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Proceeding Paper

Multi-Bioactive Potential of a Rye Protein Isolate Hydrolysate by Enzymatic Processes †

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Abstract: Foods based on plant proteins have increased in recent years. Thus, this study aimed to establish the potential of rye protein hydrolysates to release bioactive peptides to treat non-communicable diseases. Rye protein isolate was obtained using the solubilization–precipitation method and hydrolyzed with alcalase and flavourzyme. Hydrolysates were assessed for antioxidant, antihypertensive, and anti-diabetic properties. The rye protein isolate showed a protein content of 51.06 ± 1.15%, and its hydrolysates showed antioxidant, antihypertensive, and anti-diabetic capacities of 34.77 ± 0.59, 86.15 ± 1.15, and 40.62 ± 2.61% for flavourzyme, while for alcalase they were 78.58 ± 1.04, 86.76 ± 2.02, and 63.70 ± 1.27%. Hydrolysis with alcalase was the greatest, and the multifunctionality obtained was better than in similar studies.

Keywords: non-communicable diseases; hydrolysates; rye protein

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1. Introduction

Non-communicable diseases (NCDs) have been recognized as a significant challenge until 2030 in the World Health Organization (WHO) agenda for sustainable development. These illnesses are characterized by a long duration, where cardiovascular diseases (CVDs), cancers, chronic respiratory diseases, and diabetes are the prominent examples. In this context, CVDs are the most common worldwide. Indeed, it is expected that by 2030, 22.2 million deaths will be related to those illnesses [1].

As NCDs result from genetic, physiological, environmental, and behavioral factors, different approaches are considered for prevention and control. An exciting focus is using multifunctional peptides to prevent CVDs [2]. Moreover, it has been demonstrated that cereal grains such as wheat, barley, rice, rye, oat, millet, sorghum, and corn maintain health benefits to prevent several NCDSs such as CVDs, diabetes, and cancer [3].

Rye is a crop that can grow with minimal nutritional requirements, exhibits climate-resilient characteristics, and possesses valuable bioactive compounds such as phenolic acids, flavonoids, lignans, and arabinoxylans [4]. However, few studies about bioactive peptides from rye protein or about its multifunctionality have been elucidated. Therefore,

this study's aim was to determine the multi-bioactivity potential of a rye protein isolate hydrolyzed via two enzymatic processes by measuring antioxidant, antihypertensive, and anti-diabetic properties.

2. Methods

Chemicals and reagents: Whole rye grain was purchased from Tierra de colores (Edo. De México, Mexico). The HCl, NaOH, K_2SO_4, $CuSO_4 \cdot 5H_2O$, H_3PO_4, H_2SO_4, H_2O_2, H_3BO_3, KH_2PO_4, K_2HPO_4, NaCl, K_2CO_3, and methanol were acquired with J.T. Baker, Thermo Fisher Scientific (CDMX, Mexico). Methyl red and bromocresol green were from Meyer (CDMX, Mexico). Xanthan gum was supplied by Deosen Biochemical (Ordos, China). Alcalase from *Bacillus licheniformis* (>2.4 U/g Anson Units), Flavourzyme from *Aspergillus oryzae* (>500 U/g), picryl sulfonic acid solution (5%, *w/v*), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hippuryl-histidyl-leucine (HHL) hydrate, Angiotensin Converting-Enzyme (ACE) from rabbit lung (0.1 UN), Dipeptidyl Peptidase IV (DPPIV) recombinant enzyme from human (>1 unit/vial), pyridine, benzene sulfonyl chloride, and Tris (hydroxy) aminomethane were bought from Sigma-Aldrich (Saint Louis, MO, USA). Gly-Pro-p-nitroanilide was acquired from Santa Cruz Biotechnology (Dallas, TX, USA). All chemicals were ACS grade. All determinations were performed with Milli-Q deionized water at 18.2 MΩ·cm (Merck Millipore; Darmstadt, Germany)

Equipment and instrumentation: Hand grain mill (WonderMill; Pocatello, Idaho, USA); 297 µm mesh sieve (Mont-Inox; CDMX, Mexico); IEC Centra GP8R centrifuge (Artisan Technology Group; Champaign, IL, USA); FreeZone 6L Benchtop Freeze Dry System (Labconco; Kansas City, MO, USA); Kjeldahl digestor block (Sev-Prendo; Puebla, Mexico); Kjeldahl distillation unit ZDDN-II (Lab-Kits; Kowloon, Hong-Kong); Refrigerated microcentrifuge Sorvall Fresco (Thermo Fisher Scientific; CDMX, Mexico); UV-vis spectrophotometer (Thermo Fisher Scientific; CDMX, Mexico); Dry-Bath (Barnstead Thermolyne; Dubuque, IA, USA).

Rye protein isolate obtention: Rye grain was mechanically milled in a hand grain mill, and the product was sieved using 297 µm mesh. Rye flour was submitted to an acid and alkaline treatment for protein obtention [5]. Flour was mixed with NaOH (0.02 M) in a 1:14 ratio and agitated for 1 h with a magnetic bar for protein solubilization. Then, the content was centrifugated in an IEC Centra GP8R centrifuge for 25 min at 5600 rpm and at room temperature. The supernatant was recovered, and the pH was down to 5.5 with HCl (0.1 M) to precipitate the protein. Precipitate was recovered via centrifugation under the same conditions described before. After, it was lyophilized in a freeze dryer system at 0.110 mbar and −54 °C for 48 h.

Protein content: The lyophilized rye protein isolate was submitted to protein determination using the Kjeldahl method [6]. Primary digestion was carried out by blending 70 mg of the powder with 0.5 g of K_2SO_4 and digestive reactant (0.3 g of $CuSO_4 \cdot 5H_2O$; 5 mL of concentrated H_3PO_4; 43 mL of concentrated H_2SO_4; 2 mL of deionized water) at 370 °C for 15 min in Kjeldahl digestor. Afterward, the mixture was cooled, and H_2O_2 at 30% (1.5 mL) was added and returned to heating to complete digestion. Distillation was performed in two steps: neutralization and ammonia liberation took place in NaOH (50%, *w/v*) in a nitrogen distillation unit, and ammonia capture took place in 50 mL of the indicator solution (1% of H_3BO_3, 0.00075% of methyl red, and 0.001% of bromocresol green). The last volume was titrated with standardized HCl (0.02 N) to obtain the total nitrogen (%), which was converted to protein content (%) through its multiplication with the rye protein factor (5.7).

Enzymatic hydrolysis: Suspensions of the isolated rye protein at 10% (*w/v*) were prepared in phosphate buffer (0.1 M, pH = 7.5) with the addition of xanthan gum at 0.1% (*w/v*), which was sterilized in an autoclave at 121 °C for 15 min. Hydrolysis was carried out with alcalase and flavourzyme in a protein:enzyme mass ratio of 100:2.5. Reaction was performed at 60 °C for 8 h with oscillating agitation at 130 rpm. The sampling times were 0, 4, and 8 h. Enzyme inactivation was made in a boiling water bath for 10 min,

and supernatants for subsequent analysis were obtained via centrifugation at 10,000× *g* rpm/4 °C for 10 min using a refrigerated microcentrifuge.

Free amino-groups determination: The trinitrobenzene sulfonic acid method assessed amino-groups release via enzymatic hydrolysis [7]. Briefly, 0.250 mL of each sample was mixed with phosphate buffer (2 mL; 0.21 M, pH = 8.2) and picryl sulfonic acid solution (2 mL) at 0.1% (*v*/*v*) dissolved in phosphate buffer. The reaction was carried out in the absence of light for 1 h at 50 °C, and it was arrested by 0.1 N HCl addition (4 mL). Absorbance was recorded at 340 nm in a UV-vis spectrophotometer, and free amino-groups concentration was obtained from a calibration curve with glycine (0–200 ppm).

Radical scavenging activity test: The radical scavenging capacity was evaluated using the DPPH method following the technique described by Ramírez-Godínez [8]. In brief, 2.9 mL of a solution at 0.1 mM of DPPH dissolved in methanol was mixed with 0.1 mL of each hydrolysate. Afterward, the reaction was carried out without light at room temperature for 50 min. In parallel, a control was performed by substituting the sample for methanol. Absorbance from samples and control was measured in a UV-vis spectrophotometer to 515 nm, using methanol as blank. Specifically, samples were filtrated through 20 µm acrodisc syringe filters before measurements due to the presence of turbidity. Radical scavenging power was calculated from the following equation:

$$\text{Radical Scavenging Power } (\%) = 1 - \frac{\text{Sample Absorbance}}{\text{Control Absorbace}} \times 100$$

Antihypertensive activity by ACE inhibition test: In vitro ACE inhibition tested the antihypertensive capacity [9]. Control (A_{100}) and sample (A_s) systems were assessed. Control consisted of 80 µL of saline borate buffer (0.05 M, pH = 8.2 with 0.3 M of NaCl), 200 µL of HHL at 5 mM dissolved in buffer, and 20 µL of ACE from rabbit lung reconstituted in buffer to reach 0.1 U/mL concentration. The sample system contained the same volumes of HHL and ACE, but the hydrolysates were added instead of the buffer. The reaction proceeded for 80 min at 37 °C in a dry bath, and then it was inactivated via 0.1 M HCl addition (250 µL). All reaction content was transferred to test tubes, and ethyl acetate was added (1.7 mL). Organic extraction was carried out three times, and 800 µL of the organic layer was separated and evaporated by heating at 80 °C for 1 h in the same dry bath. The hippuric acid extracted was reconstituted with deionized water (500 µL). The last volume was mixed with pyridine (300 µL) and benzene sulfonyl chloride (150 µL) to record the absorbance at 410 nm in a UV-vis spectrophotometer. Finally, the ACE inhibition was calculated using the following equation:

$$\text{ACE inhibition } (\%) = \frac{A_{100} - A_s}{A_{100}} \times 100$$

Anti-diabetic capacity by DPPIV inhibition test: Anti-diabetic activity was evaluated from in vitro DPPIV inhibition analysis proposed by Nongonierma et al. [10] with slight modifications. Sample system (A_s) consisted of hydrolysates (100 µL) mixed with Gly-Pro-p-nitroanilide (100 µL) at 1.6 mM in Tris-HCl buffer (0.1 M, pH = 8). This mixture was pre-incubated at 37 °C for 10 min in a dry bath. Then, DPPIV enzyme (200 µL) diluted in the same tris buffer to reach a 0.01 U/mL concentration was added, and the reaction proceeded for 60 min. It was arrested by 0.1 M K_2CO_3 (400 µL) addition. The positive control (A_{100}) was made similarly, but hydrolysates were substituted by Tris-HCl buffer. Sample control (A_{sc}) was created to discard the hydrolysate absorbance through the blend of the sample (100 µL) with buffer (300 µL) and K_2CO_3 (400 µL). Absorbances for the three systems were measured at 405 nm in a UV-vis spectrophotometer, and the following equation determined the anti-diabetic activity:

$$\text{DPPIV inhibition } (\%) = \frac{A_{100} - (A_s - A_{sc})}{A_{100}} \times 100$$

Statistical analysis: All analyses were triplicated except for the ACE and DPPIV inhibition tests performed in duplicate. One-way ANOVA was used to determine significant differences through Tukey's contrast at a 95% confidence level using the Minitab 18 package (State College, PA, USA).

3. Results and Discussion

Protein content: Kjeldahl analysis showed that the rye protein isolate contained 51.06 ± 1.15%. This was less than reported by Drakos et al. [5], which was 63–68%. Differences are evident because those authors used a universal protein factor (6.25), while this work used a specific factor for rye (5.7). The protein content could be around 56% if the universal factor had to be employed. Nonetheless, the protein content is similar to that obtained by Vilcacundo et al. [11], where 53.59% of protein content was found for kiwicha protein concentrate.

Enzymatic hydrolysis: The hydrolysis degree and bioactive properties obtained are shown in Table 1. The results demonstrated that alcalase maintained a higher hydrolysis than flavourzyme, with significant increments in the free amino groups for each time analyzed, whereas flavourzyme only increased from 0 to 4 h, without substantial changes between 4 and 8 h. Alcalase is an endopeptidase with a high efficiency for protein hydrolysis and also with cleavage preference for diverse amino acids such as phenylalanine, tryptophan, tyrosine, glutamic acid, methionine, leucine, alanine, serine, and lysine, which explain its high hydrolysis. While flavourzyme maintains endo- and exoproteolytic properties, generating small peptides and free amino acids [12]. On the other hand, the results could be influenced by the hydrolysis conditions imposed. Both testing enzymes can work under neutral pH; however, the same experimental conditions were previously reported as optimal for alcalase under a response surface model [9]. In addition, recent work by Chirinos et al. [13] has shown similar results for the tarwi (*Lupinus mutabilis* Sweet) hydrolysis with alcalase and flavourzyme, finding a 52.3 and 19.5% hydrolysis degree, respectively.

Table 1. Free amino group concentration and bioactive properties found for rye protein hydrolysis.

Enzyme	Hydrolysis Time (h)	Free Amino Group Content (ppm)	Radical Scavenging (%)	ACE Inhibition (%)	DPPIV Inhibition (%)
Alcalase	0	2291.74 ± 155.49 ^{c}	43.78 ± 2.66 ^{c}	81.87 ± 4.90 ^{a}	51.69 ± 2.61 ^{b}
	4	4300.24 ± 203.97 ^{b}	56.50 ± 2.84 ^{b}	86.76 ± 3.17 ^{a}	63.70 ± 1.27 ^{a}
	8	5481.40 ± 409.43 ^{a}	78.58 ± 1.04 ^{a}	86.76 ± 2.02 ^{a}	57.53 ± 0.15 ^{ab}
Flavourzyme	0	812.03 ± 63.36 ^{b}	9.76 ± 1.65 ^{c}	78.82 ± 0.58 ^{b}	ND ^{b}
	4	2933.09 ± 76.80 ^{a}	31.12 ± 1.52 ^{b}	84.93 ± 0.58 ^{a}	40.62 ± 2.61 ^{a}
	8	2974.15 ± 275.39 ^{a}	34.77 ± 0.59 ^{a}	86.15 ± 1.15 ^{a}	36.62 ± 1.56 ^{a}

Different lowercase letters indicate significant differences ($p < 0.05$) between each sampling time. ND: not detected.

Both hydrolysis showed differences in the antioxidant test for each sampling time, contrasting with the other bioactive properties. Alcalase generated the hydrolysates with the best radical scavenging power, which can be explained by the number of free amino-groups obtained because the antioxidant capacity has been related to peptides with molecular weights between 500 and 1500 Da obtained from a higher hydrolysis, but also with its sequences, which contain tryptophan, tyrosine, methionine, and lysine, amino acids were used as a preferential site for alcalase cleavage as discussed before [12,14].

Both hydrolysates showed similar results regarding ACE inhibition activity, with inhibition percentages of around 86% at the final hydrolysis. Indeed, from the sample at 0 h, high antihypertensive activity was found, demonstrating the presence of sequences with this capacity from the protein isolate, as was also observed before by Vilcacundo et al. [11]. The last authors found an IC_{50} of 0.08 mg/mL in the protein concentrate before performing an in vitro digestion of kiwicha (*Amaranthus caudatus*). Also, Darewicz et al. [15] reported

an ACE inhibition of 24.49% in intact oat kernel protein. Thus, results demonstrate that processing protein isolation and treatments at previous hydrolysis could release fractions with bioactive potential.

Despite slight increments in antihypertensive capacity during the following sampling times, only flavourzyme was significant in the beginning. Thus, peptide fractions with molecular weights between 5 and 10 kDa or even <1 kDa, as well as proline, tyrosine, phenylalanine, valines, isoleucine, leucine, and tryptophan, probably were generated before and during the enzymatic hydrolysis, because these are the structural characteristics for reaching this bioactivity [14].

For DPPIV inhibition, alcalase hydrolysate showed bioactivity initially, but flavourzyme hydrolysis did not. It confirms that ACE and DPPIV inhibition can be obtained in protein isolates; however, it also depends on the free amino group content and for the faster hydrolysis propitiated by alcalase, despite its immediate inactivation. Darewicz et al. [15] found a DPPIV inhibition of 42.55% in oat protein kernels prior to digestion, and Chirinos et al. [13] did not find antidiabetic properties in flavourzyme hydrolysate with a low hydrolysis degree. Nevertheless, it was detected in alcalase early, which showed a higher hydrolysis.

In addition, alcalase and flavourzyme hydrolysates exhibited significant increments in DPPIV inhibition from 0 to 4 h, and it was lost at 8 h, demonstrating that excessive hydrolysis can delete bioactivity. Previously, Vilcacundo et al. [11] and Pérez-Escalante et al. [16] reported the loss of antihypertensive activity when pancreatin and alcalase were used. Structural conformation for DPPIV inhibition has not been completely elucidated [17]; however, it is now known that hydrophobic sequences with molecular weights lower than 1 kDa have a higher possibility of exhibiting antidiabetic activity, which explains the higher activity found with alcalase.

Finally, a few reports for multi-bioactivity in cereals have been assessed. Darewicz et al. [15] found a radical scavenging power of 35.7%, DPPIV inhibition of 89.51%, and ACE inhibition of 87.76% after gastric and duodenal digestion of oat protein. In comparison, 1–2 µmol TE/mg protein, IC_{50} of 2.14–3.65 mg/mL, and 0.16–7.85 mg/mL for antioxidant, antidiabetic, and antihypertensive bioactivities were found in tarwi hydrolysates with alcalase and flavourzyme [13]. Compared to these previous works, results found in this study are very competitive for ACE and DPPIV inhibition, even better, especially for antioxidant activity. This indicates that the rye protein hydrolysates are an excellent source for releasing multifunctional peptides.

4. Conclusions

Both enzymatic processes for rye protein hydrolysis showed competitive antioxidant, antihypertensive, and anti-diabetic properties compared to other cereal protein hydrolysates. However, the best rye hydrolysate was obtained with alcalase. Indeed, this enzyme generates peptides with comparable ACE inhibition and better radical scavenging power but less DPPIV inhibition compared to previous studies. Nonetheless, all bioactivities tested after the hydrolysis with alcalase were above 50%. Thus, in multi-bioactivity terms, rye protein hydrolysis with alcalase is better than with flavourzyme hydrolysate and even better than the hydrolysates from other cereals.

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