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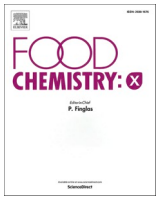

# Purified protein glutaminase from *Chryseobacterium proteolyticum* enhances the properties of wheat gluten

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ARTICLE INFO

*Chemical compounds:*
Ammonia
Nitrite
Trichloroacetic acid
Cbz-Gln-Gly
Lactose
Sodium chloride
Sodium dodecyl sulfate
Hydrochloric acid
Polyethylene glycol
Cobalt chloride

*Keywords:*
Wheat gluten
Protein glutaminase
Enzymatic deamidation
Purification
Properties
Cake

ABSTRACT

Protein glutaminase (PG), originating from *Chryseobacterium proteolyticum,* can catalyze the deamidation of glutamine residues in plant proteins into glutamic acid, thus enhancing its functional properties. However, the low yield of PG limits its industrial production. In this study, the yield of PG in *C. proteolyticum* TM1040 increased by 121 %, up to 7.30 U/mL in a 15 L fermenter after medium optimization. Subsequently, purified PG was obtained by cation exchange chromatography (CEX) coupled with hydrophobic interaction chromatography (HIC). The degree of deamidation (DD) of wheat gluten after purified PG deamidation was 87.11 %, which is superior to chemical deamidation in safety and DD. The emulsifying and foaming properties of deamidated wheat gluten were 2.67 and 18.86 times higher, and the water- and oil-holding properties were 4.23 and 18.77 times higher, respectively. The deamidated wheat gluten with enhanced functional properties was used to improve the flavor and texture in baking cakes.

## 1. Introduction

As the food industry evolves toward new protein-modified foods and plant-based protein foods, the public's awareness of functional, sustainable, futuristic, and individually customizable foods grows. PG has developed into a powerful protein-modifying enzyme in the food industry in recent years (X. Liu, et al., 2022; Zhang, et al., 2021). The PG (EC 3.5.1.44) catalytic triad (Cys-His-Asp) (Hashizume, et al., 2011) can only catalyze the deamidation of glutamine residues on proteins to glutamic acid residues and ammonia without affecting asparagine residues. Deamidation converts amide groups into highly hydrophobic carboxyl groups in proteins, which increases the negative surface charge, reduces hydrogen bonding, and increases intermolecular electrostatic repulsion (Qiu, et al., 2013), leading to a partial stretching of the spatial structure and a significant improvement in the functional properties of proteins (Liao, et al., 2016; Y. H. Liu, et al., 2018). Therefore, PG can enhance the functional properties of plant proteins such as grains, legumes, and plant processing by-products, improving their application as nutritional supplements (Chen, et al., 2021; X. Liu, et al., 2022). In addition, PG also improved the solubility of animal proteins like chicken breast myofibrillar proteins (CMPs) (Fu, et al., 2022).

In 2000, Shotaro Yamaguchi et al. discovered the strain of *C. proteolyticum* 9670 $^{T}$ that produced the PG (Yamaguchi & Yokoe, 2000). However, *C. proteolyticum* is difficult to use in industrial PG production due to its extremely low yield of PG (0.26 U/mL) (Yamaguchi, et al., 2000). Although heterologous expression is effective in increasing PG production (Kikuchi, et al., 2008; Kikuchi, et al., 2009; Lu,

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et al., 2020; Ouyang, et al., 2021; Yin, et al., 2021), it requires exogenous proteases for the activation of the PG precursor. Thus, directly increasing PG production in *C. proteolyticum*is still feasible. Wang et al. obtained mutant strain *C. proteolyticum* WG15 with a PG-yield of 2.91 U/mL, which is 48.69 % higher than the original strain by screening 1003 mutants using atmospheric and room temperature plasma (ARTP) with LiCl (L. J. Wang, et al., 2023). Moreover, adding 1.60 g/L of leucine to the medium and optimizing the fermentation conditions can improve the yield of PG in *C. proteolyticum*. Zhang et al. achieved a PG yield of 2.93 U/mL, which was 1.87 times that of the original medium (L. Wang, et al., 2023).

Wheat gluten is frequently employed as a functional additive to modulate the texture and energy of meat products (Carvalho, et al., 2019) and baked items (Umaraw, et al., 2021), such as Chinese sausage and cake. The application of wheat gluten is constrained by the poor functional characteristics brought on by its high concentration of glutamine residues (Lura & Schirch, 1988). However, non-enzymatic deamidation of proteins in high concentrations of hydrochloric acid (HCl) produces carcinogenic chloropropanols at high temperatures (Lei, et al., 2015). Peptide bond hydrolysis is difficult to control when proteins are deamidated at low concentrations of HCl, leading to protein denaturation and amino acid destruction at high temperatures (Liao, et al., 2010). The enzyme-catalyzed properties of PG have great potential for safe deamidation and expanding the applications of wheat gluten.

In this study, a high PG yield mutant strain *C. proteolyticum* TM1040 was used to optimize medium conditions for PG production. PG was purified using CEX and HIC and investigated for its enzymatic properties. The functional properties of wheat gluten were improved using PG modification and applied to superior cake production. This work provides a comprehensive set of practical suggestions for PG expression optimization, purification, and food application of PG produced in *C. proteolyticum*.

## 2. Material and methods

### 2.1. Strains and culture conditions

Table S1 in the Supplemental material contains a list of the strains utilized in this study. *C. proteolyticum* QSH1265 was isolated from the soil by Qu et al. (R. D. Qu, et al., 2018). *C. proteolyticum* strains-TM1040, A4142, 3–16, and 1–2 were obtained by nitrite- ultraviolet (NIT-UV) mutagenesis from *C. proteolyticum* QSH1265. The *C. proteolyticum* strains were cultivated in shake flasks for 16 h at 30 ℃ in seed broth (Polypeptone 10 g/L, magnesium sulfate 1 g/L, yeast extract 2 g/L, pH = 7.0). The bacteria were then subcultured in a fermentation medium (pH = 7.2, polypeptone 10 g/L, lactose 5 g/L, disodium hydrogen phosphate dodecahydrate 3.8 g/L, magnesium sulfate heptahydrate 0.25 g/L, potassium dihydrogen phosphate 0.25 g/L, ferrous sulfate heptahydrate 0.05 g/L).

### 2.2. PG enzyme activity

PG enzyme activity experiment was measured as described by Qu et al. (R. Qu, et al., 2022) and Yin et al. (Yin, et al., 2021). 10 μL of PG enzyme solution was combined with either 100 μL of 1 N trichloroacetic acid (TCA; $X_0$) or 100 μL of Cbz-Gln-Gly (10.11 g/L; $X_1$) for 30 min at 37 °C. Then, for $X_1$ or $X_0$, 100 μL of TCA or 100 μL of Cbz-Gln-Gly was added to the reaction system, respectively. 12 μL of the aforementioned reaction solution was combined with 60 μL of Color Developer A (40.46 g/L phenol, 0.15 g/L sodium nitroprusside), 48 μL of distilled water, 30 μL of Color Developer B (49.94 g/L potassium hydroxide), and 60 μL of Color Developer C (200 g/L potassium carbonate, 8.37 mL/L sodium hypochlorite) for 20 min at 37 °C. The reaction was followed by an ammonium chloride concentration gradient using the reaction system, and it was finished with 100 μL of TCA or 100 μL of Cbz-Gln-Gly, as appropriate. The absorbance was determined at OD630 using the concentration gradient of ammonium chloride in reaction with Color Developer A, distilled water, Color Developer B, and Color Developer C as a reference curve. The formula used to determine enzyme activity was as follows:

$$PGenzymeactivity(U/mL) = (A_0 - A_1) \times 21/17.03/30 \times k \quad (1)$$

In the formula, $A_0$: the absorbance value of the reaction with Cbz-Gln-Gly first; $A_1$: the absorbance value of the reaction with TCA first; k: the slope of the ammonia standard curve.

### 2.3. SP Sepharose$^{TM}$ fast flow CEX

The samples were pretreated by CEX, and the lyophilized enzyme powder after ultrafiltration concentration was dissolved in phosphate buffered saline (PBS) buffer, filtered through 0.45 μm of sterile microporous membrane, and adjusted to the proper pH with 50 mM citric acid to obtain the samples. The samples were then analyzed for the activity of the PG and the amount of protein present. The sample was added after the CEX columns had been equilibrated with 5 times the column volume of the PBS buffer. By gradient eluting the samples with PBS buffer (containing 0–2.0 M sodium chloride, NaCl) from the low to the high concentration of NaCl, with each elution using two column volumes, the PG enzyme activity and protein concentration were assessed.

### 2.4. Phenyl Sepharose HIC

The samples were pretreated by HIC, and after being concentrated through ultrafiltration, the lyophilized enzyme powder was dissolved in PBS buffer (2.0 M NaCl), filtered through a 0.45 μm sterile microporous membrane, and then its pH was adjusted to 6.5 with 50 mM citric acid. The samples were then used to calculate the concentration and activity of the PG. After equilibrating the HIC column with PBS (pH = 6.5) containing 2.0 M NaCl buffer for 5 times the column capacity, samples were added. Using PBS buffer containing 2.0–0 M NaCl to elute successively from high to low concentrations, the permeate was collected for each concentration gradient and the PG enzyme activity and protein content were assessed in the obtained permeate.

### 2.5. Temperature and pH stability

The approach of Yamaguchi et al. (Yamaguchi, et al., 2001) and Qu et al. (R. Qu, et al., 2022) were adjusted slightly to account for the stabilities of temperature and pH. A buffer with a pH range of 3 to 12 (consisting of 40 mM boric acid, acetic acid, and sodium hydroxide) was incubated with PG for 12 h and the assay was tested for remaining enzyme activity. The remaining enzymatic activity of PG was determined by incubating PG at the recommended temperature for different times.

### 2.6. Optimum reaction temperature and pH

The technique of Yamaguchi et al. (Yamaguchi, et al., 2001) and Qu et al. (R. Qu, et al., 2022) were referenced, with the ideal reaction temperature and pH being slightly changed. 40 mM boric acid/acetic acid/sodium hydroxide buffers with a pH range of 3 to 12 were used to establish the ideal reaction pH on PG activity. PG activity was measured at the specified temperature with a reaction duration of 30 min to calculate the ideal reaction temperature.

### 2.7. Deamidation degree

The deamidation degree test was measured as described by Inthawoot et al. (Suppavorasatit, et al., 2011) with a small adjustment. The content of ammonia after full deamidation of wheat gluten was

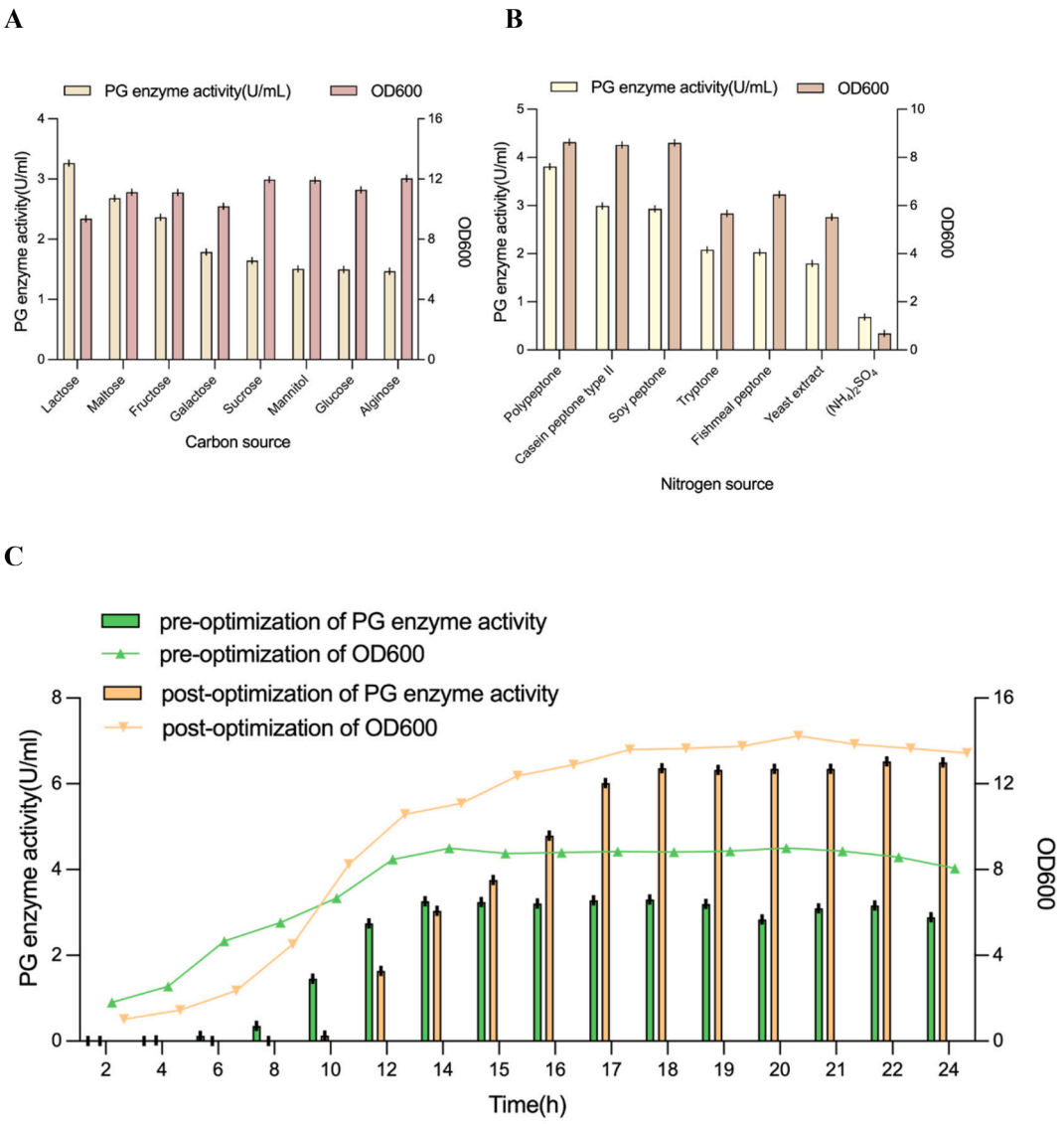


**Fig. 1. Growth and PG-producing properties of *C. proteolyticum* TM1040.** Fermentation medium of *C. proteolyticum* TM1040 optimized for (A) carbon sources and (B) nitrogen sources; (C) PG enzyme activity and OD600 detected after medium optimization of *C. proteolyticum* TM1040.

determined in the supernatant using the method for PG enzyme activity. Put 500 μL of sample in a 1.5 mL Eppendorf tube, add an equal volume of 2 N sulfuric acid solution, heat in a water bath at 100 °C for 4 h, centrifuge at 12,000 revolutions per minute for 10 min, and take an appropriate amount of supernatant. After wheat gluten had been deamidated by PG, a suitable amount of the supernatant was removed and used to measure the concentration of $NH_4^+$ in the supernatant. Deamidation is the difference between the amount of ammonia created when PG partially deaminates wheat gluten and the amount of ammonia produced when wheat gluten is completely deaminated.

### 2.8. Emulsifying activity

The methods of Hadidi et al. (Hadidi, et al., 2021) and Kunarayakul et al.(Kunarayakul, et al., 2018) were used to determine the emulsifying activity of wheat gluten. The lyophilized samples of PG-modified wheat gluten were weighed and then re-dissolved. After being dissolved in 10 mL of distilled water, the samples were added to 3 mL of soybean oil. The samples were then homogenized using a high-speed homogenizer at 20,000 revolutions per minute for 2 min. After the procedure, 50 μL of the solution was sucked up and mixed well with 50 mL of 0.1 % sodium dodecyl sulfate (SDS) solution. A spectrophotometer was used to determine the absorbance value $E_0$ at 500 nm, and the emulsifying activity index (EAI) was computed as follows:

$$EAI\left(m^2/g\right) = 2 \times 2.303 \times E_0 \times k/0.25/c/10,000.$$

k is the number of times the emulsified emulsion was sampled to a dilution of 0.1 % SDS. c is the amount of protein per ml of solution. This method is in line with the evaluation of emulsification capacity by the National Standard of the People's Republic of China (GB/T 11543–2008).

### 2.9. Foam capacity

The methods of Hadidi et al. (Hadidi, et al., 2021) and Kunarayakul et al.(Kunarayakul, et al., 2018) were used to determine the foam capacity of wheat gluten (ISO 696–1975). The wheat gluten with various degrees of deamidation was lyophilized and re-solubilized, and 20 mg of each wheat gluten was weighed and dissolved in 20 mL of distilled water. The sample was then homogenized using a high-speed tissue homogenizer at 20,000 revolutions per minute for 2 min, and after the procedure, the sample was transferred to a measuring cylinder to record

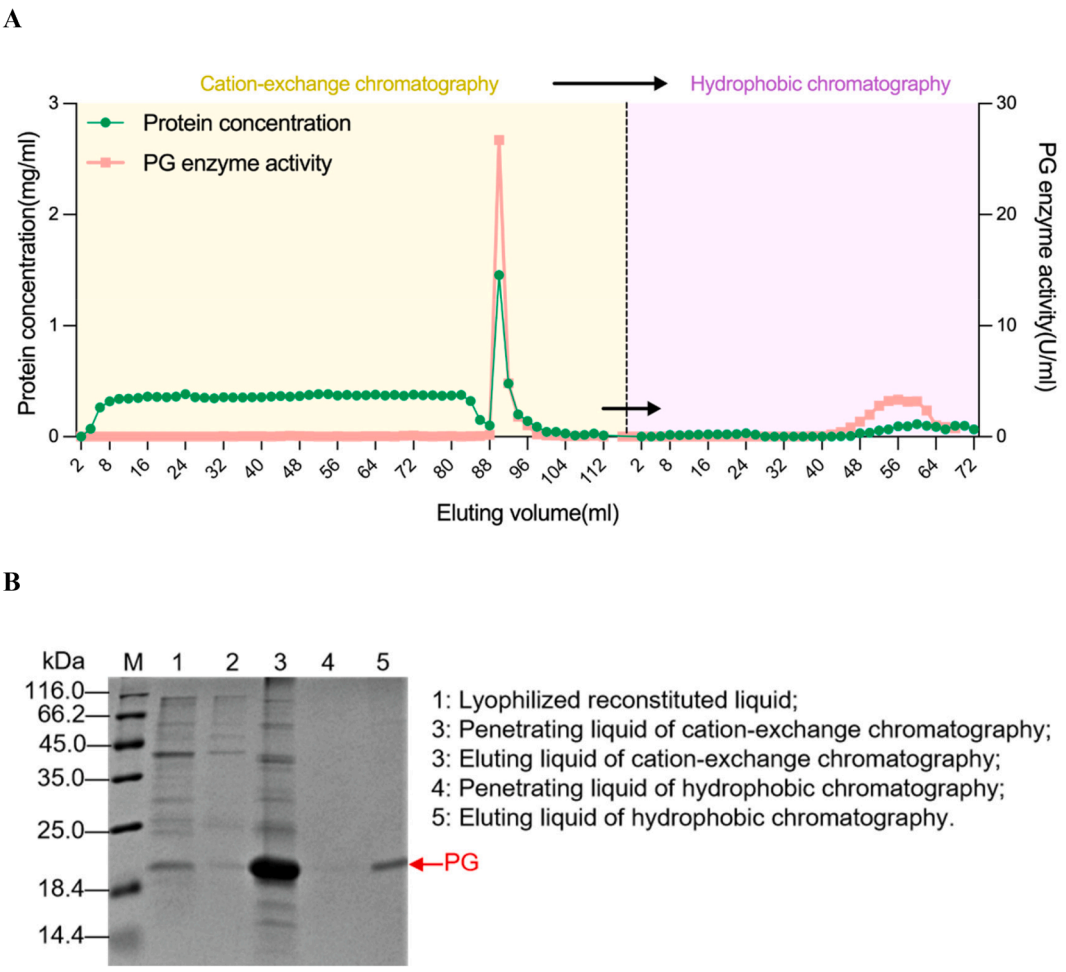


**Fig. 2. Purification of PG by CEX coupled with HIC.** PG was purified by 1 mL CEX column and 1 mL HIC column in tandem. The eluent NaCl concentration was 0 M ~ 2 M with a volume of 2 mL for each concentration for CEX, and the eluent NaCl concentration was 2 M ~ 0 M with a volume of 2 mL for each concentration for HIC. (A)Relationship between eluent volume, eluted protein concentration, and PG enzyme activity; (B) SDS-PAGE of the eluate from the PG tandem purification process.

**Table 1**
Recoveries for PG purification steps.

| Process | Total protein (mg) | PG enzyme activity(U) | Enzyme activity recovery(%) | Specific enzyme activity(U/g) |
|---|---|---|---|---|
| Centrifugation | 115.35 ± 2.06 | 92.28 ± 1.28 | 100 ± 1.39 | 800 ± 45.83 |
| Ultrafiltration | 51.64 ± 1.35 [a] | 72.81 ± 1.90 [a] | 78.90 ± 2.06 [a] | 1410 ± 98.49 [a] |
| Freeze-drying | 12.87 ± 1.02 [a] | 69.23 ± 2.06 [a] | 75.02 ± 2.23 [a] | 5380 ± 65.57 [a] |
| CEX | 3.94 ± 0.09 [a] | 66.65 ± 2.35 [a] | 72.22 ± 2.54 [a] | 16920 ± 1311.45 [a] |
| HIC | 1.87 ± 0.18 [a] | 49.69 ± 1.80 [a] | 53.84 ± 1.95 [a] | 26600 ± 995.94 [a] |

[a] , $p < 0.0001$. Each data was compared to centrifugation.

the volume of the saline solution $V_0$. The formula for foam capacity (FC) is as follows:

$$FC(\%) = (V_0 - 20)/20 \times 100.$$

### 2.10. Water-holding and oil-holding properties

Water-holding and oil-holding properties of wheat gluten were conducted based on methods from Hadidi et al. (Hadidi, et al., 2021) and Kunarayakul et al.(Kunarayakul, et al., 2018) with some modifications. A 50 mL centrifuge tube was weighed as $M_0$ and 50 mg of wheat gluten was dispersed in 10 mL of distilled water or soybean oil with varying degrees of deamidation. The mixture was then centrifuged in a water bath at 40 °C for 30 min at 10,000 revolutions per minute for 20 min. The supernatant was removed after centrifugation, and the weight of the centrifuge tube was measured once again as $M_1$. The following formula was used to calculate the water/oil holding capacity:

$$Water/oilholdingcapacity(g/g) = (M_1 - M_0 - 0.05)/0.05.$$

### 2.11. Cake baking and cake quality

This experiment used 30 g of low-gluten wheat flour, 20 g of corn oil, 15 g of fresh milk, 25 g of sugar, 30 g of egg white proteins, and a total of 30 g of egg yolk proteins with different wheat gluten to make cakes. Egg yolk proteins were replaced with 0 %, 20 % modified wheat gluten, or 20 % non-modified wheat gluten, respectively. The following variables were assessed after the cooked cakes had cooled:

Calculating the density and specific volume requires cutting the cooled cake into cubes, measuring their length (a), breadth (b), and height (h), and then weighing the result, which is represented by the number m. The following is the formula for density and specific volume:

Density ($g/cm^3$) $\rho = m \div (a \times b \times h)$.

Specific volume ($cm^3/g$) $= (a \times b \times h) \div m$.

To calculate the retraction ratio, measure the height of the cake as

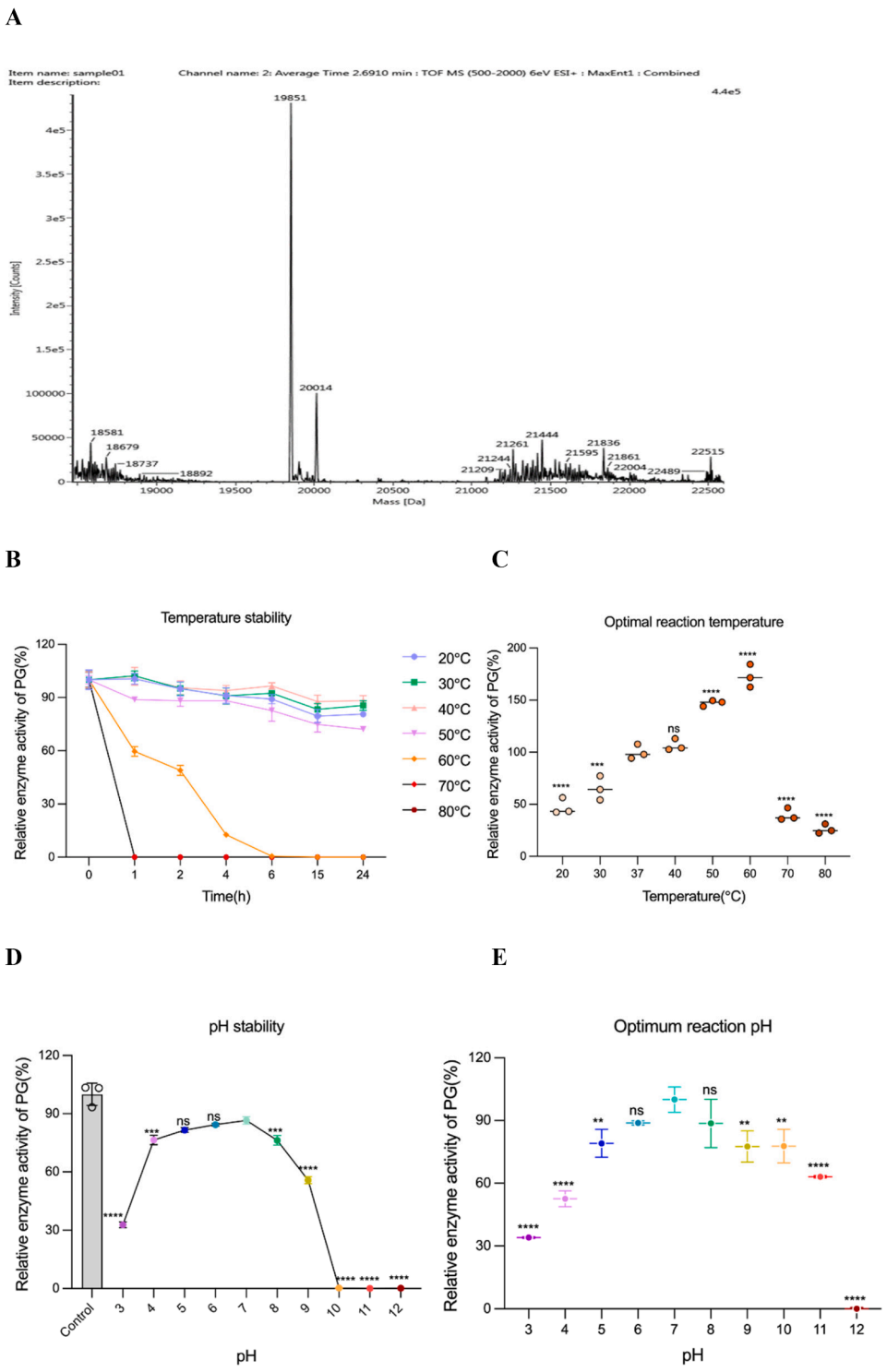


**Fig. 3. Enzymatic properties of purified PG.** Enzymatic properties of PG derived from *C. proteolyticum* TM1040 purified by CEX and HIC. (A) Molecular mass; (B) Temperature stability; (C) Optimum reaction temperature, compared to 37℃; (D) pH stability, compared to pH = 7; (E) Optimum reaction pH compared to pH = 7; (F) Effect of 2 % surfactants on PG activity incubated at 37 °C for 1 h. (G) Effect of 5 mM metal ions on PG enzyme activity incubated at 37 °C for 1 h.

soon as it is taken out of the oven ($H_0$), and again after it has been at room temperature for one hour ($H_1$). The following is the retraction ratio formula:

Retraction rate (%) = ($H_1$ - $H_0$) ÷ $H_0$ × 100.

The cake was cut into uniformly thin pieces measuring 4 cm × 4 cm × 3 cm. The texture was determined using the TA-XT plus texture analyzer with the P36R probe selected. The measurement was completed in the TPA mode with the following settings: initial speed 1 mm/s, recovery speed 1 mm/s, trigger force 5 g, and compression distance 50 %.

### 2.12. Flavor evaluation

An evaluation team of 10 trained members evaluated the odor of

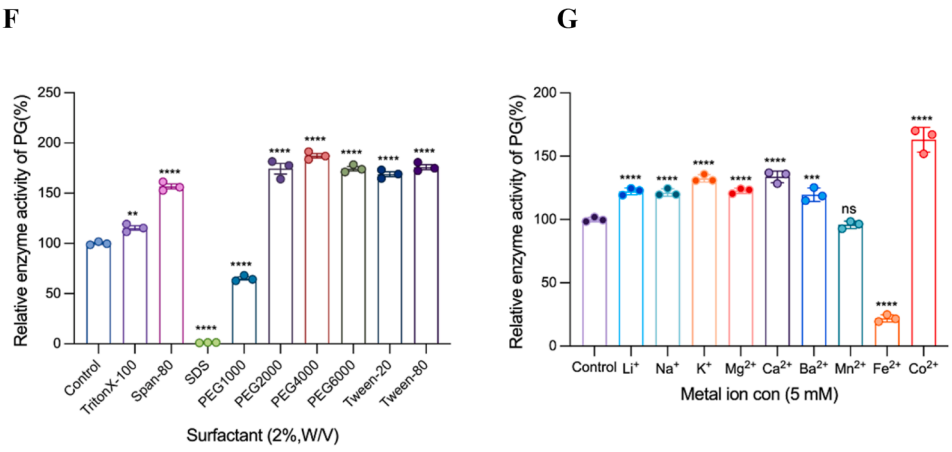


**Fig. 3.** (*continued*).

cakes of different compositions. Cake samples of different compositions were randomly distributed to the panel members. During the odor evaluation, the panel members were in the same environment and condition, and each panel member performed an olfactory evaluation on three parallel cake samples. Each evaluator was asked to assess the intensity of wheat flavor from low to high on a scale of −3, −2, −1, 0, 1, 2, and 3. Flavor evaluations from group members were tallied and presented in a heat map. The assessments were carried out in a sensory laboratory room designed according to international standards (ISO 8589–1998). The research has been approved by the University Committee on Human Research Protection (Approval No.: HR721-2022).

### 2.13. Statistical analysis

For statistical analysis, GraphPad Prism 8.0 was utilized. The information is displayed as means and ± SEM. A two-tailed *t*-test was used to determine the significance between the two groups. One-way or two-way ANOVA with Bonferroni post-test was used to analyze multiple groups. P values $\leq 0.05$ were regarded as statistically significant for all statistical tests. *: $0.01 \leq P < 0.05$, **: $0.001 \leq P < 0.01$, ***: $0.0001 \leq P < 0.001$, ****: $P < 0.0001$.

## 3. Results and discussions

### 3.1. Optimization of conditions for PG production by C. proteolyticum

The four strains of *C. proteolyticum* did not appear to have significantly different growth curves in seed mediums, as shown in Fig. S1, and all four reached a platform phase of growth after 12 h of shake flask incubation. However, the growth decline phase of *C. proteolyticum* 3–16 began much sooner than that of the other three strains in the fermentation medium (Fig. S2A). With a maximal enzyme activity of 3.30 U/mL in 18 h of shaking flasks in fermentation media, *C. proteolyticum* TM1040 produced more PG than the other three strains according to the analysis of the enzyme production curve for PG (Fig. S2B). This may be due to differences in strain growth and metabolism caused by NIT-UV mutagenesis.

Optimizing the media was an effective way to improve target protein production, but no research on improving PG production in *C. proteolyticum* by optimizing the carbon and nitrogen of media had been reported. Herein, the carbon source and nitrogen source in the fermentation medium of PG production were investigated in this study. As can be seen in Fig. S3A and Fig. 1A, adding 0.5 % lactose to the fermentation medium for TM1040 resulted in the maximum PG production of 3.27 U/mL, which increased by 122 %, compared with adding alginose. This result may be because other carbon sources mainly promote the growth of *C. proteolyticum* rather than the PG-producing metabolism (Fig. 1A). Meanwhile, as shown in Fig. S3B and Fig. 1B, the maximum PG yield was produced when 2 % polypeptone was applied, and PG enzyme activity was 3.81 U/mL, with an increase 456 % compared to ammonium sulfate. This may be due to the promotion of OD600 by the nitrogen source without affecting the enzyme-producing metabolism of *C. proteolyticum* (Fig. 1B). Shake flask culture of TM1040 in the optimized medium combining carbon and nitrogen sources resulted in higher OD600 and longer time taken to reach plateau phase, and the yield of PG was 6.52 U/mL with increased 97.6 %, compared with the pre-optimized medium (Fig. 1C). Finally, the PG yield up to 7.30 U/mL of *C. proteolyticum* TM1040 in 15L fermenter at 19 h (Fig. S4). The PG yield of *C. proteolyticum* TM1040 in this study was higher than the highest reported PG activity of 2.93 U/mL expressed in *C. proteolyticum* (L. Wang, et al., 2023) and 7.07 U/mL expressed in *B. subtilis* (Yin, et al., 2021).

### 3.2. Purification of PG by CEX and HIC in tandem

Despite these results indicating that the PG yield of TM1040 is relatively high, it is still necessary to remove proteases and other impurity proteins from the extracellular supernatant and to increase the purity of the PG for applications. In this study, a method of PG purification was performed for the first time connecting CEX and HIC in tandem from the extracellular supernatant of *C. proteolyticum* TM1040. In the CEX purification of PG, conditions were optimized by concentration and pH of sample loading buffer (Table S2 and Table S3), and 10 mM PBS was used for PG coupling with the CEX column and gradient NaCl to elute PG (Fig. S5A and S5C). In the HIC purification of PG, the concentration of sample loading buffer was optimized (Table S4), and 10 mM PBS was used for PG coupling with the HIC column and gradient NaCl to elute PG (Fig. S6A). The results showed that both CEX and HIC were effective in improving the purity of PG in the extracellular supernatant of *C. proteolyticum* TM1040 (Fig. S5B, D, and Fig. S6B). Further, CEX and HIC were used in tandem for the purification of PG in the extracellular supernatant of *C. proteolyticum* TM1040 (Fig. 2A), and the results of SDS- polyacrylamide gel electrophoresis (SDS-PAGE) showed that electrophoretically pure PG could be obtained after purification by CEX and HIC, and the hetero protein bands present in the PG purified by either CEX or HIC alone almost completely disappeared (Fig. 2B). Instead of using HIC paired with gel filtration chromatography (GFC) (Yamaguchi, et al., 2001) and CEX paired with GFC (R. Qu, et al., 2022), an approach that CEX coupled with HIC were utilized for the purification of PG in this study. The specific enzyme activity of purified PG was 26,600 ± 995.94 U/g (Table 1). The recovery of PG enzyme activity was 53.84 %, which was higher than 30.3 % for HIC paired with GFC (Yamaguchi, et al., 2001) and 32.95 % for CEX paired with GFC (R. Qu, et al., 2022). According to protein and enzyme activity recovery

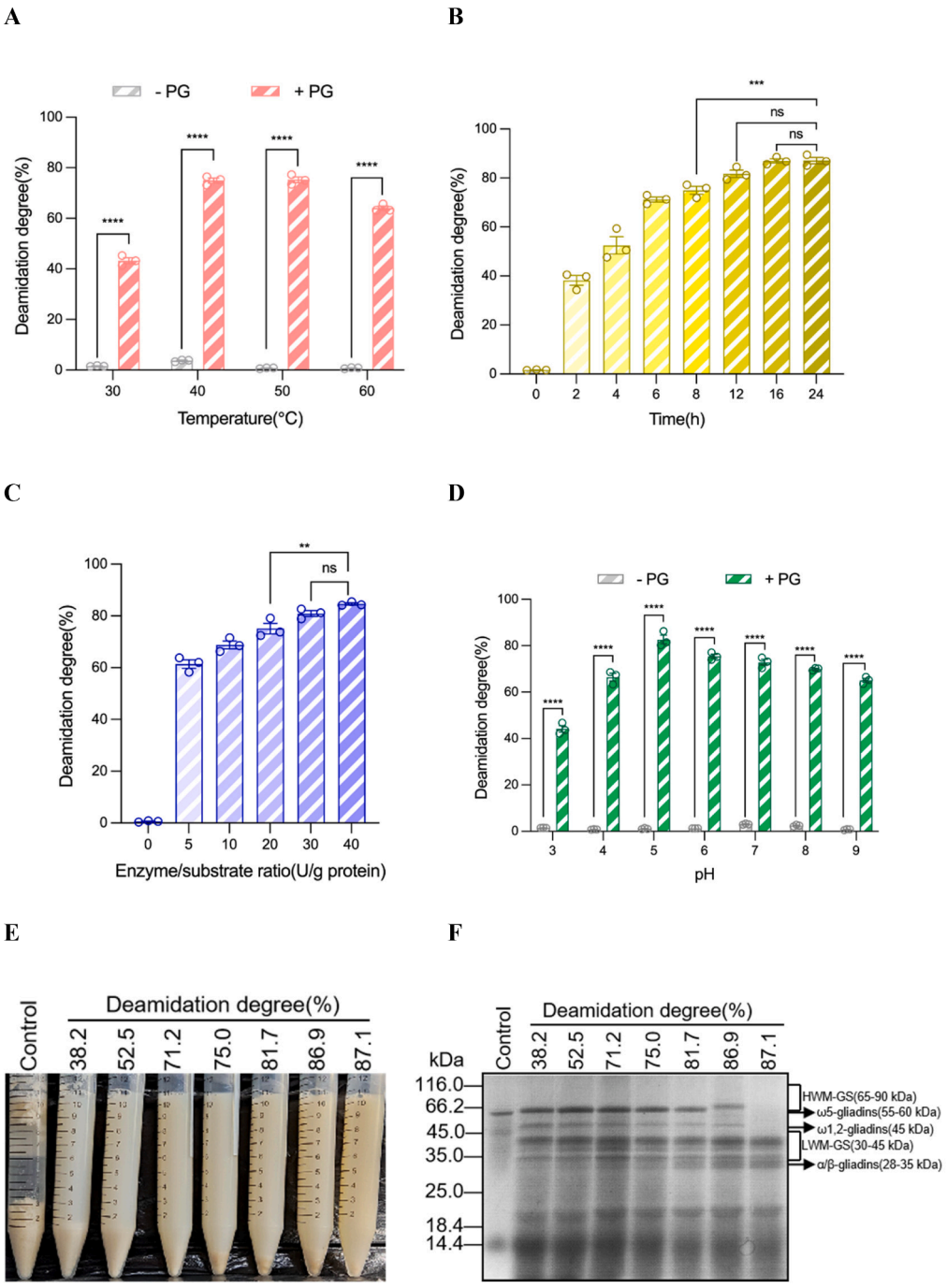


**Fig. 4. Modification of functional properties of wheat gluten by PG.** Deamidation assay of wheat gluten optimally modified by PG at, (A) different temperatures at E/S = 20 U/g, pH = 7 and 12 h reaction, (B) different times at 50 ℃, pH = 5 and E/S = 30 U/g, (C) different enzyme-to-substrate ratios at 50 ℃, pH = 5 and 12 h reaction, and (D) different pH at 50 ℃, E/S = 20 U/g and 12 h reaction. Photographs of wheat gluten modified by PG at different times for (E) wheat gluten and (F) SDS-PAGE. Wheat gluten was modified by PG at different times for (G) emulsification, (H) foaming, (I) water-holding capacity and (J) oil-holding capacity of wheat gluten changed after different times of PG modification of wheat gluten.

statistics and enzyme activity measurements for the steps of PG purification, the yields of PG enzyme activity obtained by this method were all higher than those of HIC paired with GFC (Yamaguchi, et al., 2001) and CEX paired with GFC (R. Qu, et al., 2022), which reduced the losses during PG purification.

### 3.3. Enzymatic properties of purified PG

The relative molecular mass of PG was initially assessed to confirm the enzymatic characteristics of purified PG from TM1040. The relative molecular mass of PG generated was determined to be 19851 Da using an ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometer (UPLC-TOF MS) (Fig. 3A). The theoretical relative molecular mass of PG is 19,860 Da, and the result is consistent with earlier reports that about 20000 Da (Yamaguchi, et al., 2001). According to the findings of the temperature stability tests, PG was able to keep the enzyme activity below 50 °C generally stable. 60 °C was the optimal temperature of reaction for PG and quickly inactivated at temperatures

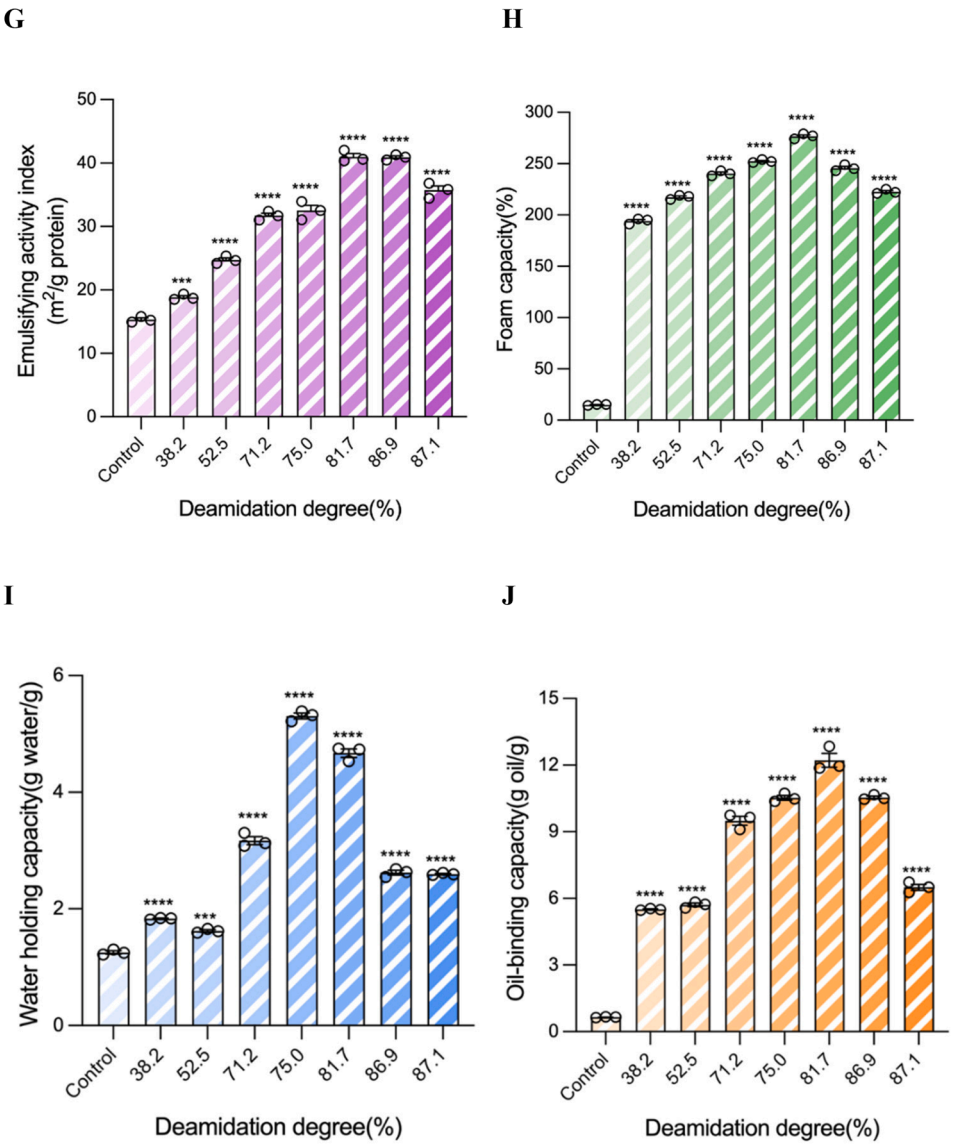


**Fig. 4.** (*continued*).

over 70 °C (Fig. 3B, 3C). The response of PG to temperature was similar to the results of Yamaguchi et al (Yamaguchi, et al., 2001). The enzyme activity of PG was reasonably stable over a pH range of 5 to 7, and it reduced when pH increased or dropped, according to pH stability studies. The enzyme activity was quickly diminished at pH 3 or pH > 10 (Fig. 3D). The optimum pH for the reaction of PG is 7 (Fig. 3E). In the study by Yamaguchi et al., the PG enzyme activity from *C. proteolyticum* 9670 $^{T}$ exhibited a broad pH optimum between pH 5 and 7, and the enzyme showed more than 90 % of the remaining activity at pH 5–8.7 after incubation at various pH for 18 h (Yamaguchi, et al., 2000). The optimal reaction temperature and pH, as well as temperature and pH stability of purified PG from TM1040, were similar to the purified PG from *Chryseobacterium cucumeris* ZYF120413-7 (R. Qu, et al., 2022).

In addition, nonionic surfactants including TritonX-100, Span-80, PEG200, PEG4000, PEG6000, Tween-20, and Tween-80 significantly improve the enzyme activity of PG (Fig. 3F). Nonionic surfactants contribute to the stability and survival of enzymes (Goswami, 2020) is because they can increase activity by encouraging partial protein defolding (de Castro, et al., 2021), particularly in the case of lipases. But because SDS, an anionic surfactant, denaturized the protein structure of PG, PG was severely inhibited by SDS. Additionally, only $Fe^{2+}$ significantly inhibited PG enzyme activity, $Mn^{2+}$ had no impact on PG enzyme activity, whereas other metal ions ($Li^{+}$, $Na^{+}$, $K^{+}$, $Mg^{2+}$, $Ca^{2+}$, and $Ba^{2+}$) slightly improved the PG enzyme activity. Similar to another study, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$ had significant inhibitory effects on PG, while $Mg^{2+}$, $Ca^{2+}$, and $Zn^{2+}$ have a slight stabilizing or even enhancing effect on PG (Ouyang, et al., 2021). Yamaguchi et al. found that $HgCl_2$ and $AgNO_3$ completely inhibited the activity, and partial inhibitions were observed with $CuCl_2$ (74.9 %), $ZnCl_2$ (69.9 %), and $FeCl_2$ (26.8 %) (Yamaguchi, et al., 2001). Interstingly, $Co^{2+}$ significantly improves the PG enzyme activity with an increase of 63 % (Fig. 3G), which had not been reported before. It has been shown that cobalt phosphate has an enhancing effect on organophosphorus hydrolase, which may be due to the enhancement of the enzymatic reaction by Cobalt(Co)-based nanozymes as the bioinorganic hybrid catalysts (Han & Liu, 2017). All of these findings provided fundamental detail characteristics of purified PG from TM1040.

### 3.4. *Effect of PG on the functional properties of wheat gluten*

5 % wheat gluten was modified using purified PG from the TM1040 fermentation supernatant. With increasing temperature, enzyme-to-substrate ratio (E/S), and reaction time, the degree of deamidation of wheat gluten increased, reaching maximal deamidation degrees of

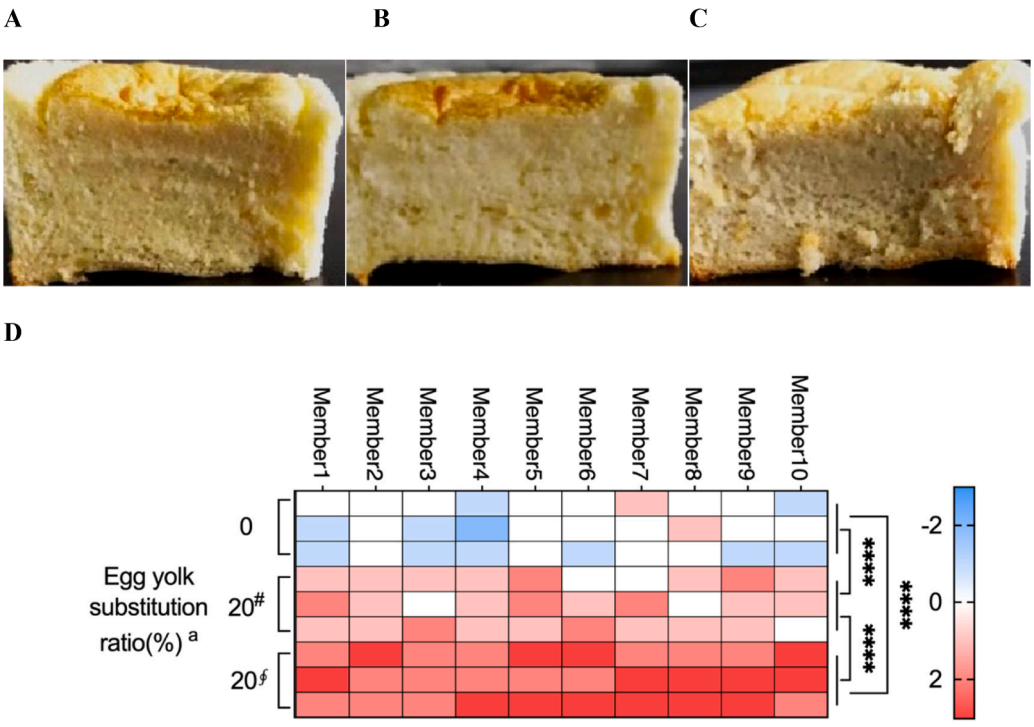


**Fig. 5. Photographs of cakes made with modified wheat gluten.** (A) Used 20 % egg yolk protein, (B) 20 % modified wheat gluten, (C) 20 % unmodified wheat gluten to make cake, and (D) heat map of cake flavor evaluation, blue: low wheat flavor, red: high wheat flavor, [a]: the initial egg yolk protein content is 40 %; [#]: modified wheat gluten substituted for egg yolk protein; [ǂ]: unmodified wheat gluten substituted for egg yolk protein. [****], $p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Effect of modified wheat gluten on cake quality.

| Egg yolk substitution ratio(%)[a] | 0 | 20 [#] | 20 [ǂ] |
|---|---|---|---|
| Densities(g/cm3) | 0.63 ± 0.10 | 0.40 ± 0.07 [ns] | 0.81 ± 0.13 [ns] |
| Specific volume(cm3/g) | 1.44 ± 0.15 | 2.14 ± 0.15 [**] | 1.23 ± 0.18 [ns] |
| Retraction ratio(%) | 9.31 ± 0.71 | 7.19 ± 0.95 * | 8.31 ± 0.67 [ns] |
| Hardness(g) | 1376.35 ± 98.39 | 1229.63 ± 95.26 * | 1570.27 ± 168.98 [**] |
| Elastic index(mm) | 0.83 ± 0.09 | 0.92 ± 0.13 [ns] | 0.88 ± 0.09 [ns] |
| Cohesiveness(g) | 0.83 ± 0.04 | 0.66 ± 0.07 [****] | 0.78 ± 0.06 [ns] |
| Chewiness(g) | 1009.22 ± 104.87 | 965.77 ± 94.46 [ns] | 1131.21 ± 112.23 * |
| Reversibility(g) | 0.32 ± 0.03 | 0.26 ± 0.02 [***] | 0.28 ± 0.04 * |

[a], the initial egg yolk protein content is 40 %; [#], modified wheat gluten substituted for egg yolk protein; [ǂ], unmodified wheat gluten substituted for egg yolk protein. ns, no significant *, $p < 0.05$; [**], $p < 0.01$; [***], $p < 0.001$; [****], $p < 0.0001$.Each data was compared to a 0 % egg yolk substitution ratio.

75.28 %, 84.61 %, and 87.11 %, respectively (Fig. 4A, 4B, and 4C). Yong et al. utilized PG to modify gluten to a degree of deamination up to 72 % (Yong, et al., 2006). Hadidi et al. (Hadidi, et al., 2021) also demonstrated that the degree of deamidation of evening primrose seed cake (EPSC) protein increased with increasing temperature (not exceeding 45 °C), time, and E/S. The deamidation degree of wheat gluten was 82.66 % at the optimal pH of 5, according to Fig. 4D. Compared to the recently reported citric acid for deamidation of wheat gluten, the deamidation of wheat gluten by PG showed shorter reaction time, lower temperature, and higher degree of deamidation (M. X. Liu, et al., 2023). In particular, the degree of hydrolysis of PG-deamidated wheat gluten was only up to 3.9 % (Fig. S7), which is an advantage of enzymatic deamidation over chemical methods (Wang, et al., 2017). As shown in Fig. 4E, when the degree of wheat gluten deamidation rose, the precipitation of wheat gluten reduced and its solubility increased. SDS-PAGE bands of deamidated wheat gluten migrated primarily upward (Fig. 4F). This upward migration of the bands of deamidated proteins is similar to that of soy protein (Suppavorasatit, et al., 2011), α-Zein (Yong, et al., 2004), and EPSC protein (Hadidi, et al., 2021). This upward migration should be due to an increase in the negative charge of deamidated proteins and intramolecular electrostatic repulsion (Yong, et al., 2006). According to functional analysis of the deamidated samples, wheat gluten with a degree of deamidation of 81.7 % had the greatest emulsification (Fig. 4G), foaming (Fig. 4H), and oil-holding capabilities (Fig. 4I), while wheat gluten with a degree of deamidation of 75.0 % had the best water-holding qualities (Fig. 4J). The results of Yong et al. (Yong, et al., 2006) showed that deamidation of wheat gluten by PG resulted in a change of the secondary structure of protein and improved the solubility and emulsification of wheat gluten at pH = 7. Deamidated wheat gluten exhibited superior functional properties than non-deamidated wheat gluten, including emulsification, foaming, and water/oil holding capacity. The functional properties of wheat gluten can be improved by controlling the conditions of the PG deamidation reaction, including temperature, pH, E/S, and time. Compared with chemical deamidation (Lei, et al., 2015; Liao, et al., 2010), PG enzymatic deamidation of wheat gluten is performed under mild reaction conditions without the need for high temperatures and the addition of chemical reagents, which exhibits great advantages in terms of safety and degree of deamidation, and effectively broadens the application of deamidated wheat gluten in the food field.

### 3.5. Impact of modified wheat gluten in cake

Wheat gluten is one of the main ingredients in cakes, noodles, and meat products. Proper addition of wheat gluten enhances properties such as viscoelasticity and firmness of cake (Y. X. Liu, et al., 2023; Squeo, et al., 2023). Based on that, cakes with egg yolk proteins substituted with modified wheat gluten with an 81.7 % deamidation

degree were baked. As the percentage of wheat gluten replacement grew from 0 % to 20 %, the cross-sectional structure of the cakes revealed better qualities of smaller pores and flatter cross-sections (Fig. 5A and 5B), but the cakes with 20 % unmodified wheat gluten had loose pores and a structure susceptible to collapsing (Fig. 5C). In terms of aroma, egg yolk, and a slight wheat flavor were both present, but the cake made with 20 % non-modified wheat gluten had a stronger wheat scent (Fig. 5D). Many studies have demonstrated the deamidation of plant protein to promote the reduction of specific plant flavors, which is beneficial for people who cannot accept specific plant protein flavors (Suppavorasatit, et al., 2013; Temthawee, et al., 2020). According to the data above, the cake with 20 % modified wheat gluten had better color, fragrance, and aesthetic appeal compared to the original recipe with a 0 % egg yolk substitution ratio. The cake with 20 % modified wheat gluten also had the lowest density, the highest specific volume, and the highest retraction ratio, which indicated that its fluffiness was significantly higher than that of the other recipes, according to a detailed analysis of the cake qualities (Table 2). When the structural properties were examined, the hardness, cohesiveness, and reversibility of 20 % modified wheat gluten cake were all lower than those of the egg yolk protein cake, indicating that the cake was softer, while the elasticity of the cakes in the different groups did not exhibit a difference (Table 2). These results demonstrated that PG-modified wheat gluten improves cake textures and opens new opportunities for wheat gluten in the food industry.

## 4. Conclusion

In this study, the fermentation process of *C. proteolyticum* was optimized to produce PG with a yield of 7.30 U/mL, which is 2.49 times higher than the highest study reported so far in *C. proteolyticum*. Deamidation of wheat gluten was increased to 87.11 % using purified PG and the deamidation reaction was more efficient, safer, and milder. Deamidated wheat gluten with great functional properties was made into cakes to improve the quality of the cakes. These results promote the progress of PG-modified proteins in the food industry.

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## Declaration of competing interest

All authors report no conflict or declaration of interest.

## CRediT authorship contribution statement

**Zheng Zhang:** Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Rui Shi:** Methodology, Investigation, Data curation, Conceptualization. **Xiaoyu Zhu:** Visualization, Software, Formal analysis, Data curation. **Lihui Zheng:** Validation, Methodology, Investigation, Data curation. **Mingfei Jin:** Resources, Formal analysis, Conceptualization. **Deming Jiang:** Writing – original draft, Visualization, Resources, Conceptualization. **Yelin Wu:** Visualization, Methodology, Formal analysis. **Hongliang Gao:** Resources, Methodology, Conceptualization. **Zhongyi Chang:** Resources. **Dongrui Wang:** Methodology, Data curation. **Jiajing Wu:** Visualization, Methodology, Data curation. **Jing Huang:** Writing – original draft, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

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

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101312.

## References

Carvalho, L. T., Pires, M. A., Baldin, J. C., Munekata, P. E. S., de Carvalho, F. A. L., Rodrigues, I., Polizer, Y. J., de Mello, J. L. M., Lapa-Guimaraes, J., & Trindade, M. A. (2019). Partial replacement of meat and fat with hydrated wheat fiber in beef burgers decreases caloric value without reducing the feeling of satiety after consumption. *Meat Science, 147*, 53–59. https://doi.org/10.1016/j.meatsci.2018.08.010

Chen, X., Fu, W. Y., Luo, Y. C., Cui, C., Suppavorasatit, I., & Liang, L. (2021). Protein deamidation to produce processable ingredients and engineered colloids for emerging food applications. *Comprehensive Reviews in Food Science and Food Safety, 20*(4), 3788–3817. https://doi.org/10.1111/1541-4337.12759

de Castro, A. S. B., de Paula, H. M. C., Coelho, Y. L., Hudson, E. A., Pires, A. C. S., & da Silva, L. H. M. (2021). Kinetic and thermodynamic of lactoferrin – Ethoxylated-nonionic surfactants supramolecular complex formation. *International Journal of Biological Macromolecules, 187*, 325–331. https://doi.org/10.1016/j.ijbiomac.2021.07.087

Fu, W. Y., Chen, X., Cheng, H., & Liang, L. (2022). Tailoring protein intrinsic charge by enzymatic deamidation for solubilizing chicken breast myofibrillar protein in water. *Food Chemistry, 385*. https://doi.org/10.1016/j.foodchem.2022.132512

Goswami, D. (2020). Lipase catalysis in presence of nonionic surfactants. *Applied Biochemistry and Biotechnology, 191*(2), 744–762. https://doi.org/10.1007/s12010-019-03212-w

Hadidi, M., Ibarz, A., & Pouramin, S. (2021). Optimization of extraction and deamidation of edible protein from evening primrose (Oenothera biennis L.) oil processing by-products and its effect on structural and techno-functional properties. *Food Chemistry, 334*. https://doi.org/10.1016/j.foodchem.2020.127613

Han, L., & Liu, A. H. (2017). Novel cell-inorganic hybrid catalytic interfaces with enhanced enzymatic activity and stability for sensitive biosensing of paraoxon. *Acs Applied Materials & Interfaces, 9*(8), 6894–6901. https://doi.org/10.1021/acsami.6b15992

Hashizume, R., Maki, Y., Mizutani, K., Takahashi, N., Matsubara, H., Sugita, A., Sato, K., Yamaguchi, S., & Mikami, B. (2011). Crystal structures of protein glutaminase and its pro forms converted into enzyme-substrate complex. *Journal of Biological Chemistry, 286*(44), 38691–38702. https://doi.org/10.1074/jbc.M111.255133

Kikuchi, Y., Itaya, H., Date, M., Matsui, K., & Wu, L. F. (2008). Production of Chryseobacterium proteolyticum protein-glutaminase using the twin-arginine translocation pathway in Corynebacterium glutamicum. *Applied Microbiology and Biotechnology, 78*(1), 67–74. https://doi.org/10.1007/s00253-007-1283-3

Kikuchi, Y., Itaya, H., Date, M., & Wu, L. F. (2009). TatABC overexpression improves Tat-dependent protein secretion in Corynebacterium glutamicum. *New Biotechnology, 25*, S224–S225. https://doi.org/10.1016/j.nbt.2009.06.191

Kunarayakul, S., Thaiphanit, S., Anprung, P., & Suppavorasatit, I. (2018). Optimization of coconut protein deamidation using protein-glutaminase and its effect on solubility, emulsification, and foaming properties of the proteins. *Food Hydrocolloids, 79*, 197–207. https://doi.org/10.1016/j.foodhyd.2017.12.031

Lei, L., Zhao, Q., Selomulya, C., & Xiong, H. (2015). The effect of deamidation on the structural, functional, and rheological properties of glutelin prepared from var. seed. *Food Chemistry, 178*, 96–105. https://doi.org/10.1016/j.foodchem.2015.01.081

Liao, L., Han, X. Y., Chen, L. P., Ni, L., Liu, Z. B., Zhang, W., & Chen, Q. (2016). Comparative characterization of the deamidation of carboxylic acid deamidated wheat gluten by altering the processing conditions. *Food Chemistry, 210*, 520–529. https://doi.org/10.1016/j.foodchem.2016.04.113

Liao, L., Liu, T. X., Zhao, M. M., Cui, C., Yuan, B. E., Tang, S., & Yang, F. (2010). Functional, nutritional and conformational changes from deamidation of wheat gluten with succinic acid and citric acid. *Food Chemistry, 123*(1), 123–130. https://doi.org/10.1016/j.foodchem.2010.04.017

Liu, M. X., Dai, S. H., Yin, L. J., Huang, Z. J., & Jia, X. (2023). Wheat gluten deamidation: Structure, allergenicity and its application in hypoallergenic noodles. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.13133

Liu, X., Wang, C., Zhang, X. W., Zhang, G. Q., Zhou, J. W., & Chen, J. (2022). Application prospect of protein-glutaminase in the development of plant-based protein foods. *Foods, 11*(3). https://doi.org/10.3390/foods11030440

Liu, Y. H., Zhang, L. L., Li, Y. X., Yang, Y., Yang, F., & Wang, S. (2018). The functional properties and structural characteristics of deamidated and succinylated wheat gluten. *International Journal of Biological Macromolecules, 109*, 417–423. https://doi.org/10.1016/j.ijbiomac.2017.11.175

Liu, Y. X., Li, M. M., Guan, E. R., Liu, Y. F., Bian, K., & Zhang, Y. Q. (2023). Modification of soft wheat protein for improving cake quality by superheated steam treatment of wheat grain. *Foods, 12*(16). https://doi.org/10.3390/foods12162992

Lu, X., Poon, T. C. W., & Zhang, H. (2020). Mass production of active recombinant Chryseobacterium proteolyticum protein glutaminase in Escherichia coli using a sequential dual expression system and one-step purification. *IUBMB Life, 72*(11), 2391–2399. https://doi.org/10.1002/iub.2358

Lura, R., & Schirch, V. (1988). Role of peptide conformation in the rate and mechanism of deamidation of asparaginyl residues. *Biochemistry, 27*(20), 7671–7677. https://doi.org/10.1021/bi00420a015

Ouyang, X., Liu, Y., Qu, R., Tian, M., Yang, T., Zhu, R., Gao, H., Jin, M., & Huang, J. (2021). Optimizing protein-glutaminase expression in Bacillus subtilis. *Current Microbiology, 78*(5), 1752–1762. https://doi.org/10.1007/s00284-021-02404-0

Qiu, C. Y., Sun, W. Z., Cui, C., & Zhao, M. M. (2013). Effect of citric acid deamidation on digestibility and antioxidant properties of wheat gluten. *Food Chemistry, 141*(3), 2772-2778. 10.1016/j.foodchem.2013.05.072.

Qu, R., Dai, T., Wu, J., Tian, A., Zhang, Y., Kang, L., Ouyang, W., Jin, C., Niu, J., Li, Z., Chang, Z., Jiang, D., Huang, J., & Gao, H. (2022). The characteristics of protein-glutaminase from an isolated Chryseobacterium cucumeris strain and its deamidation application. *Frontiers in Microbiology, 13*, Article 969445. https://doi.org/10.3389/fmicb.2022.969445

Qu, R. D., Zhu, X. Y., Tian, M., Liu, Y. J., Yan, W. J., Ye, J., Gao, H. L., & Huang, J. (2018). Complete genome sequence and characterization of a protein-glutaminase producing strain, Chryseobacterium proteolyticum QSH1265. *Frontiers in Microbiology, 9*. https://doi.org/10.3389/fmicb.2018.01975

Squeo, G., Latrofa, V., Vurro, F., De Angelis, D., Caponio, F., Summo, C., & Pasqualone, A. (2023). Developing a clean labelled snack Bar rich in protein and fibre with dry-fractionated defatted durum wheat cake. *Foods, 12*(13). https://doi.org/10.3390/foods12132547

Suppavorasatit, I., De Mejia, E. G., & Cadwallader, K. R. (2011). Optimization of the enzymatic deamidation of soy protein by protein-glutaminase and its effect on the functional properties of the protein. *Journal of Agricultural and Food Chemistry, 59*(21), 11621–11628. https://doi.org/10.1021/jf2028973

Suppavorasatit, I., Lee, S. Y., & Cadwallader, K. R. (2013). Effect of enzymatic protein deamidation on protein solubility and flavor binding properties of soymilk. *Journal of Food Science, 78*(1), C1–C7. https://doi.org/10.1111/j.1750-3841.2012.03012.x

Temthawee, W., Panya, A., Cadwallader, K. R., & Suppavorasatit, I. (2020). Flavor binding property of coconut protein affected by protein-glutaminase: Vanillin-coconut protein model. *Lwt-Food Science and Technology, 130*. https://doi.org/10.1016/j.lwt.2020.109676

Umaraw, P., Chauhan, G., Mendiratta, S. K., & Verma, A. K. (2021). Optimization of processing conditions for development of chicken meat incorporated whole wheat bread. *Journal of Food Science and Technology-Mysore, 58*(7), 2792–2805. https://doi.org/10.1007/s13197-020-04788-6

Wang, L., Lyu, Y., Miao, X., Yin, X., & Zhang, C. (2023). Enhanced protein glutaminase production from Chryseobacterium proteolyticum combining physico-chemical mutagenesis and resistance screening and its application to soybean protein isolates. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.12535

Wang, Y. Q., Gan, J., Zhou, Y., Cheng, Y. Q., & Nirasawa, S. (2017). Improving solubility and emulsifying property of wheat gluten by deamidation with four different acids: Effect of replacement of folded conformation by extended structure. *Food Hydrocolloids, 72*, 105–114. https://doi.org/10.1016/j.foodhyd.2017.04.013

Yamaguchi, S., Jeenes, D. J., & Archer, D. B. (2001). Protein-glutaminase from Chryseobacterium proteolyticum, an enzyme that deamidates glutaminyl residues in proteins – Purification, characterization and gene cloning. *European Journal of Biochemistry, 268*(5), 1410–1421. https://doi.org/10.1046/j.1432-1327.2001.02019.x

Yamaguchi, S., & Yokoe, M. (2000). A novel protein-deamidating enzyme from Chryseobacterium proteolyticum sp nov., a newly isolated bacterium from soil. *Applied and Environmental Microbiology, 66*(8), 3337–3343. https://doi.org/10.1128/Aem.66.8.3337-3343.2000

Yin, X. X., Zhang, G. Q., Zhou, J. W., Li, J. H., & Du, G. C. (2021). Combinatorial engineering for efficient production of protein-glutaminase in Bacillus subtilis. *Enzyme and Microbial Technology, 150*. https://doi.org/10.1016/j.enzmictec.2021.109863

Yong, Y. H., Yamaguchi, S., Gu, Y. S., Mori, T., & Matsumura, Y. (2004). Effects of enzymatic deamidation by protein-glutaminase on structure and functional properties of alpha-zein. *Journal of Agricultural and Food Chemistry, 52*(23), 7094–7100. https://doi.org/10.1021/jf040133u

Yong, Y. H., Yamaguchi, S., & Matsumura, Y. (2006). Effects of enzymatic deamidation by protein-glutaminase on structure and functional properties of wheat gluten. *Journal of Agricultural and Food Chemistry, 54*(16), 6034–6040. https://doi.org/10.1021/jf060344u

Zhang, G., Ma, S., Liu, X., Yin, X., & Du, G. (2021). Protein-glutaminase: Research progress and prospect in food manufacturing. *Food Bioscience, 43*(13), Article 101314. https://doi.org/10.1016/j.fbio.2021.101314