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Research Article

Enzymatic hydrolysis of porcine fibrinogen by trypsin and flavourzyme: structural characterization and functional properties of low-molecular-weight peptides

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Abstract: This study investigated the enzymatic hydrolysis of porcine fibrinogen using trypsin, flavourzyme, and their sequential combination to clarify how hydrolysis conditions influence hydrolysates' structural and functional properties. The dual-enzyme treatment (trypsin followed by flavourzyme) produced the highest degree of hydrolysis (21.64%) and soluble protein recovery efficiency. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel permeation chromatography revealed extensive cleavage of the α-, β-, and γ-chains and a marked shift toward low-molecular-weight peptides, with fragments below 1.5 kDa comprising the majority of the hydrolysate. Amino acid analysis showed that the resulting peptide fraction of dual-enzyme treatment contained 31.39% essential amino acids and a higher cysteine content than single-enzyme hydrolysates. Moreover, enzymatic hydrolysis by complex proteases significantly increased surface hydrophobicity and reactive sulfhydryl group content, indicating greater exposure of nonpolar residues and active thiols that may favor disulfide bond formation during gelation. Overall, the improved structural and functional characteristics of dual-enzyme fibrinogen hydrolysates may provide a theoretical basis for their potential use in the development of gel-based foods.

Keywords: fibrinogen; enzymatic hydrolysis; structural characterization; functional properties

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1 Introduction

Fibrinogen is a plasma glycoprotein composed of two sets of three non-identical polypeptide chains, e.g., Aα (66 kDa), Bβ (52 kDa), and γ (46.5 kDa)[1–2]. *In vivo*, fibrinogen is converted by thrombin into fibrin monomers through the release of fibrinopeptides A and B, and these monomers spontaneously assemble into a three-dimensional gel network[3–4]. This well-organized, cross-linked structure provides a useful model system for understanding protein assembly, intermolecular interactions, and the mechanisms underlying gel network formation.

The structural and functional properties of proteins can be significantly modified through physical, chemical, or enzymatic treatments. Compared with physical or chemical treatments, enzymatic hydrolysis offers mild and controllable reaction conditions, allowing for targeted cleavage of peptide bonds without destroying the protein's essential structure[5]. Hydrolysis typically induces protein unfolding, peptide chain fragmentation, and exposure of reactive amino acid residues, e.g., thiol and hydrophobic groups. These structural transitions can substantially alter solubility, gelation, and other physicochemical parameters that govern protein behavior in aqueous systems[6]. Trypsin is a member of the serine endopeptidases of family S1, which plays an important role in diverse physiological processes, and its structure makes trypsin an ideal system to explore general features of protein folding and function[7]. As for flavourzyme, this is a microbial protease characterized by low cost, safe usage, and a relatively high hydrolysis yield compared to proteases from other sources[8]. Numerous studies have demonstrated that enzymatic treatments using these proteases can markedly improve the functional and physicochemical properties of food proteins. Gomes et al.[9] found that rice protein hydrolysates treated with flavourzyme exhibited improved emulsion stability and enhanced amphiphilicity. Shuai et al.[10] found that the solubility of pea protein treated with flavourzyme and trypsin was distinctly boosted.

Porcine blood, an abundant byproduct of meat processing, is a rich source of high-quality proteins but remains underutilized, often processed into low-value animal feed. Among its protein components, fibrinogen is particularly attractive for structural studies because of its high Cys content and disulfide-linked architecture, which make it sensitive to enzymatic modification[11–12]. Previous research has reported that enzymatic hydrolysis of animal blood proteins can yield peptides with desirable structural and physicochemical properties. For example, Laosam et al.[13] found that the products obtained from duck blood protein after treatment with neutrase or papain exhibited antioxidant and angiotensin-converting enzyme-inhibitory properties. Lafarga et al.[14]

found that the number of novel angiotensin-I-converting enzyme and renin inhibitory peptides was obtained from bovine fibrinogen after treatment with papain. However, systematic investigations on the structural and physicochemical characteristics of porcine fibrinogen hydrolysates obtained using trypsin and flavourzyme, particularly in dual-enzyme systems, remain limited.

In this study, we employed trypsin and flavourzyme for both individual and combined enzymatic hydrolysis of porcine fibrinogen. Optimal conditions were determined for each enzymatic system. The hydrolysates were then evaluated based on their degree of hydrolysis (DH), soluble protein recovery efficiency (SPRE), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography (GPC) profiles, and molecular weight distribution. To further characterize the functional potential of the hydrolyzed peptides, ultrafiltration was conducted using a 3 kDa molecular weight cut-off membrane. The resulting low-molecular-weight peptide fractions were analyzed for amino acid composition, surface hydrophobicity, and free thiol group content.

2 Materials and methods

2.1 Extraction of fibrinogen

After blood collection, 3.8 g/100 mL sodium citrate and 0.05 g/100 mL NaCl were added to the sample. The mixture was centrifuged at 4 000 × *g* for 10 min, and the supernatant was collected and frozen for 12 h at –20 °C. After thawing, the sample was centrifuged again at 4 000 × *g* for 15 min. The resulting precipitate was dissolved in physiological saline and centrifuged once more at 4 000 × *g* for 15 min. The final supernatant was supplemented with 1.5 g/100 mL mannitol and subsequently lyophilized for further enzymatic hydrolysis.

2.2 Enzymatic hydrolysis of fibrinogen

Fibrinogen was enzymatically hydrolyzed using three different protocols. In group 1 (FTP), fibrinogen was hydrolyzed with trypsin at pH 8.0 and 37 °C for 6 h. In group 2 (FFP), fibrinogen was hydrolyzed with flavourzyme at pH 6.0 and 50 °C for 6 h. In group 3 (FTFP), fibrinogen was sequentially hydrolyzed by two enzymes, where trypsin hydrolysis was first conducted at pH 8.0 and 37 °C for 3 h, followed by flavourzyme hydrolysis at pH 6.0 and 50 °C for an additional 3 h. In all groups, the enzyme-to-substrate ratio was maintained at 3% (*m*/*m*, based on the weight of fibrinogen). After enzymatic hydrolysis, the pH of the reaction mixtures was adjusted to 7.0, and the hydrolysates were then freeze-dried to yield peptide powders.

2.3 Determination of the DH and SPRE

The DH was determined as the ratio of amino nitrogen content to total nitrogen content. The amino nitrogen content was measured according to the Chinese national standard GB 5009.235–2016. Amino nitrogen reacted with acetylacetone and formaldehyde in a sodium acetate-acetic acid buffer (pH 4.8), forming yellow amino acid derivatives. The absorbance was measured at 400 nm for quantification. The total nitrogen content was determined by the Kjeldahl method, using a nitrogen-to-protein conversion factor of 6.25. The SPRE was calculated using the following equation:

$$\text{SPRE}\ (\%) = \frac{m}{m_0} \times 100 \quad (1)$$

Where m is the protein mass (g) after enzymatic hydrolysis and centrifugation and m_0 is the initial protein mass (g) in raw material.

2.4 Determination of SDS-PAGE profile

The SDS-PAGE analysis of three hydrolysates was performed following the methods of Du et al.[15]. A gradient precast gel was used for electrophoretic separation. The hydrolysate was mixed with the sample loading buffer, followed by heating at 70 °C for 10 min. A volume of 10 μL of the resulting supernatant was loaded into each well. Electrophoresis was carried out under constant voltage at 120 V. After electrophoresis, the gel was stained in Coomassie brilliant blue staining solution for 1 h, then destained for 24 h. The gel was subsequently imaged and analyzed using a gel documentation system.

2.5 Determination of molecular weight distribution

The molecular weight distribution of the hydrolysates was determined by GPC according to the method described by Jayaprakash et al.[16]. The hydrolysate solution (5 mg/mL) was filtered through a 0.22 μm microporous membrane prior to injection. Chromatographic separation was performed on a Superdex™ 30 increase column with phosphate buffer (pH 7.0) as the mobile phase, at a flow rate of 0.5 mL/min. A 500 μL aliquot of the sample was injected, and detection was conducted at 220 nm. Molecular weight calibration was performed using standard compounds, including bacitracin (1 422 Da), Gly-Gly-Tyr-Arg (451 Da), reduced glutathione (307 Da), and glutamic acid (145 Da), which were used to estimate the molecular weight distribution of the peptide components in the hydrolysates.

2.6 Determination of amino acid composition

The free amino acid compositions of the samples were analyzed using an automatic amino acid analyzer according to the method described by Chen et al.[17]. Fibrinogen hydrolysates with different enzymes, followed by section 2.2, were ultra-filtered using a 3 kDa centrifugal filter to obtain peptide fractions smaller than 3 kDa (designated as FTP, FFP, and FTFP). The hydrolysate was mixed with sulfosalicylic acid at a volume ratio of 4:1, followed by centrifugation at 12 000 × *g* for 15 min. The supernatant was collected and centrifuged again under the same conditions. The resulting supernatant was diluted, filtered through a 0.22 μm membrane, and subjected to amino acid analysis.

2.7 Determination of surface hydrophobicity

Surface hydrophobicity of the myofibrillar proteins was determined using a modified method described by Zhang et al.[18]. The protein concentration of the above hydrolysates (FTP, FFP, and FTFP) was first adjusted by mixing with buffer and quantified using a BCA protein assay kit (Biorigin, Beijing, China). A 1 mL aliquot of protein solution (adjusted to 2 mg/mL) was mixed with 80 μL of 1 mg/mL bromophenol blue (BPB) and incubated at room temperature for 10 min. The mixture was then centrifuged at 10 000 × *g* for 3 min. In the control group, 80 μL of 1 mg/mL BPB was added directly to the buffer without protein. The supernatant from the sample group was diluted 10-fold with buffer, and the

absorbance was measured at 595 nm. The BPB binding amount was calculated using the following equation:

$$\text{BPB binding amount } (\mu g/\text{mg prot}) = 80 \times \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}} \times 2} \quad (2)$$

Where A is the absorbance at 595 nm, 80 is the mass of BPB (μg), and 2 is the mass of protein (mg).

2.8 Determination of free sulfhydryl content

The free sulfhydryl content was determined according to the method described by Han et al.[19]. A 0.5 mL aliquot of the above hydrolysates (FTP, FFP, and FTFP) with concentration of 1 mg/mL was mixed with 4.5 mL of Tris-glycine buffer (0.086 mol/L Tris, 0.09 mol/L glycine, 4 mmol/L ethylene diamine tetraacetic acid, pH 8.0), followed by the addition of 0.5 mL of Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The mixture was vortexed thoroughly and incubated at 30 °C for 30 min. Absorbance was then measured at 412 nm. The free sulfhydryl content was calculated using the following formula:

$$\text{Free sulfhydryl content } (\text{nmol}/\text{mg}) = \frac{A_{412\text{ nm}} \times 11}{13\,600 \times \rho} \times 10^6 \quad (3)$$

Where ρ is the protein concentration (mg/mL), 11 is the dilution factor, and the molar absorptivity of the DTNB-thiol adduct is 13 600 L/(mol·cm).

2.9 Statistical analysis

Statistical analysis was determined according to the method described by Bie et al.[20]. Each experiment was replicated at least three times. The comparisons among experimental groups were performed using one-way analysis of variance in SPSS 22, followed by Duncan's multiple range test, with a significance level of $P < 0.05$.

3 Results and discussion

3.1 DH and protein recovery efficiency analysis

The DH is defined as the extent to which peptide bonds in proteins are cleaved during the hydrolysis process[21]. Higher DH often indicates that proteins are cleaved into a greater number of low-molecular-weight polypeptides. Figure 1A shows the DH of fibrinogen with different enzymatic hydrolysis. The DH of fibrinogen was 12.55% when hydrolyzed by trypsin, 17.48% by flavourzyme, and 21.64% by complex protease, which was hydrolyzed by trypsin first and then by flavourzyme. Samples of complex protease hydrolysis had the best hydrolysis degree, and this might be due to the greater number of cleavage sites accessed by the dual-enzyme system. To the best of our knowledge, DH primarily depends on the protease's accessibility to cleaved peptide bonds. The catalytic efficiency of an enzyme is determined by several factors, including the affinity of the enzyme to bind to the substrate, the shape and structure of the active site, and the stereospecific orientation of the peptide bonds. The DH of fibrinogen was limited when trypsin was used alone; however, the combination with flavourzyme significantly enhanced the hydrolysis of hydrophobic peptide sequences within the fibrinogen, leading to a marked improvement in overall hydrolysis efficiency[22].

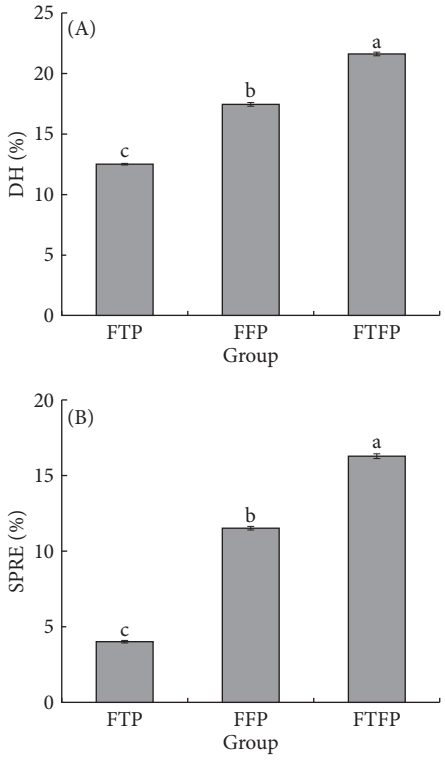

Figure 1 (A) DH of fibrinogen by different enzymes and (B) SPRE of fibrinogen enzymatic hydrolysates by different enzymes. Bars labeled with different lowercase letters indicate statistically significant differences ($P < 0.05$) between groups.

The SPRE indicated the ratio of recovered hydrolysis products to the initial protein mass, thus providing an indicator of enzymatic hydrolysis efficiency[23]. As summarized in Figure 1B, the FTFP group had the highest SPRE, reaching 16.27%, followed by the FFP group at 11.52%, whereas FTP group exhibited the lowest SPRE at 4.02%. This observation is consistent with the DH results presented in Figure 1A, confirming that dual or sequential enzymatic hydrolysis can effectively enhance the solubilization and degradation of fibrinogen. Trypsin could facilitate the initial breakdown of fibrinogen's tightly folded structure, exposing potential enzymatic sites. Flavourzyme could further degrade intermediate peptides, thus improving the overall hydrolysis degree and peptide recovery of porcine fibrinogen. Similarly, Ma et al.[24] found that soy protein treated with alkaline followed by flavourzyme processing increased hydrolysis by 3.5% and improved protein recovery by 0.8%, as compared with alkaline alone.

3.2 SDS-PAGE analysis

To examine the composition of fibrinogen in trypsin-, flavourzyme-, and complex protease-treated samples, SDS-PAGE analysis was employed. As shown in Figure 2, the electrophoretic profiles revealed clear differences among treatments. Lane 1 corresponds to the molecular weight marker, while lane 2 shows the typical electrophoretic bands of fibrinogen. Three major bands were observed, representing the α- (66 kDa), β- (52 kDa), and γ-chains (46.5 kDa) of fibrinogen, consistent with previous reports[25–26]. Lanes 3–5 represent the electrophoretic profiles of fibrinogen hydrolysates with different proteases. Notably, in lane 5 (complex protease treatment), several high-molecular-weight bands, particularly at

52 and 46.5 kDa, were no longer visible, indicating extensive degradation of the β- and γ-chains. This indicates that the sequential treatment by trypsin followed by flavourzyme enabled more comprehensive cleavage of peptide bonds, yielding a higher proportion of small peptides. The formation of these low-molecular-weight fractions agrees well with the higher DH (21.64%) and SPRE (16.27%), demonstrating that the dual-enzyme system effectively disrupted the fibrinogen network structure and enhanced peptide solubilization. Similar observations have been reported by Purschke et al.[27], where protein bands of migratory locust (*Locusta migratoria* L.) protein flour after treatment by flavourzyme in the 37–45 kDa range gradually decomposed following enzymatic hydrolysis and completely disappeared after 24 h. It was also worth noting that there were many new bands that appeared at 10 kDa. While the protein flour was treated with neutral protease, the band here did not completely disappear. This further confirms that many small molecular weight peptide fragments appeared after the enzymatic degradation of fibrinogen by the complex enzyme.

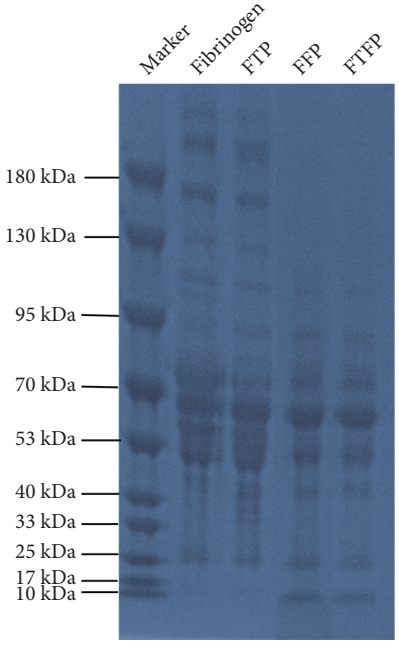

Figure 2 SDS-PAGE profile of fibrinogen enzymatic hydrolysates by different enzymes.

3.3 Molecular weight distribution analysis

Peptides of lower molecular weight, which are highly relevant to bioactivity and solubility, cannot be effectively visualized by SDS-PAGE. To address this limitation, GPC was conducted to characterize peptides below 10 kDa further. GPC normally refers to the separation of polymers on a separation column according to their molecular hydrodynamic volume, thereby facilitating the analysis of molecular weight distribution within fibrinogen enzymatic hydrolysate[28]. The GPC chromatograms of fibrinogen hydrolysates obtained by different enzymatic treatments are presented in Figure 3, and the corresponding molecular weight distributions are summarized in Table 1.

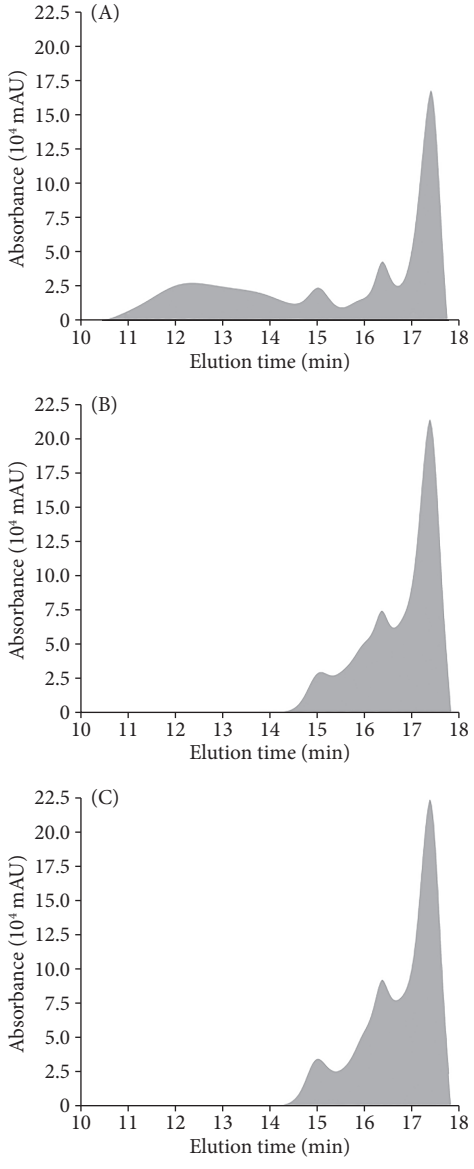

Figure 3 GPC chromatograms of fibrinogen enzymatic hydrolysates (A) FTP, (B) FFP, and (C) FTFP.

Table 1 Molecular weight distribution of different enzymatic hydrolysates.

Molecular weight (Da)	Relative content (%)		
	FTP	FFP	FTFP
> 3 000	54.28 ± 0.73^a	35.74 ± 0.78^b	34.46 ± 0.29^c
1 500–3 000	6.93 ± 0.43^c	14.83 ± 0.10^a	13.65 ± 0.19^b
1 000–1 500	10.78 ± 0.15^c	14.53 ± 0.28^b	15.23 ± 0.10^a
500–1 000	27.44 ± 0.14^c	32.60 ± 0.51^b	34.23 ± 0.16^a
< 500	0.58 ± 0.03^b	2.30 ± 0.08^a	2.43 ± 0.22^a

Note: Values within a row marked with different lowercase superscript letters indicate statistically significant differences ($P < 0.05$).

In the GPC chromatograms, the horizontal axis represents elution time, where shorter elution times indicate higher molecular weight species, while the vertical axis reflects absorbance, corresponding to protein concentration. For the FTP samples (Figure 3A), the elution time was short, and the concentration of small molecular weight proteins was low. In the GPC, the larger the peak area for a given time interval, the higher the protein

concentration during that interval. The protein elution time of FTFP (Figure 3C) is similar to that of FFP (Figure 3B). As shown in Table 1, the molecular weights of fibrinogen hydrolysates were primarily distributed in two major ranges: peptides larger than 3 000 Da and those between 500 to 1 000 Da, together accounting for over 70% of the total protein content. Importantly, compared with single enzymatic hydrolysis, the proportion of peptides within the 500–1 000 Da increased markedly, while the proportion of peptides larger than 3 000 Da decreased substantially following hydrolysis with the complex protease ($P < 0.05$). This indicates that sequential enzymatic hydrolysis progressively cleaves fibrinogen into smaller peptide fragments. The complementary results from GPC provide more detailed evidence of peptide size distribution below the detection limit of SDS-PAGE. Rezvankhah et al.[29] found that when using only alcalase or flavourzyme to hydrolyze lentil protein, the chromatogram of the resulting hydrolysate showed a reduction in peak area for molecular weights of > 9.4 kDa, whereas sequential treatment with alcalase followed by flavourzyme resulted in the near-complete disappearance of peaks with molecular weights > 9.4 kDa and a significant increase in peak areas for molecular weights < 4.4 kDa, in agreement with our experimental results.

3.4 Amino acid composition analysis

Amino acid composition plays a critical role in determining the functional properties and nutritional quality of proteins[30]. The detailed composition of each treatment group is presented in Table 2. Glu is one of the umami amino acids, its content was significantly raised to 4.38% when fibrinogen was treated by dual enzymes. Thr, Ser, Gly, Ala, and Pro all belong to sweet amino acids, and the content of Gly, Ala, and Pro was significantly raised when fibrinogen was treated with dual enzymes. Met, Ile, Leu, Tyr, Phe, Lys, and Arg belong to the bitter amino acids, and their contents were significantly reduced when fibrinogen was treated by dual enzymes ($P < 0.05$). This could demonstrate that fibrinogen treated with dual enzymes might enhance sweetness and saltiness while reducing bitterness. These shifts might be attributed to the broader specificity of complex protease, which effectively cleaves peptide bonds at sites less accessible to single enzymes, thereby releasing more Ala-, Gln-, Glu-, and Gly-rich fragments while reducing the release of Leu- and Lys-rich sequences. Furthermore, trypsin (endo-type) and flavourzyme (exo-type) act synergistically during combination hydrolysis, generating more cleavage sites and causing deeper structural unfolding of fibrinogen. This enhanced proteolysis would expose previously buried Cys residues and release Cys-containing peptides, thereby increasing the detectable Cys content[31]. The relatively higher Cys contents in the FTFP group suggest an increased abundance of reactive sulfhydryl groups, which may promote disulfide bond formation, potentially influencing gelation, emulsification, and antioxidant properties of the hydrolysate. Conversely, the relative reduction in Lys, although lowering the basic amino acid content could reduce Maillard reactivity during thermal processing, thereby influencing flavor stability. Essential amino acids refer to amino acids that the human body (or other vertebrates) cannot be synthesized or are synthesized at a rate that is far from sufficient to meet the body's needs and must be supplied by dietary protein[32]. In the FTFP group, essential amino acids accounted for 31.39% of the total amino acids, a ratio consistent with the Food and Agriculture Organization/World Health Organization recommended intake levels for adults[33]. This finding indicates that the amino acid composition of the complex protease hydrolysate meets the requirements for high-quality dietary protein. Senadheera et al.[34] found that the essential amino acids in the protein hydrolysates from body parts of North Atlantic sea cucumber (*Cucumaria frondosa*) treated with flavourzyme were more exposed than those in the unhydrolyzed protein.

Table 2 Amino acid composition of fibrinogen hydrolysates from different enzymes.

Amino acid	Relative content (%)		
	FTP	FFP	FTFP
Ala	1.79 ± 0.13^{b}	1.33 ± 0.12^{c}	10.57 ± 0.33^{a}
Arg	12.44 ± 0.15^{b}	14.10 ± 0.09^{a}	7.89 ± 0.37^{c}
Asn	2.61 ± 0.04^{a}	1.84 ± 0.12^{b}	0.54 ± 0.11^{c}
Asp	1.54 ± 0.06^{a}	0.93 ± 0.09^{b}	1.27 ± 0.22^{a}
Gln	10.20 ± 0.10^{b}	8.65 ± 0.11^{c}	22.18 ± 0.05^{a}
Glu	1.45 ± 0.04^{b}	0.96 ± 0.10^{a}	4.38 ± 0.13^{c}
Gly	0.98 ± 0.07^{b}	0.87 ± 0.09^{b}	5.94 ± 0.17^{a}
His	2.59 ± 0.05^{c}	3.05 ± 0.10^{b}	4.16 ± 0.12^{a}
Ile	5.24 ± 0.02^{a}	3.93 ± 0.09^{b}	2.88 ± 0.12^{c}
Cys	0.99 ± 0.06^{b}	1.18 ± 0.11^{b}	1.72 ± 0.16^{a}
Leu	9.63 ± 0.08^{a}	8.04 ± 0.15^{b}	4.87 ± 0.15^{c}
Hyp	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}	0.41 ± 0.15^{a}
Trp	4.70 ± 0.04^{b}	7.09 ± 0.10^{a}	3.63 ± 0.17^{c}
Lys	17.34 ± 0.28^{b}	20.20 ± 0.26^{a}	8.36 ± 0.11^{c}
Met	2.44 ± 0.12^{a}	2.07 ± 0.10^{b}	0.83 ± 0.13^{c}
Phe	5.67 ± 0.03^{b}	6.47 ± 0.09^{a}	2.67 ± 0.20^{c}
Pro	3.59 ± 0.10^{b}	2.80 ± 0.11^{c}	5.19 ± 0.16^{a}
Ser	4.03 ± 0.03^{a}	2.81 ± 0.10^{b}	1.59 ± 0.14^{c}
Thr	3.13 ± 0.05^{a}	2.74 ± 0.08^{b}	2.29 ± 0.12^{c}
Tyr	5.31 ± 0.04^{b}	7.78 ± 0.10^{a}	2.76 ± 0.18^{c}
Val	4.28 ± 0.05^{b}	3.15 ± 0.12^{c}	5.86 ± 0.20^{a}

Note: Values within a row marked with different lowercase superscript letters indicate statistically significant differences ($P < 0.05$).

3.5 Surface hydrophobicity analysis

Surface hydrophobicity is an important indicator of protein stability and solubility, as it reflects the exposure of hydrophobic residues, which in turn affects protein interactions, aggregation, gelation, and emulsification[35–36]. Enzymatic hydrolysis alters protein conformation by cleaving peptide bonds, which can induce peptide chain unfolding or fragmentation[37]. Typically, the higher the DH, the higher the surface hydrophobicity, as more hydrophobic amino acid residues become exposed to the aqueous environment[38]. As shown in Figure 4, the surface hydrophobicity of fibrinogen hydrolysates varied significantly among treatments. The highest value was observed in the FTFP group (complex protease), followed by the FFP group, while the lowest value was found in the FTP group. This trend is consistent with the DH data presented in Figure 1A, further supporting the notion that extensive cleavage by complex proteases exposes a larger number of hydrophobic residues. These findings also align with the previous study, which reported that tree peony seed protein treated with composite enzymes generally exhibited higher surface hydrophobicity compared with single-enzyme treatments such as flavourzyme alone[39]. The exposure of hydrophobic patches could enhance the potential for intermolecular interactions, which may promote aggregation, affect solubility, and alter gelation behavior. This can be advantageous in certain food applications, such as emulsification, where hydrophobic interactions at the oil-water interface stabilize emulsions.

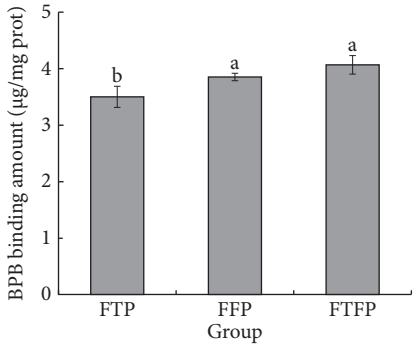

Figure 4 Surface hydrophobicity of fibrinogen enzymatic hydrolysates. Bars labeled with different lowercase letters indicate statistically significant differences ($P < 0.05$) between groups.

3.6 Free sulfhydryl content analysis

The content of free sulfhydryl is an important indicator of the structural state and functional behavior of proteins, particularly in relation to the formation of protein networks[40]. Reactive sulfhydryl groups can be oxidized to form disulfide bonds, which serve as covalent cross-links that stabilize the three-dimensional structure of protein gels. Thus, a higher content of free sulfhydryl generally reflects greater potential for disulfide bond formation, contributing to stronger and more stable protein networks[24]. As shown in Figure 5, this result is consistent with amino acid composition analysis (Table 2), where the FTFP hydrolysates contained a relatively higher proportion of Cys residues, providing more potential free sulfhydryl donors. The elevated free sulfhydryl content in FTFP can be attributed to the higher DH achieved by complex protease treatment, which promotes protein unfolding and cleavage, thereby exposing buried Cys residues. Similar trends have been reported previously, where egg yolk protein treated with flavourzyme unfolding and structural extension led to increased exposure of free sulfhydryl[41]. The increase in free sulfhydryl shows practical implications for gel formation and protein functionality. Upon heating or oxidative processing, these free sulfhydryl can undergo disulfide bond formation, which strengthens protein-protein interactions and stabilizes gel networks. This suggests that hydrolysates produced by complex protease treatment may act as effective functional additives to improve the gelation properties[18].

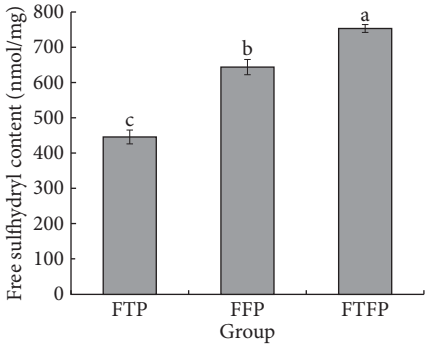

Figure 5 Free sulfhydryl content of fibrinogen enzymatic hydrolysates. Bars labeled with different lowercase letters indicate statistically significant differences ($P < 0.05$) between groups.

4 Conclusion

In this study, porcine fibrinogen was hydrolyzed using different enzymatic methods, among which complex proteases of trypsin and flavourzyme exhibited the most efficient performance, achieving the highest DH of 21.64%. This treatment generated abundant small peptides, many of which had molecular weights below 1.5 kDa. These small peptides showed a balanced profile of essential amino acids and a higher Cys content. Moreover, enzymatic hydrolysis by complex proteases significantly increased surface hydrophobicity and free sulfhydryl content, both of which could potentially facilitate intermolecular interactions and the formation of stable gel networks. These findings demonstrate that enzymatic hydrolysis, particularly with dual-enzyme systems, improved structural and functional characteristics of porcine fibrinogen. From an application perspective, considering the vast annual production of pig blood and its current low utilization rate, this strategy offers an effective pathway to upgrade its value-added use.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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