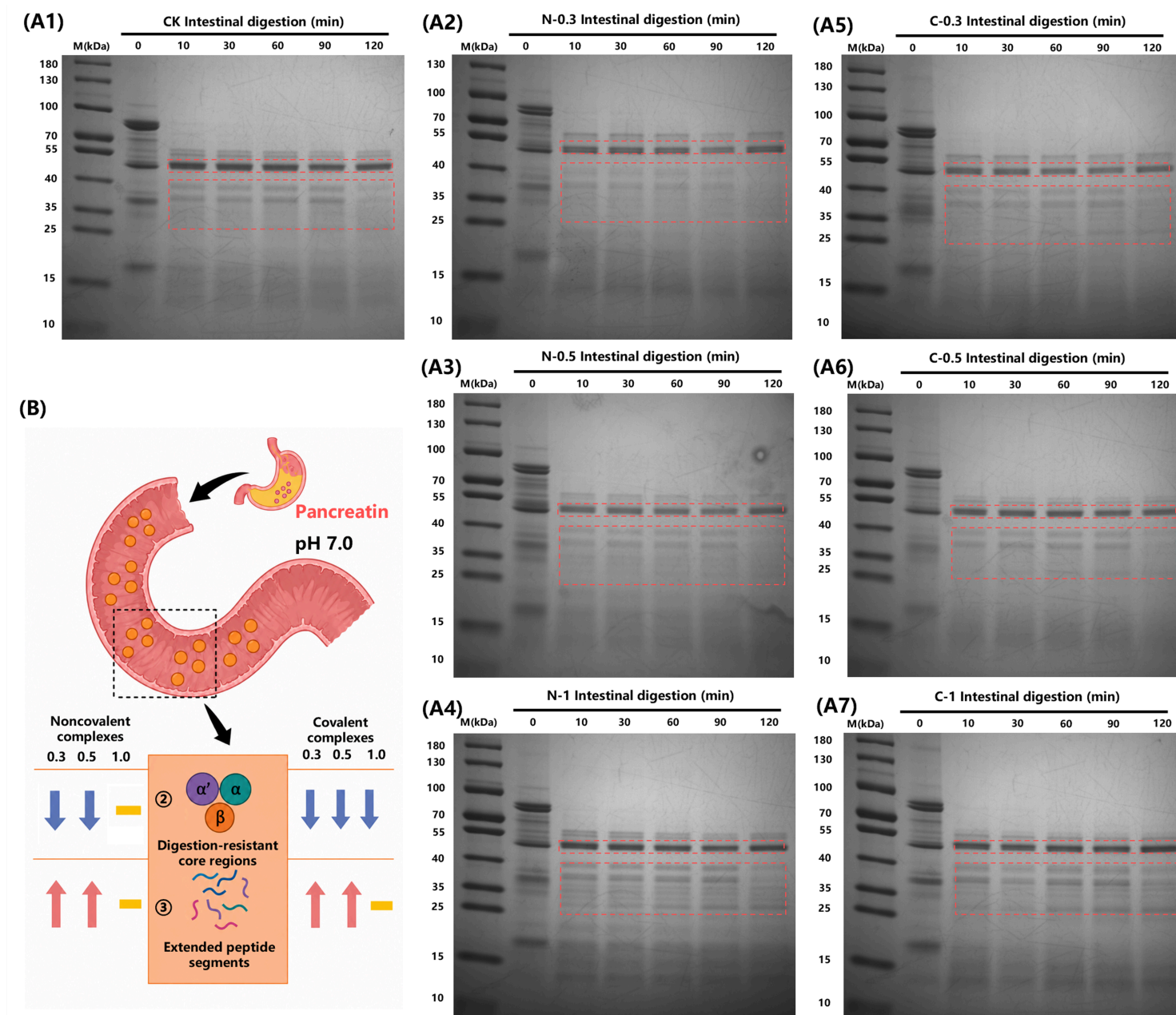


**Figure 3**

SDS-PAGE profiles and schematic representation of the intestinal degradation of soybean 7S globulin-CHA complexes. (A1-A7) SDS-PAGE profiles of CK, N-0.3, N-0.5, N-1, C-0.3, C-0.5, and C-1 during intestinal digestion. (B) Schematic summary of the effects of CHA interaction mode and concentration on the intestinal degradation of soybean 7S globulin.

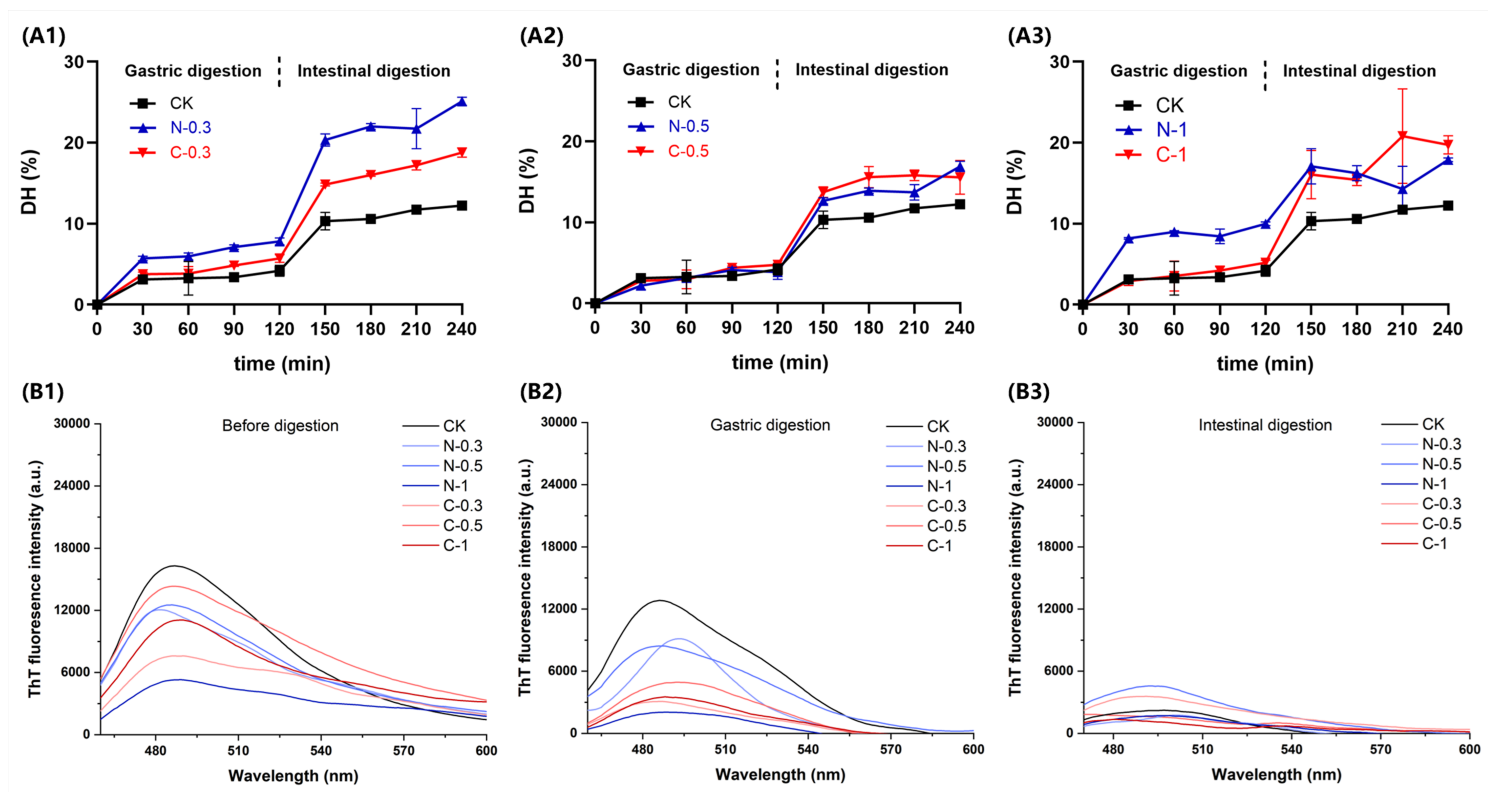


**Figure 4**

Degree of hydrolysis and retention of ThT-responsive amyloid-like aggregate structures during gastrointestinal digestion. (A1–A3) DH profiles at 0.3, 0.5, and 1.0 mM CHA, respectively. (B1–B3) ThT fluorescence spectra before digestion and after gastric and intestinal digestion, respectively.

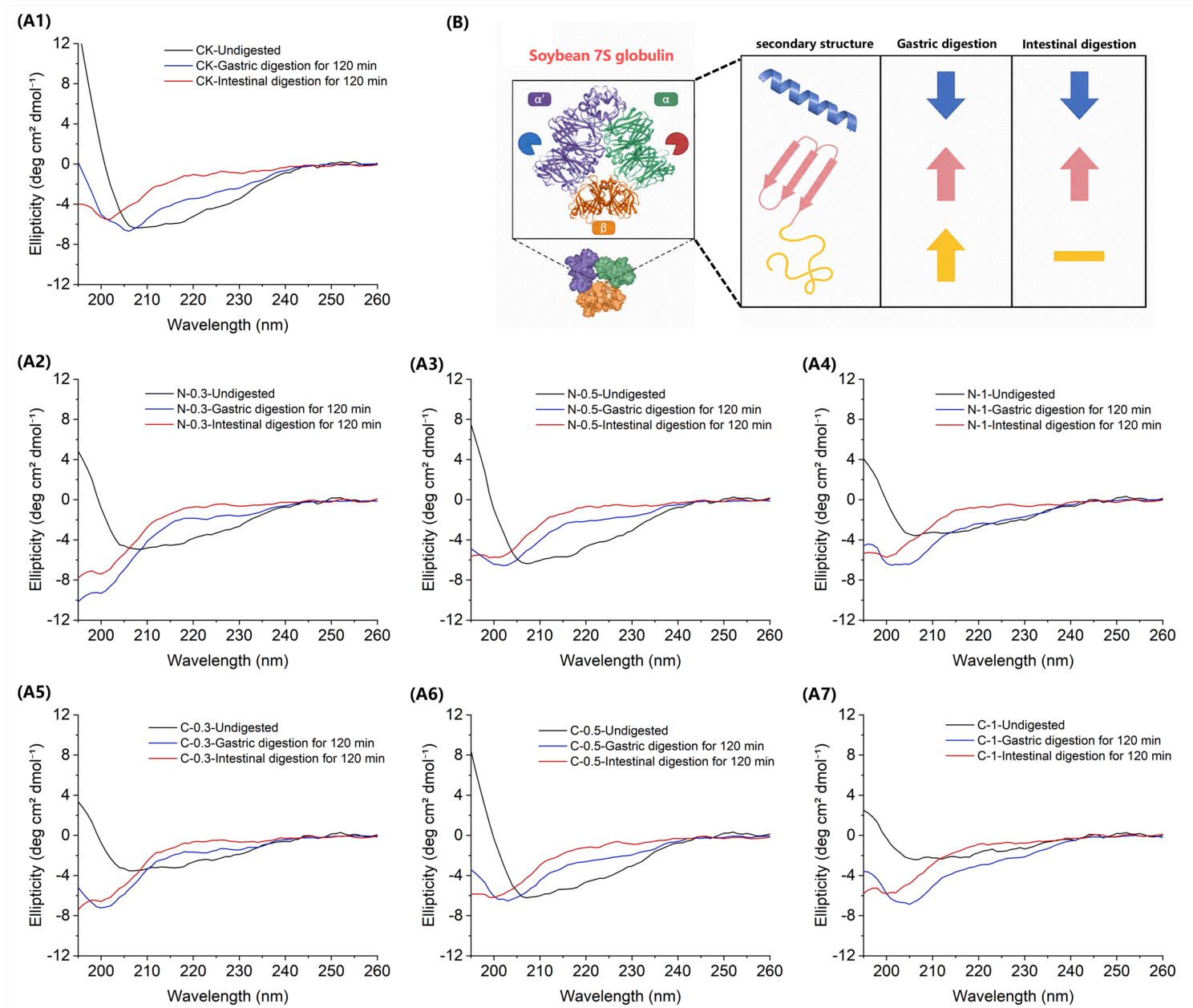


**Figure 5**

CD spectra and schematic representation of secondary-structure changes in soybean 7S globulin-CHA complexes during gastrointestinal digestion. (A1–A7) Far-UV CD spectra of CK, N-0.3, N-0.5, N-1, C-0.3, C-0.5, and C-1 before digestion and after gastric and intestinal digestion. (B) Schematic summary of the changes in  $\alpha$ -helix,  $\beta$ -sheet, and random-coil structures during digestion.



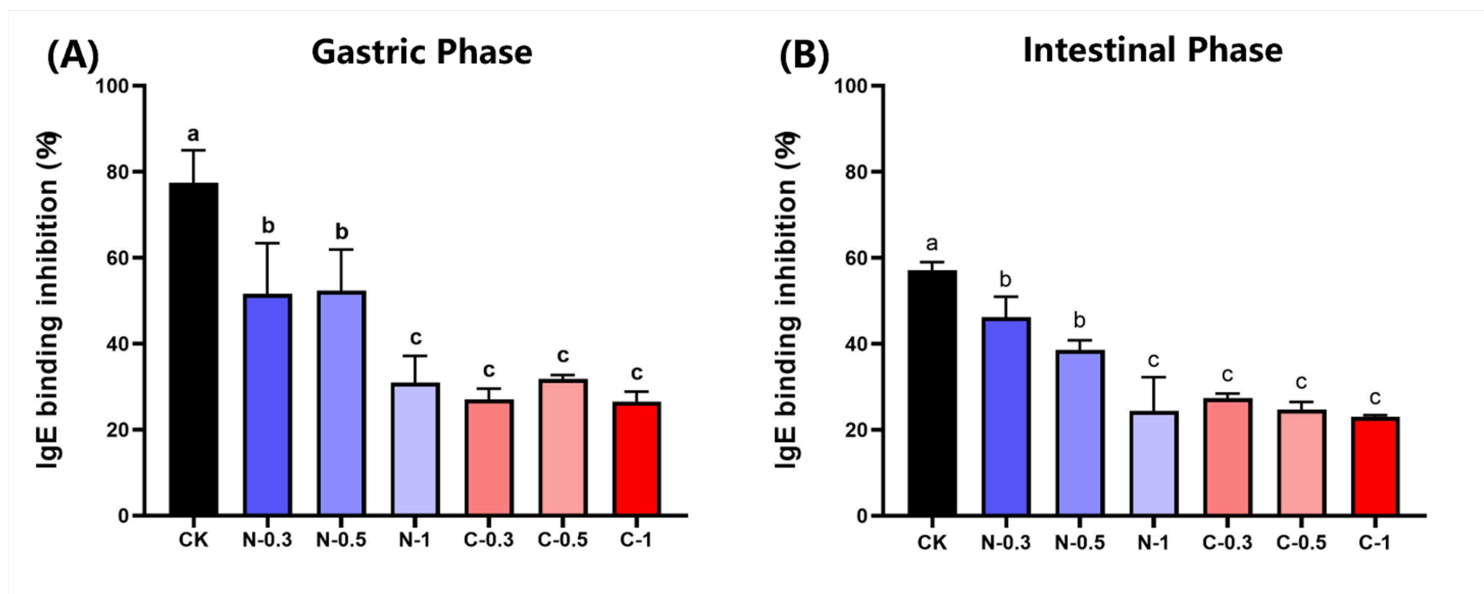


**Figure 6**

IgE-binding inhibition rates of soybean 7S globulin-CHA complexes after gastric and intestinal digestion. (A) Gastric digestion for 120 min. (B) Intestinal digestion for 120 min. Different lowercase letters indicate significant differences among groups ( $p < 0.05$ ).

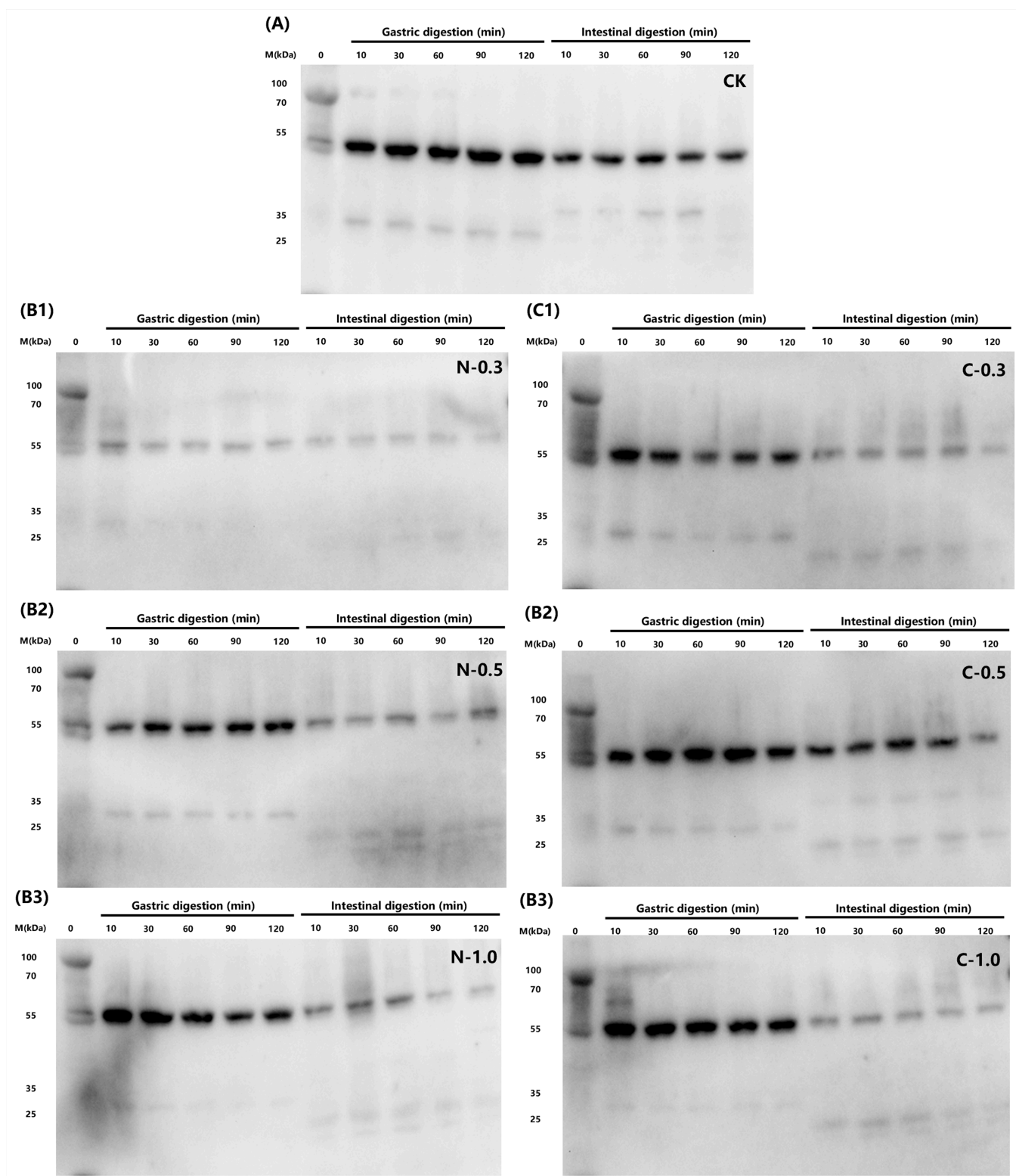


**Figure 7**

Western blot profiles of IgE-reactive digestion products from soybean 7S globulin-CHA complexes during gastrointestinal digestion. (A) CK; (B1-B3) N-0.3, N-0.5, and N-1.0; (C1-C3) C-0.3, C-0.5, and C-1.0.

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