



Research article

Purification and kinetic characterisation of extracellular glutaminase from *Priestia aryabhatai* B8W22 with deamidation activity on wheat gluten

Ha Phuong Trang^a, Dam Sao Mai^a, Ngoc Nam Trinh^b, Tran Thi Huyen^{a,*}

^a Institute Biotechnology and Food Technology, Industrial University of Ho Chi Minh city, Ho Chi Minh city 72700, Vietnam

^b Office of Science Management and International Affairs, Industrial University of Ho Chi Minh City, Ho Chi Minh City 72700, Vietnam

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Abstract

Importance of the work: Glutaminase, an enzyme catalyzing the conversion of glutamine to glutamate and ammonia, has garnered considerable interest due to its role in enhancing the physical and functional properties of proteins in food processing industries.

Objectives: To isolate and characterize an extracellular glutaminase from strain B1, evaluate its enzymatic properties and assess its effect on improving the functionality of gluten proteins.

Materials and Methods: An extracellular glutaminase was isolated from a bacterial strain (designated B1), based on 16S rRNA gene sequencing and showed 97% homology to the strain *Priestia aryabhatai* B8W22. Screening was carried out for influential factors for glutaminase production (incubation time, temperature, pH, carbon and nitrogen sources, concentration of glutamine). Partial glutaminase purification using ammonium sulfate was performed based on 10 kDa molecular weight cut-off (MWCO) dialysis followed by 3 kDa MWCO concentration. The molecular weight was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Glutaminase activity and kinetic parameters (K_m and V_{max}) were measured under various temperature and pH conditions. The glutaminase's deamidation ability was evaluated by treating wheat gluten and using Fourier-transform infrared spectroscopy (FTIR) analysis, followed by analysis of the functional properties (solubility, foaming capacity, water-holding and oil-holding capacities).

Results: Glutaminase displayed maximal activity $\pm$ SE of 161.571 ± 0.704 U/mL. Optimal catalysis occurred at 40°C and pH 7, with a K_m value of 0.9611 mM and a V_{max} value of 193.3 U/mL. The molecular weight of the partially purified glutaminase was approximately 60 kDa, based on SDS-PAGE. Based on the FTIR results, there was a substantial influence of glutaminase in all the gluten samples. Compared to the untreated controls, the wheat gluten treated with partially purified glutaminase had significantly enhanced solubility (33.4-fold), foaming capacity (163.2-fold), water-holding capacity (3.1-fold) and oil-holding capacity (28.0-fold), outperforming conventional chemical treatments.

Main finding: Partial purified glutaminase was an effective biocatalyst for wheat gluten modification, offering promising applications to improve protein functionality in food processing industries.

* Corresponding author.

E-mail address: tranthihuyen@iuh.edu.vn (T.T. Huyen)

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Introduction

Wheat gluten is a natural protein complex that constitutes approximately 85–90% of the total protein content in wheat grains and is primarily composed of two main types of proteins: gliadin and glutenin (Wang et al., 2020)). Gluten is utilized widely in the food industry for its ability to improve the texture, elasticity and nutritional value of products such as sausages, vegetarian meat alternatives and baked goods ((Mengli Zhang et al., 2023)). However, gluten's functionality remains limited because of its high glutamine residue content, resulting in poor water solubility and inadequate foaming and emulsification properties (Day, 2011). Deamidation is an effective method for enhancing the solubility of gluten by removing amide groups from glutamine residues, converting them into their corresponding glutamic acid, as this transformation greatly improves the solubility in neutral pH environments (Zhong et al., 2021). Traditional chemical methods, typically involving treatment with acid or alkaline solutions at elevated temperatures, can increase gluten solubility but may lead to the formation of potentially carcinogenic chloropropanol compounds. Furthermore, the presence of edible organic acids in these processes raises serious food safety concerns (Batey and Gras, 1983; Lei et al., 2015).

Glutaminase (glutamine aminohydrolase, E.C. 3.5.1.2) is a hydrolase enzyme that catalyzes the hydrolysis of glutamine into glutamic acid and ammonia, playing a crucial role in cellular nitrogen metabolism in animals, plants and especially microorganisms (Curthoys and Watford, 1995). Various bacteria, such as *Bacillus* sp. and *Trichoderma koningii*, are capable of producing both extracellular and intracellular glutaminase (El-Sayed, 2009; Singh et al., 2013). Among these, *Bacillus* sp. stands out as a particularly promising candidate for glutaminase production. The *Bacillus subtilis* JK-79 strain has demonstrated strong potential, achieving a maximum glutaminase activity of 507 Units (U)/mL (Thenmozhi Kulasekaran et al., 2024). In 2006, Yong et al. (2006) investigated the effect of glutaminase on α -zein-soybean protein, observing a slight increase in solubility (Li and Zhao, 2012). Additionally, glutaminase is used extensively in soy sauce fermentation to generate the umami flavor and to enhance the natural taste profile of food products (Iyer and Singhal, 2008) while also serving as a therapeutic enzyme in medical applications. (Orabi et al., 2020; Francis and Chakraborty, 2021). Microbial sources are preferred increasingly for glutaminase production in biotechnological applications (particularly in the pharmaceutical and food industries) because

of their various advantages, including rapid synthesis rates, low cost, mild cultivation conditions and high compatibility with extraction and purification processes (Patel et al., 2019). For example, ion exchange chromatography has been used to purify various glutaminases from *Capsicum annuum*, achieving up to a four-fold increase in purity compared with cell-free extracts (Suhail Zbar, 2022). Notably, a glutaminase with an apparent molecular mass of 44 kDa, produced by *Streptomyces canarius* FR (KC460654), not only exhibited specific catalytic activity on glutamine but also demonstrated safety in liver and kidney function, as well as in hematological indices, following 10 d of injection in rabbits (Reda, 2015)

A specialized enzyme recognized widely for its extensive applications in the food processing industry to enhance the functional properties of food proteins is Protein-glutaminase (PG), which was first discovered in bacteria such as *Chryseobacterium proteolyticum*. PG catalyzes the deamidation of amide groups at glutamine residues on proteins and peptides without causing other side reactions. PG acts exclusively on proteins and peptides and does not deamidate free glutamine or asparagine residues. However, glutaminase primarily converts free glutamine into glutamate and ammonia, acting mainly on free glutamine rather than protein-bound glutamine. Therefore, there remains some skepticism regarding the deamidation capability of glutaminase. Furthermore, alongside deamidation of glutamine side chains in proteins, uncontrolled protein hydrolysis may also occur, leading to the loss of native protein structure (Amobonye et al., 2019), which could negatively affect its safety and applicability in product development. Therefore, maintaining protein stability, the degree of hydrolysis and quality and structural integrity after glutaminase-mediated deamidation remains a major challenge and requires further investigation. Furthermore, high purity and favorable kinetic parameters of glutaminases are essential for assessing their effective enzymatic activity in applications aimed at enhancing the functional properties of food proteins. These factors highlight key research gaps, particularly in the optimization of enzyme production from novel, safe microbial sources, and in identifying suitable conditions for the deamidation process. To help address these limitations, the present study focused on optimizing the cultivation medium for glutaminase, using a promising bacterial strain isolated from soil with strong potential for industrial application. The findings should not only offer valuable insights into the capabilities of glutaminase but also propose an optimized process for the purification and effective application of glutaminase in the modern food industry.

Materials and Methods

Sample collection, isolation and screening of glutaminase-producing bacteria

One soil sample was collected from Tien Giang province, Vietnam and stored under sterile conditions at -4°C in the laboratory until analysis. For the isolation of glutaminase-producing bacteria, 1 g of soil was homogenized in 9 mL of sterile 0.9% NaCl solution. Then, 100 μL of the resulting suspension was spread onto screening agar plates prepared with the following composition (g/L): NaCl 0.5, KCl 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, KH_2PO_4 1.0, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1, ZnSO_4 0.1, glutamine 0.5 (as the nitrogen source) and phenol red 0.002 (as an indicator of glutaminase activity). The plates were incubated at 37°C for 24–48 hr and monitored for colonies that caused a color change in the surrounding medium, indicating alkalization due to glutaminase activity (Saleem and Ahmed, 2020). The bacterial strain marked B1, which exhibited the highest glutaminase activity during screening, was chosen for further in-depth enzyme production studies. The bacterial strain B1 was repeatedly streaked twice on nutrient agar plates for purification. Extracellular glutaminase production from strain B1 was achieved by inoculating the strain into 250 mL Erlenmeyer flasks containing fermentation medium composed of: glucose 10.0 g/L, glutamine 5.0 g/L, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 6.0 g/L, KH_2PO_4 3.0 g/L, MgSO_4 0.49 g/L and CaCl_2 0.002 g/L. Cultures were incubated at temperatures: 37°C . Samples were collected daily, centrifuged at $4,025 \times g$ at 4°C for 15 min and the supernatants were stored at 4°C for subsequent glutaminase activity assay.

Glutaminase activity assays

The glutaminase activity (expressed in Units per milliliter) was measured following the method described by Imada et al. (1973), with slight modifications. The reaction mixture was prepared by combining 0.5 mL 20mM Tris-HCl buffer (pH 8), 0.5 mL of 100 mM glutamine, 0.5 mL crude enzyme and 0.5 mL distilled water. The mixture was incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 30 min. Then, 0.5 mL of trichloroacetic acid (TCA) was added to stop the reaction. Subsequently, 0.1 mL of the reaction mixture was transferred into a test tube containing 3.7 mL distilled water, followed by the addition of 0.2 mL Nessler's reagent. Absorbance was measured at 450 nm and enzyme activity was calculated using a standard curve of ammonium

chloride (NH_4Cl). One unit (U) of glutaminase activity was identified as the amount of enzyme that liberated 1 μmol of ammonia under optimal assay conditions.

Morphological and molecular identification of B1 strain

The B1 bacterial strain was initially subcultured on nutrient agar plates and incubated at 37°C for 24 hr. Distinct colonies were selected and characterized based on morphological traits such as size, color, shape, margin and elevation. A representative colony was subjected to Gram staining using standard reagents (crystal violet, iodine solution, ethanol, safranin) and examined under a light microscope at $100\times$ magnification to determine cell shape and Gram reaction. For molecular identification, genomic DNA was extracted using the 95°C heat-shock method and used for polymerase chain reaction amplification of the 16S rRNA gene with universal primers 27-F and 1492-R (Dada et al., 2013). The reaction mixture (25 μL) included 1 μL of the template. The polymerase chain reaction products were verified on a 1.5% agarose gel (approximately 1500 bp) and sequenced. The obtained sequence was compared with entries in the NCBI GenBank database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). A phylogenetic tree was constructed using the MEGA 7.0 software (<https://www.megasoftware.net/>) with the neighbor-joining method and 1,000 bootstrap replicates to assess sequence similarity and evolutionary relationships. Bootstrap values were interpreted as indicators of confidence based on $>85\%$ (high), 65–85% (moderate) and $<65\%$ (low).

Screening for influential factors for glutaminase production from strain B1

The yield of glutaminase produced by strain B1 was enhanced based on optimizing the culture conditions by evaluating various factors (incubation time, temperature, pH, carbon and nitrogen sources, the concentration of glutamine added to the fermentation medium). The cultures were incubated at 37°C and glutaminase activity was measured at 0 d, 1 d, 2 d, 3 d, 4 d, 5 d and 6 d. The effect of temperature on glutaminase production was evaluated at 20°C , 25°C and 37°C . The effect of pH was tested at pH 6, pH 7, pH 8 and pH 9. Glucose, sucrose, lactose, starch and fructose (each at 1%) were tested as carbon sources. In addition, evaluation was carried out of various nitrogen sources at 0.5% concentration: tryptone, yeast extract, ammonium sulfate [$(\text{NH}_4)_2\text{SO}_4$] and sodium nitrate (NaNO_3).

Finally, the effects of different concentrations of glutamine (0.5–3.0%) were tested to determine the optimal level. Each factor was tested in triplicate. All data were presented as mean $\pm$ SD values ($n=3$). Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test to determine significant differences among means. Statistical significance was accepted at $p < 0.05$.

Glutaminase partial purification

Glutaminase was purified using ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ precipitation, following the method of (Mothe and Sultanpuram, 2016) with sulfate concentrations in the range 40–80%. After 96 hr of cultivation, the fermentation broth was centrifuged at $11,180 \times g$ for 30 min. The supernatant was supplemented with ammonium sulfate at the specified concentrations, gently stirred and incubated overnight at 4°C . Then, following centrifugation, the precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7). Next a dialysis membrane (10-kDa molecular weight cutoff) was used to remove residual salts from the precipitate, followed by incubation at 25°C 16 hr. The enzyme solution was concentrated from 47 to 17 mL using a 3 kDa ultrafiltration device. The protein concentration was determined using the Bradford assay (Bradford, 1976). The molecular weight was estimated using SDS-PAGE with 12% resolving and 5% stacking gels, stained with Coomassie Brilliant Blue G-250. A PageRuler prestained protein ladder (10–250 kDa; Thermo Scientific; Waltham, MA, USA) was used as a molecular weight marker. The purified enzyme was used for subsequent studies. The total protein content was measured using the Bradford assay (Bradford, 1976), with bovine serum albumin as the standard reference.

Effects of temperature and pH on the biochemical properties of glutaminase

The biochemical properties of glutaminase were examined based on incubating the enzyme with 35 mM glutamine at different reaction temperatures (20– 70°C , with intervals of 10°C between each point). The effect of pH was studied covering a pH range of 3–12 using different buffers: acetate buffer (pH 3–5), Tris-HCl (pH 6–9) and glycine-NaOH (pH 10–12). Enzyme reactions were carried out for 30 min. The optimal reaction conditions identified from these experiments were selected for further studies. Glutaminase activity was measured as described previously. Each factor was tested in triplicate. Results were presented as mean $\pm$ SD or mean $\pm$ SE.

Kinetics

Glutaminase kinetics were analyzed by preparing the enzyme in 20 mM Tris-HCl buffer (pH 7) and incubating it with varying concentrations of glutamine (0–50 mM). The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined using Michaelis-Menten plots. Glutaminase activity was measured as described above. Enzyme kinetic parameters were calculated using Prism 8.0.2 (GraphPad Software Inc.; San Diego, CA, USA).

Influences of investigated factors on degree of deamidation of wheat gluten by glutaminase

The degree of deamidation was assessed using the method described by (Kunarayakul et al., 2018), with minor modifications. Following partial deamidation of the wheat gluten, the ammonia present in the supernatant was measured using enzyme assay. Specifically, 500 μL of sample was mixed with an equal volume of 2 N H_2SO_4 , incubated at 100°C for 4 hr and centrifuged at $16,100 \times g$ for 10 min, after which the supernatant was analyzed. The degree of deamidation was calculated as the difference between the ammonia released from partial and complete deamidation. The four factors of temperature (30°C , 35°C , 40°C , 45°C , 50°C), pH (4, 5, 6, 7, 8, 9, 10), enzyme-to-substrate ratio (E/S; 20, 30, 40, 50) and deamidation time (0 hr, 3 hr, 6 hr, 9 hr, 12 hr, 18 hr, 24 hr, 30 hr, 38 hr) were investigated sequentially for their effects on the deamidation ability of partially purified glutaminase on wheat gluten and the effects of temperature and glutaminase treatment on the degree of deamidation. Data were presented as mean $\pm$ SD values ($n = 3$). Statistical differences between samples with and without glutaminase at each temperature were analyzed using two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test.

Foaming capacity

Foaming capacity was evaluated according to (Kunarayakul et al., 2018) and; Hadidi et al. (2021). Dried gluten samples with different levels of deamidation were rehydrated and 20 mg of each sample was dissolved in 20 mL of distilled water. The samples were homogenized at $25,155 \times g$ for 2 min and then transferred into graduated cylinders and the initial foam volume was recorded. The foaming capacity (FC) percentage was calculated using Eq. (1):

$$\text{FC} = (V_0 - 20) / (20 \times 100) \quad (1)$$

where V_0 is the initial foam volume.

The experiment was done in triplicate. All data were presented as mean $\pm$ SD ($n=3$) and were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. Differences were considered statistically significant at $p < 0.05$.

Water-holding and oil-holding capacities

The water- and oil-holding capacities were determined following (Kunarayakul et al. (, 2018) and ; Hadidi et al. (, 2021), with slight modifications. A 50 mL centrifuge tube was weighed (M_0) and 50 mg of gluten sample was added to 10 mL of either distilled water or soybean oil. The mixture was incubated at 40°C and centrifuged at 11,180 $\times$ g for 20 min. After discarding the supernatant, the tube was re-weighed (M_1). The water- and oil-holding capacity were both measured in grams per gram and calculated using Eq. (2):

$$\text{Water/Oil holding capacity} = (M_1 - M_0 - 0.05) / 0.05 \quad (2)$$

where M_0 is the weight of the 50 mL centrifuge tube and M_1 is the weight of the test tube after processing and removing the excess supernatant, with all measurements in grams.

The experiment was done in triplicate. All data were presented as mean $\pm$ SD($n=3$) and were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test.

Method of sample preparation for Fourier-transform infrared spectroscopy analysis

Wheat gluten deamidation ability was determined using glutaminase under the conditions presented in the experiment above. A sample of 5% wheat gluten powder was prepared in a 250 mL glass beaker contained phosphate-buffered saline (PBS) buffer at pH 6, followed by adding glutaminase to make $n \text{ E/S} = 40$. The deamidation process was carried out at 45°C and pH 6 for 24 hr. After the reduction time, the mixture was centrifuged at 11,180 $\times$ g for 20 min to collect samples of the precipitant (insoluble gluten) and the liquid (soluble gluten) for Fourier-transform infrared spectroscopy (FTIR) analysis. The negative control sample was prepared similarly, but without adding glutaminase. Spectral measurements were taken for the wavelength range 4000–500 cm^{-1} using an FTIR spectrometer (FT-IR 47000; Jasco; Tokyo, Japan). The spectrum results responding at spectral ranges of

3300–3400 cm^{-1} , 1550 cm^{-1} and 1000 cm^{-1} were used to determine changes in the three functional groups of amine (NH), amide II (N-H bend + C-N stretch) and carboxyl (CO) in each sample.

Results

Isolation, screening and identification of potential glutaminase-producing bacteria

Bacterial strains capable of producing glutaminase were isolated from a soil sample collected in Tien Giang province, Vietnam. Initial screening identified five distinct strains, designated B1, T1, L8, L9 and L10, based on their potential glutaminase activity. During growth and enzyme production, glutaminase catalyzed the conversion of glutamine to glutamic acid, leading to a decrease in the medium's pH and causing phenol red to change color from yellow to pink (Gulati et al., 1997). Among the five strains, B1 had the highest glutaminase activity, as evidenced by the intense pink coloration on the agar plate after incubation (Fig. 1). Consequently, strain B1 was chosen for further investigation, including optimization of the enzyme production conditions, determination of kinetic parameters and evaluation of its ability to deamidate wheat gluten.

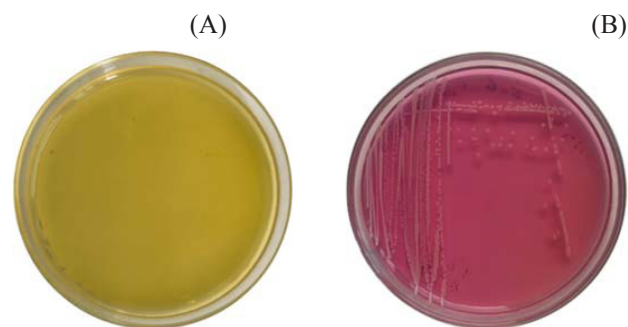


Fig. 1 Color change of agar plate by glutaminase-producing strain B1: (A) control agar plate; (B) color change in agar plate caused by B1 strain.

The B1 bacterial strain was studied in detail regarding its morphological characteristics and molecular identification to accurately determine its taxonomic position. When cultured on nutrient agar medium, the B1 colony exhibited a circular shape with a yellowish-white color, slightly wavy edges and a smooth, dry surface (Fig. 1SA). Combining these morphological features with the Gram staining results (revealing rod-shaped cells characteristic of a Gram-positive bacterium) provided preliminary identification of strain B1 (Fig. 1SB).

Molecular analysis based on 16S rRNA gene sequencing was performed to determine accurately its taxonomic classification. After successful amplification, the obtained sequence was compared with entries in the NCBI GenBank database using the BLASTn tool. The analysis showed that the 16S rRNA sequence of strain B1 had a high similarity (97%) with the sequence under accession number NR115953.1 and was identified as *Priestia aryabhatai* B8W22 of the Bacillaceae family. The evolutionary relationship between strain B1 and other bacterial species was illustrated through a phylogenetic tree constructed using the neighbor-joining method (Fig. 1SC). This identification was important because it provided a clearer understanding of the biological characteristics and glutaminase-producing potential of *Priestia aryabhatai* B8W22.

Screening for influential factors for glutaminase production from strain B1

Finding the best culture medium is one of the most effective strategies for enhancing the production of target enzymes. The current study evaluated the effects of various factors on the activity of glutaminase (incubation time, temperature, pH, carbon source, nitrogen source, glutamine concentration). The statistical analysis revealed that all these tested factors

had a significant effect on glutaminase activity, as shown in Table 1S. As shown in Fig 2A and 2B, the highest glutaminase activity occurred on the fourth day of incubation at 37°C, with a value of 102.4 ± 0.799 U/mL. In addition, each pH value significantly impacted enzyme activity, with a notable difference between pH 6–8 and pH 9 (Fig. 2C). After 4 d of incubation, glutaminase activity reached its peak within pH 6–8, with the highest value recorded at pH 7 (139.74 ± 1.819 U/mL). The effects of different carbon and nitrogen sources are illustrated in Figs. 2D and 2E. Among the carbon sources tested at 1% concentration, starch and fructose led to a sharp decline in activity, yielding only 9.81 ± 1.815 U/mL, which was the lowest value recorded. In contrast, the addition of glucose, lactose or sucrose (each at 1%, weight per volume, w/v) enhanced enzyme production, with glucose yielding the highest activity (159.87 ± 0.690 U/mL). With respect to nitrogen sources, replacing glutamine with alternatives such as yeast extract, tryptone, ammonium sulfate or sodium nitrate (0.5%, w/v) reduced glutaminase activity across the board. However, when varying the concentration of glutamine, the enzyme produced the highest activity of 161.57 ± 0.704 U/mL at 2% (w/v) glutamine (Fig. 2F). These findings are important for optimizing the medium composition to maximize enzyme production efficiency.

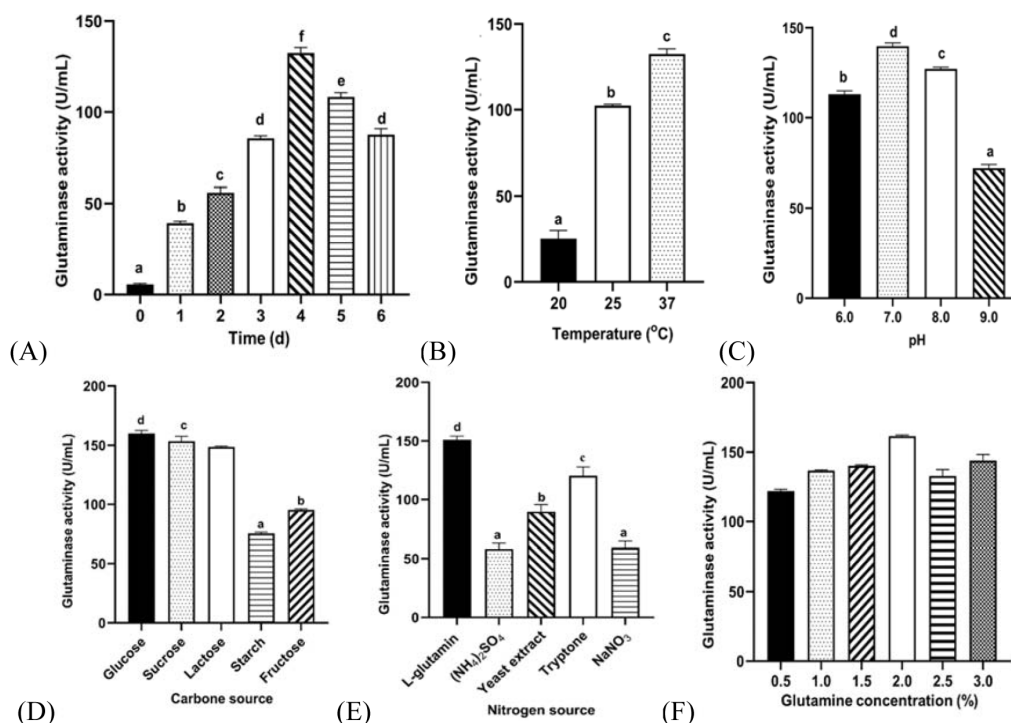


Fig. 2 Screening for influential factors of the fermentation medium for *Priestia aryabhatai* B8W22: (A) incubation time; (B) temperature; (C) pH; (D) carbon source; (E) nitrogen source; (F) glutamine concentration. Error bars indicate $\pm$ SD from three independent experiments. Columns represent mean values and different lowercase letters above the columns indicate significant differences ($p < 0.05$) according to Tukey's multiple comparisons test.

Partial purification, enzymatic properties and kinetics of glutaminase

Partial purification and enzymatic properties of glutaminase

The partial purification of glutaminase using ammonium sulfate precipitation at concentrations in the range 40–80% revealed that 70% saturation was the most effective step for obtaining a highly purified enzyme. At this stage, the yield increased to 80.474% with a specific activity of 37.451 U/mg (Table 1). The overall purity of glutaminase increased progressively throughout the purification process, although the total protein content, overall enzymatic activity and yield decreased to some extent. Following the final concentration step using a 3 kDa molecular weight cut-off (MWCO) concentrator, the yield and specific activity were 49.795% and 52.224 U/mg, respectively. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis after the last purification stage revealed a distinct protein band corresponding to an approximate molecular weight of 60.0 kDa (Fig. 3), confirming the high purity of the partial purified glutaminase.

After successful purification of the enzyme, the operational characteristics of the partially purified glutaminase were investigated. The effect of temperature on the specific activity was assessed over the range 20–70°C. The statistical analysis showed that both temperature and pH significantly influenced the biochemical properties of glutaminase (Table 2S).

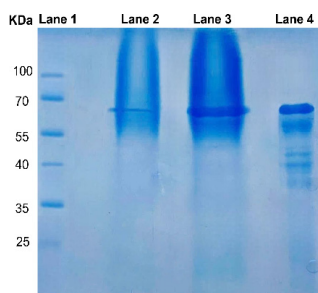


Fig. 3 Partial purification of glutaminase from *Priestia aryabhatai* B8W22 on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1 = Page Ruler protein ladder (10–250 kDa); Lane 2 = partial purified enzyme after using 10 kDa molecular weight cut-off (MWCO) dialysis membrane; Lane 3 = partial purified enzyme using 3 kDa MWCO concentrator; Lane 4 = bovine serum albumin (2 mg/mL).

The results in Fig. 4A show that the activity of glutaminase increased as the temperature rose from 20 to 40°C. Its activity peaked at 40°C (178.070 ± 5.705 U/mL). When the temperature exceeded 50°C, the activity dropped to 143.831 ± 7.137 U/mL. The decline was more pronounced at 60°C (74.615 ± 2.063 U/mL), with the lowest activity occurring at 70°C (43.294 ± 2.526 U/mL). These findings suggested that 40°C was the optimal temperature for the enzyme and indicating that partially purified glutaminase has good thermal stability at moderate temperatures, which is important for industrial applications. Regarding the influence of pH at 40°C, the partially purified glutaminase exhibited relatively stable activity in the pH range 5–7, with the highest activity recorded at pH 7 (185.683 ± 2.585 U/mL). The activity decreased markedly at pH values below 5 or above 9 and was almost completely lost at the extreme pH values of 3 and 12 (37.224 ± 1.482 U/mL and 31.167 ± 2.590 U/mL, respectively), as shown in Fig. 4B. Based on these results, the partially purified glutaminase functioned optimally in slightly acidic-to-neutral environments.

Kinetics of glutaminase

The kinetic results provided evidence of effective enzyme activity, following the Michaelis-Menten kinetic model. As shown in Fig. 4C, partial purified glutaminase activity increased gradually as the glutamine concentration increased, reaching saturation at 10 mM. Beyond this concentration, no significant increase in activity was observed, indicating that all enzyme active sites were saturated with the substrate. From the Michaelis-Menten plot, key kinetic parameters of purified glutaminase were determined with high reliability (coefficient of determination = 0.9741). The maximum velocity (V_{max}) of the reaction was 193.3 U/mL, while the Michaelis constant (K_m) was 0.9611 mM. This K_m value suggested that the partially purified glutaminase had reasonably good affinity for the glutamine substrate because lower K_m values indicate higher enzyme-substrate affinity (Alazmi, 2026). These kinetic parameters are essential for understanding the mechanism of purified glutaminase action and provide valuable insight for optimizing its use in practical applications. In particular, the precise determination of the saturating substrate concentration at 10 mM has practical relevance for designing efficient production processes and avoiding unnecessary substrate waste.

Table 1 Overall partial purification of glutaminase from *Priestia aryabhatai* B8W22.

Procedure or step	Fraction volume (mL)	Total protein (mg)	Activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification (-folds)
Crude cellular extract	200	82.947	636.827	7.678	100	1
Precipitation with ammonium sulfate 70%	60	13.684	512.480	37.451	80.474	4.88
Dialysis 10 kDa MWCO	47	12.599	393.364	31.222	61.769	4.07
Concentration 3 kDa MWCO	17	6.072	317.106	52.224	49.795	6.80

MWCO = molecular weight cut-off.

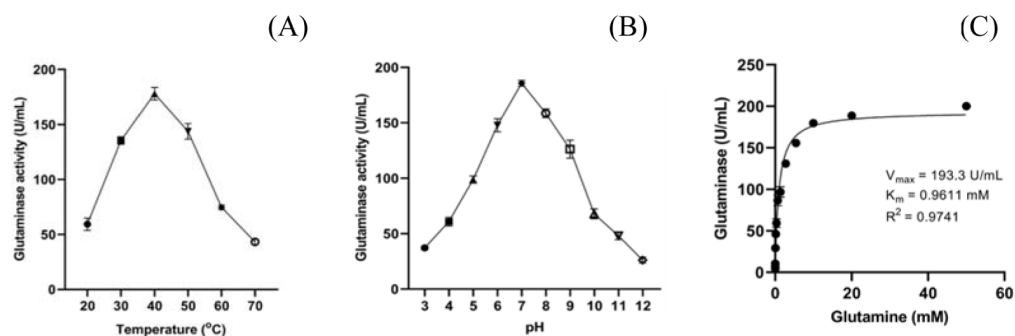


Fig. 4 Enzymatic properties and kinetics of partial purified glutaminase from *Priestia aryabatai* B8W22: (A) effect of temperature; (B) effect of pH; and (C) substrate saturation kinetics based on the Michaelis-Menten plot. Error bars indicate $\pm$ SE; U = Unit.

Influence of investigated factors on degree of deamidation of wheat gluten by glutaminase

The modification of functional properties was evaluated in 5% wheat gluten by the partially purified glutaminase through the degree of deamidation. Specifically, the deamidation capacity of wheat gluten was investigated based on varying the factors temperature, E/S, pH and reaction time. The deamidation degree of the wheat gluten treated with glutaminase was highly

significantly greater than that of samples without glutaminase ($p < 0.0001$), as determined based on two-way ANOVA followed by Sidak's multiple comparisons test, as shown in Table 3S. The degree of deamidation reached maximum values of 60.54%, 66.04% and 72.11%, corresponding to a temperature of 45°C, a pH of 6 and an E/S value of 30 U/g, respectively. Under these conditions, the highest deamidation (data not presented) was achieved at 24 hr (Figs. 5A–5D).

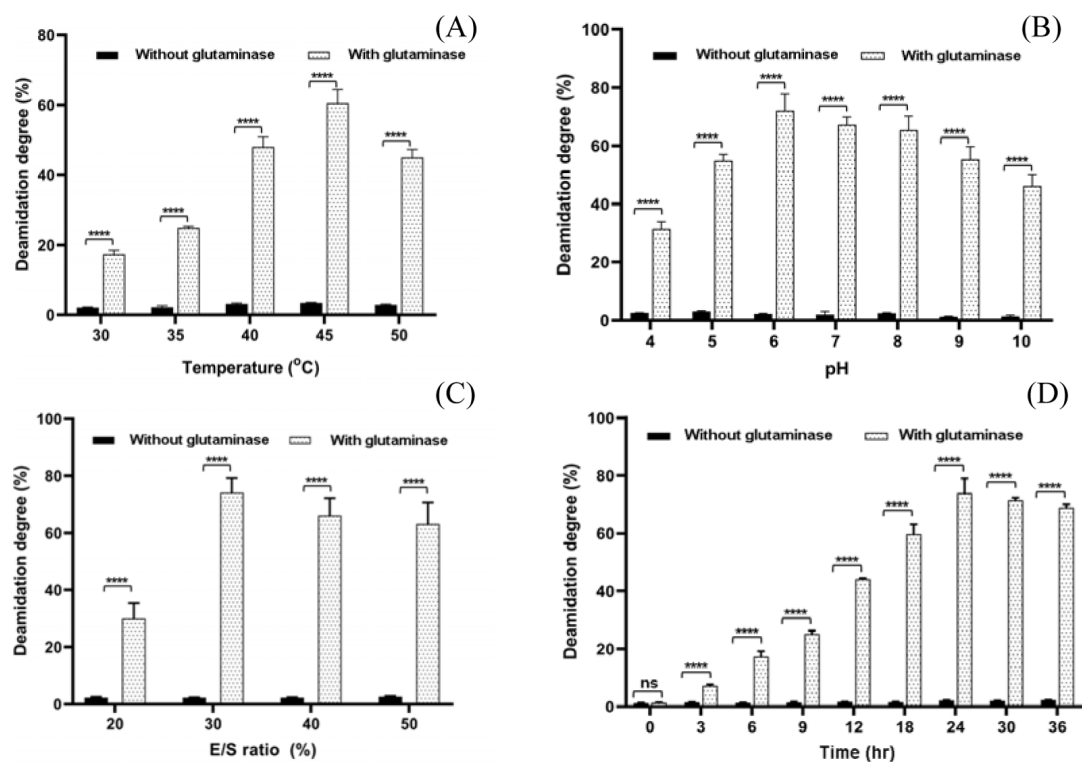


Fig. 5 Deamidation assay of wheat gluten optimally modified by glutaminase at: (A) different temperatures (enzyme-to-substrate ratio, E/S = 30 Units/g, pH 7.0, 24 hr reaction); (B) different pH values (45°C, E/S = 30 U/g, 24 hr reaction); (C) different enzyme-to-substrate ratios (45°C, pH 6, 24 hr reaction); (D) different reaction times (45°C, pH 6, E/S = 30 Units/g), where statistical differences between samples with and without glutaminase at each temperature were analyzed using two-way analysis of variance followed by Sidak's multiple comparisons test, statistical significance was defined as $p < 0.05$ and ns = not significant, *** = $p < 0.001$, **** = $p < 0.0001$.

Determining structural changes of buffer dissolved gluten (Glu-C) and buffer solved gluten (Glu-L) using Fourier-transform infrared spectroscopy

FTIR serves as a sensitive analytical technique for characterizing molecular peaks in the transmittance spectra (Esfanjani et al., 2015; Sarmiento et al., 2006). The results of the FTIR spectral analysis clearly confirmed the existence of typical peaks at wavenumbers of 3300 cm^{-1} , 1550 cm^{-1} and 1000 cm^{-1} , indicating the presence of the NH, Amide II and CO groups respectively, in both the insoluble gluten and soluble gluten samples (Fig. 2S). In addition, the transmittance of the characteristic peaks for three functional groups (NH, Amide II, CO) were used to assess changes in their contribution in all gluten samples. For the NH group, transmittance in Glu-C1 (insoluble gluten treated with glutaminase) decreased to 1.26% compared to Glu-C0 (insoluble gluten untreated with glutaminase), as shown in Fig. 2S(A). Similarly, NH transmittance in Glu-L1 (soluble gluten treated with glutaminase) was approximately one-half of Glu-L0 (soluble gluten untreated with glutaminase), according to Fig. 2S(B). For the Amide II group, transmittance in Glu-C0 was 64.17%, which was lower than the 81.12% in Glu-C1. In contrast, Amide II transmittance did not vary greatly between Glu-L0 and Glu-L1. For the CO group,

there were minimal spectral differences between Glu-C1 and Glu-C0 and between Glu-L1 and Glu-L0. In summary, among the three groups measured, only the NH transmittance declined consistently in both glutaminase-treated insoluble and soluble gluten. These findings indicated the successful deamidation by the glutaminase in both Glu-C1 and Glu-L1 compared to their untreated controls (Glu-C0 and Glu-L0).

Effect of glutaminase on three functional properties of wheat gluten

Wheat gluten treated with partially purified glutaminase exhibited significant improvements in solubility, foaming capacity and water- and oil-holding capacities, as presented in Table 4S. As shown in Fig 6A, foaming capacity increased markedly, reaching a peak improvement of 163.22-fold when the gluten was deamidated to 76.41%, compared with non-deamidated wheat gluten. In addition, the water- and oil-holding capacities increased from their initial values, peaking when the wheat gluten was deamidated to 58.47% (Figs. 6B and 6C). As the degree of deamidation increased, precipitation declined and solubility improved over time. At 24 -hr, the gluten samples treated with 30 U/g of enzyme had significantly better solubility than the control without enzyme treatment (Fig. 6D).

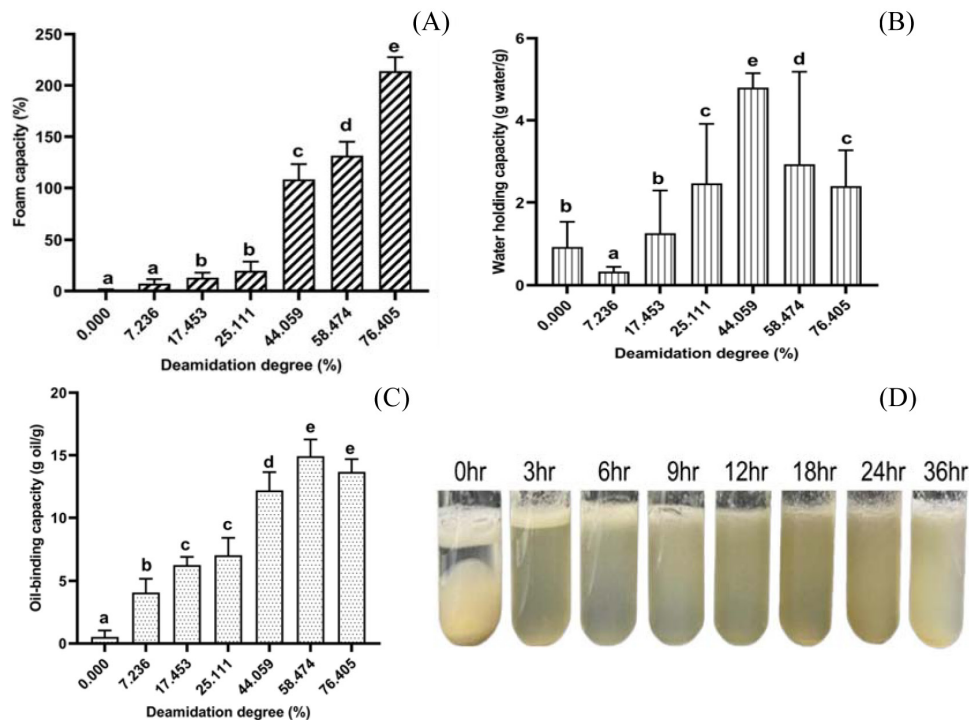


Fig. 6 Functional property changes of wheat gluten following deamidation by glutaminase: (A) Foaming capacity; (B) Water-holding capacity; (C) Oil-binding capacity (D) Solubility over time. Columns represent mean values and error bars represent $\pm$ SD. Different lowercase letters above columns denote statistical differences ($p < 0.05$, Tukey's multiple comparisons test).

Discussion

Screening for influential factors for glutaminase production from strain B1

Following the isolation, screening and optimization of the cultivation conditions for glutaminase production, the bacterial strain *Priestia aryabhatai* B8W22 was identified that was capable of synthesizing glutaminase and considered safe for industrial-scale production. By adjusting media components (sources of nitrogen and carbon) and physical factors (pH, temperature), the enzyme content and activity were determined at 96 hr, which was the same incubation time used in fermentation processes for glutaminase production by *Bacillus* sp. DV2-37 (Gomaa, 2022). Under similar fermentation conditions (pH 7 and 37°C with 1% glucose and 2% glutamine) glutaminase activity reached 69.10 U/mL, which was superior to that of *Bacillus* sp. DV2-37 (47.12 U/mL; (Gomaa, 2022) and *Acinetobacter junii* (21.5 U/mL; (Abdel-Hafez et al., 2025). Furthermore, because the temperature and pH significantly influence the bioavailability of trace minerals and enzymatic metabolic processes (Amobonye et al., 2019), the observed optimal activity of glutaminase at 37°C and pH 7 aligned well with its thermophilic nature. Collectively, these results demonstrated that the careful control of parameters—particularly the pH, temperature and nutrient composition—is critical for maximizing enzyme yield and activity. The highest glutaminase yield in the current study was achieved using glucose as the carbon source at 1% and glutamine at 2%, which likely served not only as effective nitrogen sources but also as inducers to enhance enzyme synthesis.

Partial purification, enzymatic properties and kinetics of glutaminase

Precipitation using 70% ammonium sulfate was the most effective method for harvesting high-quality glutaminase. Based on the current results, there was a single purified glutaminase band at approximately 60 kDa, confirming the high purity of the preparation. This molecular weight was consistent with that for glutaminase produced by *Halomonas meridiana* (57.0 kDa; (Mostafa et al. (, 2019) and *Bacillus* sp. LKG-01 (60 kDa; (Kumar et al. (, 2013). The recovery yield of partially purified glutaminase in the current study was 61.769%, which was higher than that reported for glutaminase from *Halomonas meridiana* (42.05%; (Mostafa et al. (, 2019)

and from *Bacillus cereus* strain LC13 (49.4%; (More et al., 2018). Using a two-step purification process (ammonium sulfate precipitation followed by dialysis) there was 4.07-fold purification of the partially purified glutaminase, which was superior to the 2-fold purification reported for glutaminase from *Bacillus subtilis* OHEM11 (Orabi et al., 2020), even though that enzyme was purified through three steps (ethanol precipitation, dialysis, ion-exchange chromatography). Overall, the purification process in the current study preserved the structural integrity and functional quality of the purified glutaminase, with substantial recovery across each step.

The enzyme had high activity and stability at pH 7, with optimal activity at 40°C. These characteristics were comparable to those of glutaminase from *Streptomyces canarius* FR, which also functions best at pH 8.0 and 40°C (Reda, 2015). The current kinetic parameters were determined, with a K_m of 0.9611 mM and a V_{max} of 193.3 U/mL. Notably, the partially purified glutaminase had a higher V_{max} value than glutaminase purified from *Bacillus cereus* LC13 (V_{max} of 61.34 U/mL; (More et al. (, 2018), indicating potentially greater catalytic efficiency under similar conditions.

Effect of partially purified glutaminase on functional properties of wheat gluten

Solubility, foaming and the water- and oil-holding capacities are key contributors to the structural and functional performance of plant proteins in general and wheat gluten in particular (Foegeding and Davis, 2011). Compared with animal proteins, such as bovine milk or egg white, often plant proteins are more cost-effective and stable; however typically, they exhibit inferior functional properties (Dayanand and Mousumi, 2013). The use of hydrolytic enzymes offers a meaningful approach to improve limitations in solubility, foaming capacity and oil and water retention.

Based on the FTIR results, changes in the transmittance degree of peaks attributed to NH (amide groups containing nitrogen and hydrogen), Amide II (secondary amide groups related to protein structure) and CO (carbonyl groups, C=O) at typical wavenumbers (cm^{-1}) demonstrated the important influence of glutaminase in all gluten samples. Taken together, the decreased transmittance degree of the NH groups and the negligible transmittance degree of the CO groups provided clear evidence regarding the deamidation activity (removal of amide groups, which converts glutamine to glutamic acid) of glutaminase on both soluble (Glu-L1) and insoluble (Glu-C1)

gluten samples. Furthermore, the additional presence of the NH group could help to maintain the stability, quality and structural integrity of all gluten samples. Next, the current study focused on evaluating the impact of deamidation on 5% wheat gluten (w/v) using glutaminase produced from a specific strain of *Priestia aryabhatai* BSW2. Based on these results, the partially purified glutaminase effectively catalyzed deamidation, significantly enhancing the functional properties of the gluten. A maximum deamidation degree of 73.89% was achieved after 24 hr, which compared favorably with the findings of Yong et al. (2006) who reported approximately 72% deamidation after 30 hr using PG from *Chryseobacterium proteolyticum* (Yong et al., 2006). Compared to citric acid-based deamidation methods, partially purified glutaminase exhibited superior efficiency, operating under milder conditions, with lower temperature requirements and higher deamidation levels (Liu et al., 2023). During the deamidation process, purified glutaminase altered the protein chains, improving gluten solubility in water. As a result, protein molecules more readily migrated to the air-water interface, enhancing foaming capacity—especially within a neutral pH range (6–8). Notably, a deamidation degree in the range 44.51–76.41% was sufficient to significantly improve solubility, foaming capacity and oil- and water-holding capacities. By contrast, PG from *Chryseobacterium proteolyticum* TM1040 required a higher deamidation degree of 81.7% to achieve comparable functional improvements (Zhang et al., 2024), suggesting that purified glutaminase induces only partial conversion of amide groups, enabling moderate structural changes without extensive degradation and thereby preserving the essential structural integrity and nutritional value of wheat gluten. These results aligned with the study by (Li and Zhao, 2012), who performed deamidation of 5% (w/v) soybean protein using glutaminase. Although the enzymatic deamidation effect was weak, with only 6.6% deamidation achieved under 400 U/kg glutaminase supplementation at 37°C for 36 hr, the SDS-PAGE analysis showed that glutaminase could catalyze peptide bond hydrolysis in soybean proteins. In addition, there were beneficial changes in increased solubility and viscosity compared to the original non-deamidated soybean protein. These findings highlight the promising potential of purified glutaminase as a cost-effective, efficient and safe tool for improving gluten functionality in modern food processing.

Conclusion

Priestia aryabhatai B8W22 was reported for the first time to produce glutaminase via fermentation under optimized conditions of 37°C for 4 d, at pH 7, using 1% glucose as the carbon source and 2% glutamine as the nitrogen source. Under these conditions, the highest enzyme activity achieved was 161.57 ± 0.704 U/mL. The kinetic parameters V_{max} and K_m were determined as 193.3 U/mL and 0.9611 mM, respectively. Partial purified glutaminase, with an approximate molecular size of approximately 60 kDa, demonstrated the ability to deamidate wheat gluten to a degree of 73.89% using optimized reaction conditions of 45°C, pH 6, an enzyme-to-substrate ratio of 30 U/g and 24 hr of reaction time. The FTIR results demonstrated the significant influence of glutaminase by the additional presence of the NH group in all gluten samples. This significantly improved the functional properties of gluten by deamidation of the glutamine side chains. These findings underline the great potential of glutaminase in enhancing wheat gluten performance and offer a new perspective on its application compared to previously validated protein-glutaminase enzymes. This study contributed valuable insights to the advancement of food technology, particularly in plant protein modification.

Conflict of Interest

The authors declare there are no competing interests. The corresponding author, declares, on behalf of all authors of the paper, that no financial conflict of interest exists in relation to the work described.

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